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DOTTORATO DI RICERCA IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

A NEW CONFIRMATORY ASSAY FOR THE DIAGNOSIS OF CYSTIC FIBROSIS: DETERMINATION OF CHLORIDE ION BY INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

DOCTORAL THESIS

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“Being the richest man in the cemetery doesn’t matter to me. Going to bed at night saying we’ve done something wonderful… that’s what matters to me.”
Steve Jobs

This milestone is dedicated to those who have believed in me and have always supported me: my family, my wonderful husband and children, and to a great Professor, Alessandro Saba.

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SUMMARY

The current guidelines for sweat chloride analysis identify the procedures for sweat collection, but not for chloride assay, which is usually performed by methods originally not aimed at the low concentrations of chloride found in sweat. To overcome this limitation, we set up, characterized, and adopted an original inductively coupled plasma mass spectrometry (ICP-MS) method for sweat chloride determination, which was designed for its easy use in a clinical laboratory. The method was linear in the range 8.5E−3 to 272.0E−3 mM, precision exhibited a relative standard deviation < 6%, and accuracy was in the range 99.7–103.8%. Limit of blank, limit of detection, and limit of quantitation were 2.1 mM, 3.2 mM, and 7.0 mM, respectively, which correspond to real concentrations injected into the mass spectrometer of 3.9E−3 mM for LOD and 8.5E−3 mM for LOQ. At first, the method was assessed on 50 healthy volunteers who exhibited a mean chloride concentration of 15.7 mM (25–75th percentile 10.1–19.3 mM, range 2.8–37.4 mM); then, it was used to investigate two patients with suspected cystic fibrosis, who exhibited sweat chloride values of 65.6 mM and 81.2 mM, respectively. Moreover, the method was cross-validated by assaying 50 samples with chloride concentration values in the range of 10–131 mM, by both ICP-MS and coulometric titration, which is the technology officially used in Tuscany for cystic fibrosis newborn screening. The reference analytical performances and the low cost of ICP-MS, accompanied by the advantageous cost of a single sweat chloride assay, make this technology the best candidate to provide a top reference method for the quantification of chloride in sweat. The method that we propose was optimized and validated for sweat samples ≥ 75 mg, which is the minimum amount requested by the international protocols. However, the method sensitivity and, in addition, the possibility to reduce the sample dilution factor, make possible the quantification of chloride even in samples weighting < 75 mg that are discarded according to the current guidelines. In general, ICP-MS is highly cost-effective, and the operating costs of the clinical laboratory could greatly benefit from its high versatility, which allows performing different assays, usually conducted by several machines, by just one instrument. For instance, one of these assays could be the quantification of metal elements in biological fluids that are usually quantified individually by atomic absorption and that can be assessed simultaneously by ICP-MS multi-elemental analysis.

INTRODUCTION

Cystic fibrosis

Cystic fibrosis (CF), an autosomal recessive disorder occurring in 1/2000–3000 livebirths among the Caucasian populations, [1] is caused by mutations of the gene encoding for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a cyclic adenosine monophosphate–regulated ion channel, which primarily acts as a chloride channel and controls the movement of salt and water into and out of the epithelial cells of several glands.

Cystic Fibrosis has been recognized as a distinct clinical entity for overwore 60 years. [1] Early references include a Northern European folklore motto: "Woe betides those children who when you kiss them on the forehead have a salty taste. They are bewitched and will die soon". The first comprehensive description of Cystic Fibrosis was given in 1938 by Dorothy Andersen. [1] She described cystic fibrosis of the pancreas in forty-nine patients. [1] In 1948, a heat wave in New York allowed St. Agnes to discover that patients with Cystic Fibrosis lose an excessive number of salts with sweat. [2] This historic discovery led in 1959 to the development of the sweat test for diagnosis, by Gibson and Cooke. [3] Quinton reported that the sweat gland ducts of cystic fibrosis patients were relatively impermeable to chlorine. [4] The genetic basis was confirmed by the discovery of the CFTR gene in 1989. [5]

The incidence of cystic fibrosis varies according to ethnicity; in Europe is estimated at 1: 2000 -1: 3000 Caucasian births; mutations in the CFTR gene are more frequent in populations of Northern Europe, where relative homogeneity of mutations has been observed (absolute predominance of DF508), differing considerably with other European regions characterized by a different frequency of many other mutations. [6]

The high frequency of the mutation in Caucasians could be due to an advantage of the heterozygote, which seems to have greater resistance to secretory diarrhea from cholera or typhoid fever, increased fertility, reduced asthma episodes, and resistance to a variety of infectious agents [5,7]. However, these theories lack confirmatory evidence.

In Italy, the average prevalence of the disease at birth, considering all the data collected from 1988 to 2014, is 1: 4079 live births, therefore lower than what is reported in the literature

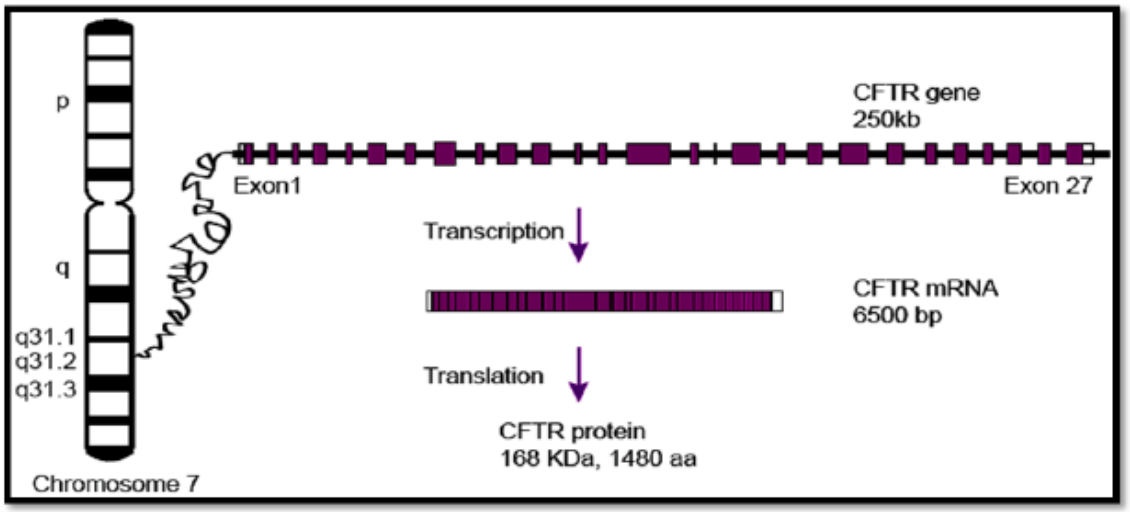
for the Caucasian population (1: 2500 –1: 3000), with considerable differences between regions that have been conducting newborn screening for many years and regions where the diagnosis is almost made exclusively for symptoms [8].

As well as for other European regions, [9] also in Italy there was a notable genetic` variability, as shown by the molecular analysis results available for 82% of patients: the most` frequent mutation (DF508) affects only 49% of all CF chromosomes and the remaining is characterized by numerous other mutations, with significant differences concerning the Region of origin [10].

Mutations of the CFTR gene

The Cystic Fibrosis Transmembrane Conductance Regulator gene (CFTR) is located on chromosome arm 7. It extends over 250,000 bases and contains 27 exons; the product is a cAMP-dependent channel responsible for the secretion of chloride ions and other anions. [5]

Figure 1: CFTR gene and its transcription



More than 2000 genetic variants have been defined for CFTR. Mutations more commonly involve certain regions of the protein, such as the nucleotide-binding domain and the regulatory domain [11].
The diverse types of mutations discovered include missense (46%), frameshift mutations (22%) split-site (16%), non-sense (14%) and frame deletion (2%).
Most of these mutations (83%) are associated with the disease, while the remaining are classified as polymorphisms referred to as "non-pathological" mutations: an example is DF508C (T-C conversion in position 508) which can have the same electrophoretic appearance as DF508.68. Other polymorphisms may have a slight dysfunction that in some circumstances can alter CFTR function in such a way as to cause disease (e.g., M470V).
Alterations in the CFTR gene to be designated as disease-causing mutations should meet at least one of the criteria suggested in the declaration of consent of the FC foundation [12].
Mutations in the CFTR gene were initially classified into 5 classes based on the effects they have on the function of the CFTR and recently included a sixth group [13-15]. However, these classes are not
mutually exclusive and some mutations can have characteristics of more than one class.

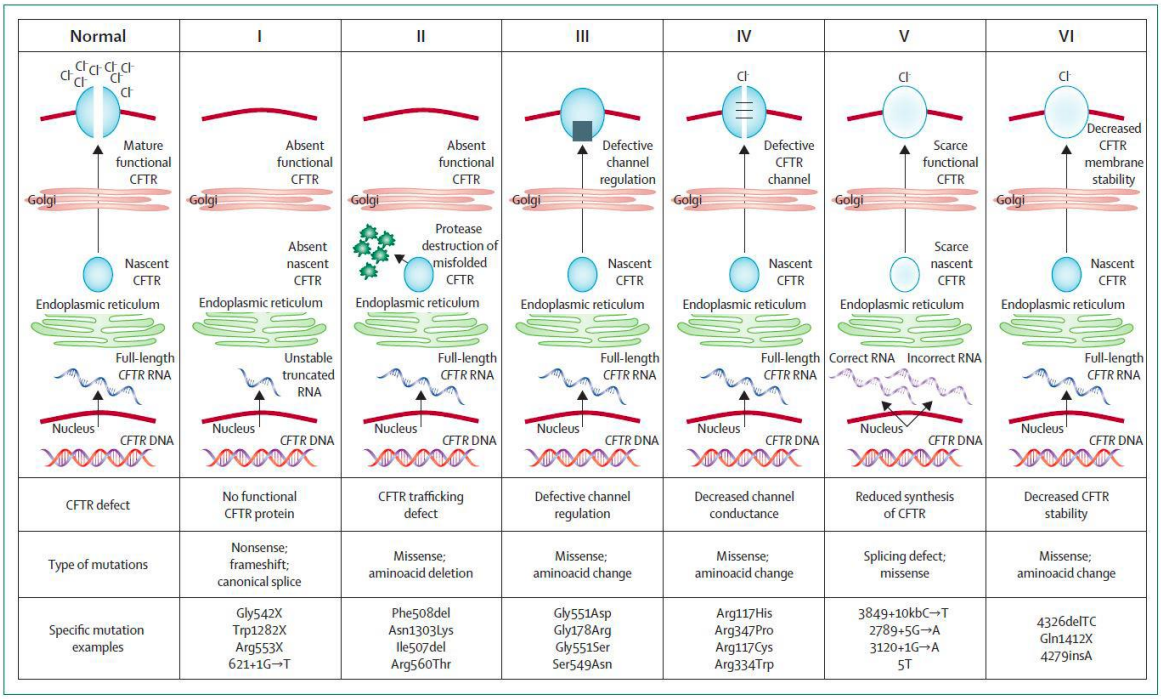


Figure 2. Mutation classes of CFTR gene.

Class I: Defective protein synthesis

Mutations of this class do not allow for protein synthesis and include more severe CF phenotypes. [16,17]. The most common class I mutation G542X prevents the synthesis of a stable protein or results in the production of a protein truncated due to the synthesis of a premature stop codon [17].

Class II: impaired processing of the protein

Mutations of this class translate to a CFTR protein structurally abnormal which, due to incorrect folding, fails to reach a correct cellular localization. This class includes the most common and earliest recognized mutation DF508 (elimination of phenylalanine in position 508). Other missense mutations associated with processing errors are present throughout the CFTR. In recombinant cells, CFTR containing the DF508 mutation fails to mature to its completely glycosylated form; therefore, it is degraded by the cell before it can perform its function. The protein, therefore, is missing or present in small quantities in the apical membrane of the intestinal, respiratory, and hepatobiliary epithelia. Some mutations, such as P574H, have a less severe folding defect than DF508 and as a result, the protein reaches the plasma membrane and maintains some function [18].

Class III: Deficit in regulation

Mutations of this class produce a protein that is transferred to the cellular membrane but does not respond to cAMP stimulus. Up to now the mutations of class III cause an alteration at the level of the binding domain of the nucleotide. Since intracellular ATP regulates the opening of CFTR chloride channels through the direct interactions with the binding domain of the nucleotide, it is not surprising that the mutations in these domains can alter channel function. In some mutations, for example, G551D, there is a minimum function and, in some cases, e.g., S125P, the ATP is less powerful in stimulating channel activity.

Class IV: Defective conduction

Many missense mutations have been identified in the gene and code for altered membrane domains; in these cases, the CFTR gene encodes a protein that reaches the cell membrane correctly and responds to stimuli but generates a reduced chloride current [16]. Some examples include mutations in which arginine is replaced by histidine at residue 117 (R117H), tryptophan at 334 (R334W), or proline at 347 (R347P); when these mutant CFTRs are expressed in heterologous epithelial cells, CFTR proteins are processed correctly, are present on the apical membrane, but have a reduced ion conductance [19]. This is due to the reduced velocity of ion flow through a single open channel. Furthermore, at least for R117H, the amount of time the channel is open is also reduced [19].

Class V: The reduced presence of protein on the cell membrane

Mutations in this group include missense mutations, e.g. A455E (replacement of glutamic acid with alanine). The most frequent mutations are at the level of exon 9 and intron 8. However, even mutations in the promoter of the CFTR gene can produce pathological phenotypes by reducing transcript levels.

These mutations produce a reduced amount of CFTR gene transcript and low functional protein levels at the cell membrane level. In some cases, it comes produced a small amount of normal-length mRNA and this could explain a milder phenotype.

Class VI: Reduced stability of the protein

Mutations of this new class include alterations in the stability of the protein mainly due to the absence of residues 70-98 at the C-terminal end [15, 20].

Although this portion is not necessary for the biological function of the canal it is essential to maintain the stability of the glycosylated CFTR complex. CFTR mutations are classified as severe or mild depending on the impact on the functionality of the protein and the clinical picture. Generally, severe mutations cause a lack of synthesis or blocking of the process (Class I, II, III), while mild mutations
lead to altered conductance or reduced synthesis (Classes IV, V, VI).

Functions and dysfunctions of the CFTR

The CFTR gene encodes a transmembrane glycoprotein of 1480 amino acids and has a molecular mass of 168 kDa [21]. CFTR, which is found on the apical membrane of the epithelial cells that line the respiratory tract, the biliary tree, the intestine, the vas deferens ducts, sweat glands, and pancreatic ducts, is a member of the family of transporters that bind ATP.

CFTR is composed of 5 domains: 2 domains interact with the plasmatic membrane, each consisting of 6 subunits; two nucleotide-binding domains, and a cytoplasmic regulatory domain. (Figure 3)

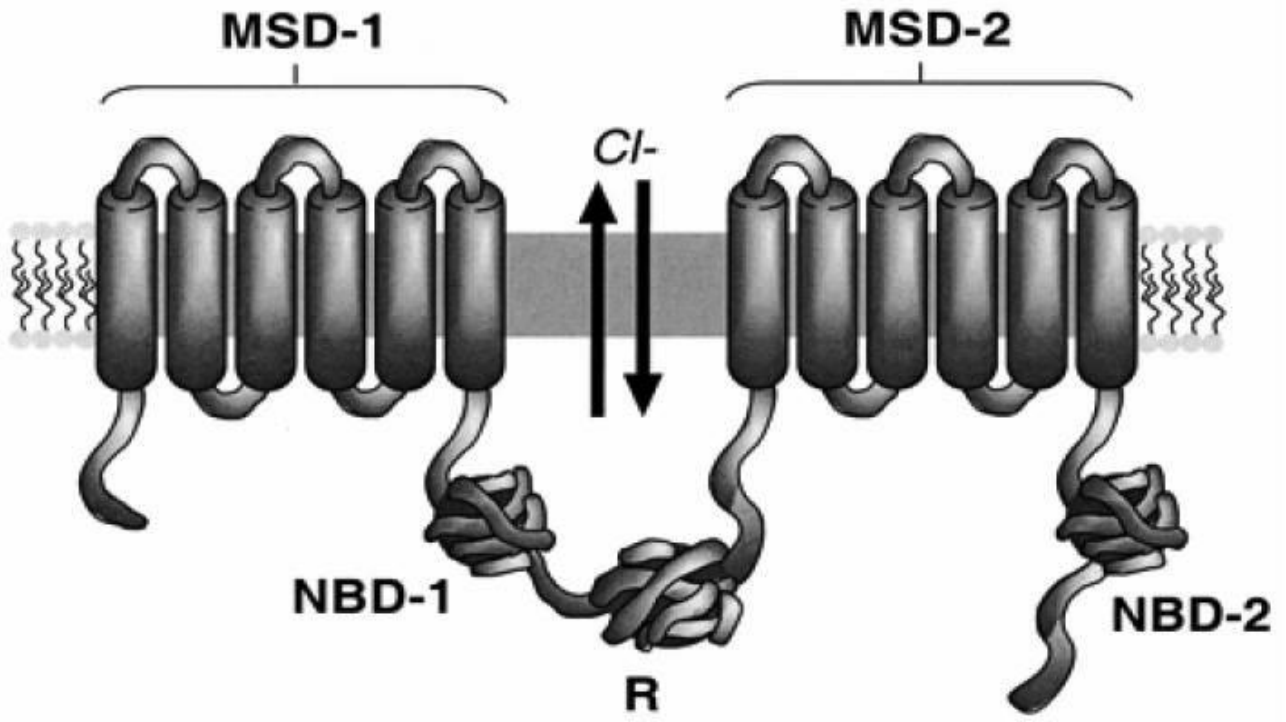
The domains present at the plasmatic membrane level seem to contribute to the formation of a channel that allows the passage of chloride: it has been seen that mutations of specific residues within the first domain alter the anionic selectivity of the channel [22,23].

The nucleotide-binding domain of CFTR is responsible for binding and hydrolysis of the ATP that provides the energy necessary for the activity of the channel. The regulatory cytoplasmic domain modulates the activity of the CFTR channel and can have both stimulatory and inhibitory effects.

Scientific evidence has shown that CFTR is a cAMP-dependent chlorine channel. [24].

However, the chloride transport function alone cannot explain the entire pathogenesis of the disease; many other described functions are not directly associated with the channelopathy mechanism. The CFTR interacts with several other membrane channels (sodium, potassium, and chlorine activated by calcium), with transporters (ATP and glutathione), and with proteins bound to the cytoskeleton of epithelial cells [25,26]. These regulatory roles cannot be easily related to each other but suggest that CFTR modulates multiple cellular pathways. CFTR co-regulates the transport of sodium through an epithelial sodium channel (ENaC): the Normal CFTR inhibits ENaC-mediated transport, while the mutated one allows for a better sodium transport [27]. CFTR also stimulates a conductance of the chloride outwards (ORCC), increasing its chlorine transport activity [27,28].

Figure 3. CFTR protein



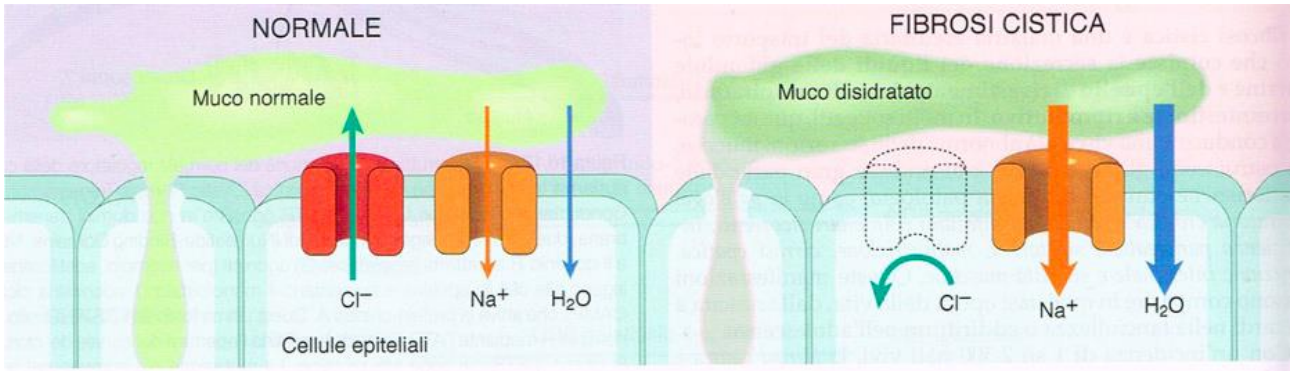
A current hypothesis also suggests that the transporter facilitates the release of ATP, which stimulates a purinergic receptor; the latter therefore acts through a second messaging route to regulate the ORCC [29]. In several tissues, CFTR plays a key role in modulating bicarbonate secretion: [29,30].
The apical epithelial membrane also contains another calcium-activated chloride channel (CaCC).
CaCC is present in airway and epithelial cell sweat glands; in the latter, muscarinic agonists increase the intracellular calcium concentration and stimulate the production of sweat through the opening of the calcium-activated chloride channels present at the membrane level [30].
CFTR is therefore not just a chloride channel, but a protein complex multifunctional.
The main effect of channel failure is impaired ion transport of chloride and bicarbonate [31,32]. The interaction between CFTR and other ion channels, in particular the epithelial

sodium channel (ENaC), and with related cell pathways inflammation (inflammasome) could all be important in the pathophysiology of cystic fibrosis [32].
It is possible that CFTR dysfunction has multiple consequences on hydration, mucociliary clearance, the binding and function of mucus, the impaired innate immunity, and could also predispose to an increased intrinsic cellular inflammation. The relative impact of these effects could
vary with age and disease progression.

Clinical features

CF affects the epithelial cells of various organs, including the respiratory tract, the exocrine pancreas, the intestine, the vas deferens, the hepatobiliary system, and exocrine sweat glands.
The affected epithelia present, in their original state, different functions: some are responsible for the absorption of liquids (such as the epithelium of the airways and the distal intestine), others are deputed to the absorption of salts but not liquids (such as the epithelium of the proximal intestine and sweat ducts), others are responsible for the secretion of liquids (such as the epithelium of the pancreatic ducts). Given the diversity of native activities, it is not surprising that CF produces different organ-specific effects on the transport of electrolytes and water.
This results in a multi-organ disease characterized by suppurative lung disease, pancreatic insufficiency, multifocal biliary cirrhosis, infertility, and high loss of electrolytes with sweat.
Most CF patients present with signs and symptoms of the disease in childhood; approximately 20% of patients have gastrointestinal obstruction, said Meconium ileus, within the first 24 hours of life. Other common modalities of presentation, over the first 2 years of life include symptoms related to the respiratory system, such as persistent cough and/or recurrent pulmonary infiltrates and delayed growth. In a considerable proportion of patients (approximately 5%), the diagnosis is made after 18 years of age.
Pulmonary manifestations are the most serious complications of CF. The epithelium of the airways of FC subject expresses alterations both in the active absorption of Na + and in the active secretion of Cl-. An important observation is that there is also the CaCC channel expressed on the apical membrane which can replace CFTR concerning secretion of Chlorine and a potential therapeutic target.

Figure 4. Airways mucus production in healthy and CF patients



The central hypothesis of airway pathophysiology in CF is that altered regulation of Na uptake and the inability to secrete Cl through the CFTR reduces the volume of fluid on the surface of the airway; the airway is "dehydrated." Both mucus thickening and periciliary fluid depletion led to mucus adhesion to the airway surface. The latter leads to an inability to clear mucus from the airways via both cilia and airflow-dependent mechanisms (cough). This causes small airway obstruction and promotes airway infection. Recurrent infections and resulting inflammation led to submucous gland hypertrophy, excessive mucus secretion, and airway damage to the point of bronchiectasis formation.

Early in life, patients are infected with a limited spectrum of bacteria, most commonly Staphylococcus aureus and Hemophilus influenzae, and with disease progression, Pseudomonas aeruginosa becomes the most common pathogen [33]. The first symptom of lower respiratory tract involvement is cough, which over time becomes persistent and is accompanied by viscous, purulent sputum, often greenish. Inevitably, periods of clinical stability are interspersed with "flare-ups", characterized by increased cough and sputum volume, weight loss, fever, and decreased respiratory function.

As the years go by, relapses become increasingly frequent and the recovery of pulmonary function less and less complete, with an inevitable progression to respiratory failure and pulmonary heart disease.

The first alteration in lung function observed in children with CF is an increase of residual volume to total lung capacity ratio; this suggests that the first functional alteration in CF is lower airway disease. As the disease process progresses, reversible changes in forced vital capacity and FEV1 (forced expiratory volume in 1 second) are observed; the reversible component reflects an accumulation of intra-luminal secretions and/or airway hyperresponsiveness, which occurs in 40-60% of patients, whereas the irreversible component reflects chronic airway wall destruction and bronchiolitis. The earliest visible change on chest radiography is hyperinflation, reflecting small airway obstruction; in a more advanced stage, signs of luminal accumulation of mucus, bronchial wall thickening, and eventually bronchiectasis (ring shadows) are observed.

Several gastrointestinal effects of CF have been registered. In the exocrine pancreas, the absence of a Cl- channel in the apical membrane of pancreatic ductal epithelia alters the function of a Cl-HCO3- exchanger of the apical membrane in carrying out the secretion of bicarbonate and Na+ (by a passive process) into the duct; the deficit of secretion of Na+HCO3- and water leads to the retention of enzymes in the pancreas up to the almost destruction of pancreatic tissue. Exocrine pancreatic insufficiency is present in 85% of patients with cystic fibrosis and is the only clinical feature that correlates well with genotype. [30] Insufficient release of pancreatic enzymes gives rise to the typical picture of protein and lipid malabsorption with steatorrhea and poor nutrition; overcome today with diet and pancreatic enzyme replacement.

Beta-pancreatic cells are spared in preliminary stages, but their function declines with age. This effect, in addiction to insulin resistance induced by inflammation, causes hyperglycemia with the need for insulin administration in more than 15% of older CF patients (>35 years of age). Patients with pancreatic sufficiency (15%) usually have milder pulmonary disease and electrolyte values.

In CF, the intestinal epithelium, due to the lack of Cl- and water secretion, is unable to remove mucins and other macromolecules secreted by the intestinal crypts. Decreased CFTR-mediated secretion of fluid may be exacerbated by excessive fluid absorption, reflecting alterations in CFTR-mediated regulation of Na+ absorption. Both dysfunctions lead to dehydration of intestinal lumen contents and obstruction. Meconium ileus

syndrome occurs in 10-20% of infants with cystic fibrosis and has a wide range of presentations from a delayed passage of meconium to frank intestinal obstruction. [34,35] Recurrent constipation and distal bowel obstruction syndrome are more frequently seen in older cystic fibrosis patients.
Delayed onset of puberty is common in both males and females with CF; the picture of delayed development is likely secondary to the effects of chronic lung disease and impaired endocrine reproductive function secondary to inadequate nutrition. Approximately 97% of males with CF have azoospermia, related to the obliteration of the vas deferens due to altered fluid secretion. Infertility may be the initial presentation for some males with mild disease; diagnosis of CF in these men is problematic [36].
Approximately 20% of females with cystic fibrosis are infertile because of the effects of the chronic lung disease on the menstrual cycle, the presence of thick cervical mucus that blocks sperm migration, and possibly alterations in fluid transport at the uterine tube wall and uterus. Most pregnancies lead to healthy babies, and women with CF can breastfeed normally.
Defective reabsorption of sodium and chlorine in the sweat glands leads to excessively salty sweat and in some cases electrolyte imbalance, with metabolic alkalosis, dehydration, and risk of death [35]. Infants with CF are much more sensitive to heat [37]. However, once the diagnosis is made, with additional salt intake, this problem rarely occurs.
Although CF is a multi-system disease, pulmonary involvement is the major cause of morbidity and more than 90% of mortality.
With a life expectancy of less than one year in 1940, median survival has increased to more than 35 years. Despite these advances, current therapies mostly treat the symptoms rather than the primary cause of the disease; as a result, CF remains a fatal and life-threatening disease.

Diagnosis

A conclusive diagnosis of CF should involve a combination of clinical presentation, genetics, and laboratory testing. The standard laboratory assays for CF consist of the measurement of chloride in sweat [8–11], which is often collected by the absorption method of Gibson and Cooke (proposed in 1959), based on the use of pilocarpine iontophoresis for sweat stimulation, that is generally regarded as the reference method for sweat testing. [12] More recently, a sweat stimulation and collection procedure (1982) employing the Wescor (Logan, UT, USA) Macroduct system has been introduced [13]. Despite the significant differences between these two methods that have an impact on both the sample preparation procedure and the chloride quantification method, a good agreement between them has been observed.
The discovery of the CFTR gene and laboratory techniques capable of detecting mutations in it have facilitated the diagnosis of cystic fibrosis and greatly expanded the clinical spectrum of the disease allowing the inclusion of milder, atypical forms as well [38].
In 1998 [39], a new definition of cystic fibrosis was given based on multiple criteria:
- presence of at least one of the phenotypic features or a history of CF in a sibling or a positive neonatal screening,
- evidence of CFTR abnormalities documented by at least one of the following conditions:
1) elevated chlorine concentrations in sweat,
2) identification of 2 CFTR mutations associated with cystic fibrosis.

3) in vivo demonstration of abnormalities of electrical potential at the level of the nasal mucosa.

These criteria have been updated by the Cystic Fibrosis Foundation CFF which published in 2008 the Guidelines for the diagnosis of cystic fibrosis in both neonatal and adult age. The new diagnostic criteria consider not only forms of cystic fibrosis with pancreatic insufficiency and sufficiency, but also phenotypically fewer classic forms classified as CFTR-related disorders.

In 2015, the CF Foundation decided to revise the 2008 diagnostic guidelines [40]: it convened an international committee of experts in CF diagnosis to update the diagnostic guidance and achieve standardization in definitions worldwide. The mission of this committee was to develop clear and actionable consensus guidelines for the diagnosis of CF and other conditions associated with mutations in the CFTR gene, such as CFTR-related metabolic syndrome (CRMS) [41] or positive screening for CF, inconclusive diagnosis (CFSPID) [42], and CFTR-related disorders [43]. The committee approved 27 consensus statements (Table 1) in 4 overlapping categories that apply to:

1) selected and nonelected populations.

2) populations and fetuses undergoing neonatal screening and prenatal testing.

3) children with an uncertain diagnosis and patients with CRMS or CFSPID (now considered the same)

4) patients with typical clinical manifestations of CF who did not have neonatal screening, including older subjects, infants born at home or born in regions where NBS was not performed, and those with false-negative screening tests.

Figure 5 provides a simplified algorithm for how these consensus statements should be applied to individuals with suspected CF because of a positive NBS result, the presence of signs or symptoms typical of the condition, or recognition of a family history of CF (most often sibling). It should be noted that a positive NBS result does not mean that the child has CF; the likelihood of a CF diagnosis after a positive result varies greatly depending on the NBS method used.

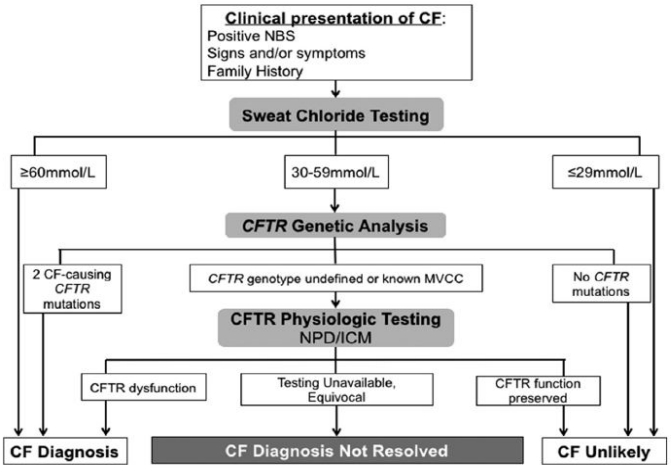
Although many individuals enter this algorithm through a positive newborn screening in which CFTR genetic testing was subsequently conducted, the diagnosis of CF is primarily based on direct demonstration of abnormal CFTR function by measuring chloride concentration in sweat [44]. Although being able to obtain an adequate sweat sample for chloride measurements can be difficult, particularly in young children, experience and studies have shown that this is often feasible in term infants during the first postnatal month (i.e., during the neonatal period) [45,46].

Table I. Consensus recommendations for diagnosis of CF*

Statement numbers	Consensus statements	Vote	Abstain (n)
1	Sweat chloride testing should be performed according to approved procedural guidelines published in established, international protocols such as the CLSI 2009 Guidelines.	100%	0
2	Newborns with a positive CF newborn screen, to increase the likelihood of collecting an adequate sweat specimen, should have the test performed bilaterally and when the infant weighs >2 kg, and is at least 36 wk of corrected gestational age.	87%	0
3	Newborns greater than 36 wk gestation and >2 kg body weight with a positive CF newborn screen, or positive prenatal genetic test, should have sweat chloride testing performed as soon as possible after 10 d of age, ideally by the end of the neonatal period (4 wk of age).	93%	1
4	In infants with presumptive CF identified through NBS, CF treatment should not be delayed while efforts to establish a diagnosis of CF are initiated.	83%	1
5	Sweat chloride analysis should be performed within a few hours of sweat collection and the results and interpretations should be reported to clinicians and parents or patients, as soon as possible and certainly on the same day.	90%	0
6	In individuals presenting with a positive newborn screen, clinical features consistent with CF, or a positive family history, a diagnosis of CF can be made if the sweat chloride value is ≥60 mmol/L.	93%	0
7	Individuals who are screen-positive and meet sweat chloride criteria for CF diagnosis should undergo *CFTR* genetic testing if the *CFTR* genotype was not available through the screening process or is incomplete.	100%	0
8	In individuals with a positive newborn screen, a sweat chloride <30 mmol/L indicates that CF is unlikely.	82%	2
9	Individuals with clinical features that may be consistent with CF who have a sweat chloride <30 mmol/L indicates that CF is less likely. It may, however, be considered if evolving clinical criteria and/or *CFTR* genotyping support CF and not an alternative diagnosis.	80%	0
10	Individuals presenting with a positive newborn screen, symptoms of CF, or a positive family history, and sweat chloride values in the intermediate range (30-59 mmol/L) on two separate occasions may have CF. They should be considered for extended *CFTR* gene analysis and/or CFTR functional analysis.	90%	0
11	The latest classifications identified in the CFTR2 project (http://www.cftr2.org/index.php) should be used to aid with CF diagnosis: • CF-causing mutation: individuals with 2 copies on separate alleles will likely have CF (clinical sweat confirmation needed) • Mutation of varying clinical consequence (MVCC): a mutation that in combination with a CF-causing mutation or another MVCC mutation may result in CF • Uncharacterized mutation/mutation of UNK: mutation that has not been evaluated by CFTR2 and may be disease causing or of variable clinical consequence or benign • Non-CF-causing mutation: individuals with 1 or more are unlikely to have CF (as a result of that allele)	100%	0
12	In individuals presenting with a positive newborn screen, symptoms of CF, or a positive family history, the identification of 2 CF-causing mutations (defined by CFTR2) is consistent with a diagnosis of CF. Sweat chloride testing is necessary, though, to confirm the diagnosis.	87%	0
13	The absence of detection of 2 CF-causing *CFTR* mutations does not exclude a diagnosis of CF.	93%	1
14	If further CF functional testing is needed (NPD and ICM), it should be performed in a validated reference center with trained staff certified by the CF Foundation TDN or ECFS Clinical Trial Network.	100%	0
15	In individuals with a positive newborn screen but variable or uncharacterized *CFTR* mutations (<2 CF-causing mutations), the diagnosis of CF can be made by demonstrating CFTR dysfunction (a sweat chloride ≥ 60 mmol/L or CF-typical NPD or ICM).	93%	0
16	The term CRMS is used in the US for healthcare delivery purposes and CFSPID is used in other countries, but these both describe an inconclusive diagnosis following NBS.	96%	2
17	The term CRMS/CFSPID is reserved for individuals who screen positive without clinical features consistent with a diagnosis of CF.	83%	1
18	The definition of CRMS/CFSPID is an infant with a positive NBS test for CF and either: • A sweat chloride value <30 mmol/L and 2 *CFTR* mutations, at least 1 of which has unclear phenotypic consequences *OR* • An intermediate sweat chloride value (30-59 mmol/L) and 1 or 0 CF-causing mutations	86%	1
19	Children designated as CRMS/CFSPID should undergo at least one repeat sweat chloride test at CF centers with suitable expertise, such as an accredited CF center.	86%	1
20	Children designated as CRMS/CFSPID should have clinical evaluation performed by CF providers to identify the minority that may develop clinical symptoms.	83%	1
21	Children designated as CRMS/CFSPID can be considered for extended *CFTR* gene analysis (sequencing and or deletion duplication testing), as well as CFTR functional analysis (NPD/ICM) testing to further define their likelihood of developing CF.	80%	0
22	The decision to reclassify children designated as CRMS/CFSPID as CF is an integrated decision that should take into account functional assessment of CFTR (sweat chloride, and possibly NPD/ICM), *CFTR* genetic analysis, and clinical assessment by the CF clinicians caring for the patient.	90%	0
23	Genetic counseling should be offered to families of individuals followed for CRMS/CFSPID, including a discussion of the risk in future pregnancies.	100%	1
24	Research Recommendation: Infants with a designation of CRMS/CFSPID (by definition) do not have clinical features consistent with a diagnosis of CF and further research is needed to determine the prognosis and best practices for frequency and duration of follow-up.	96%	0
25	For individuals presenting with CF symptoms, the same diagnostic criteria recommended for the screened population for sweat chloride testing, *CFTR* genetic analysis, and CFTR functional testing should be used to confirm a CF diagnosis.	93%	0
26	The diagnosis of CFTR-related disorder has been defined as a monosymptomatic clinical entity (CBAVD/pancreatitis/bronchiectasis) associated with CFTR dysfunction that does not fulfill the diagnostic criteria for CF.	86%	2
27	Clinicians should avoid the use of terms like classic/nonclassic CF, typical/atypical CF, delayed CF, because these terms have no harmonized definition and could be confusing for families or caregivers.	83%	1

CBAVD, congenital bilateral absence of the vas deferens; *CLSI*, Clinical and Laboratory Standards Institute; *CTN*, Clinical Trial Network; *ICM*, intestinal current measurement; *MVCC*, mutation of varying clinical consequence; *NPD*, nasal potential difference; *TDN*, Therapeutics Development Network; *UNK*, unknown clinical consequence.
*In each of the 2 surveys distributed for reviewing the consensus statements drafted and voting, 1 committee member, a different person each time, did not respond.

Figure 5. Simplified diagnosis algorithm



Although NBS is now widely implemented, the diagnosis of CF is not always clear. A sweat test is required to confirm the diagnosis of CF: a sweat chloride level ≥60 mmol/L indicates a diagnosis of CF, and a sweat chloride level <30 mmol/L indicates that CF is unlikely. For individuals who fall in the intermediate level of 30-59 mmol/L, genetic analysis is required. This is a considerable change from previous guidelines which, for subjects over 6 months old, considered 40 mM as the cutoff value; this change was determined by the diagnosis of CF in patients with chloride values in the 30–39 mM range [14]

Additional tests to assess CFTR function such as NPD (nasal potential difference) and ICM (measurement of intestinal current) may also be indicated but should be performed in a specialized center approved for such studies. Some children with positive NBS and sweat chloride levels of 30 to 59 mmol/L or even ≤29 mmol/L and inconclusive genetic testing may be designated as CRMS/CFSPID. Further research is needed to determine their prognosis; restricted follow-up is recommended.

Guidelines have been developed by many groups to standardize the sweat collection procedure and ensure the quality of the test results [47,48]. Sweat testing is a reliable test for the diagnosis of cystic fibrosis in approximately 98% of affected patients.

However, cases of patients with clinical manifestations ascribable to CF but normal or borderline sweat electrolyte values have been reported [19, 13, 14]; this occurs in only 1-2% of cases [19, 14, 46]. Available data suggest that healthy adults may have sweat electrolyte values equivalent to those present in CF patients; though they are not CF patients, no data have been collected regarding the normal reference values in adults. [17-18] Performing the test during childhood has limitations, because of the insufficient collection of sweat and the transient increase in chloride levels in the sweat during the first 24 hours of life [17, 20].

Newborn screening

Newborn screening (NBS) for cystic fibrosis became practically feasible with the advent of a radioimmunoassay [49,50] for the assay of immunoreactive trypsin (IRT) on blood adsorbed on filter paper and dehydrated (Guthrie card), a procedure already routinely used since the 1960s for other neonatal screenings. The rationale for the use of this test to select patients with CF at birth derives from the finding of high values of this enzyme in the

blood of newborns with CF in the first months of life, probably due to reflux of trypsin into the bloodstream for an obstruction of the pancreatic ducts. However, high IRT values can also be found in non-CF infants, and to overcome the problem of reduced specificity of the test in the first days of life (the first blood sample is collected on the 3rd-5th day of life), a second blood sample is taken from subjects who tested positive in the first test at 20-30 days of age to confirm the condition of hypertyrosinemia. In fact, in non-CF subjects, the IRT tends to normalize in the first month, while in CF subjects this parameter tends to remain high over time.
Since the '80s screening laboratories started to use enzyme immunoassays (EIA, ELISA, DELFIA, etc.) for IRT assay implemented on automated or semi-automated analyzers: these assays, compared with conventional radioimmunoassay, offer the advantages of reduced errors due to sample handling, use of non-radioactive reagents, reduced working time and higher specificity and sensitivity.
To date, in Italy, neonatal screening for cystic fibrosis is active throughout the national territory except for Friuli Venezia Giulia.
In the neonatal screening programs of the Veneto-Trentino-Alto Adige and Tuscany regions, the lactase assay on dried meconium sent together with the IRT card has been used for a long time in IRT positive cases, in conjunction with the genetic test, with an appreciable increase in sensitivity and specificity. For the genetic analysis, different molecular biology techniques and different commercial kits are used to investigate 30-31 mutations of the CFTR gene.
CF NBS programs have a false-negative rate of approximately 5% of unexpected CF cases, which results from falsely low neonatal IRT or the presence of CFTR mutations absent in the selected mutation panel. [50,51]. There is also a rate of "false positives" with increased identification of healthy CF carriers [51,52]. This has been estimated at 1.5-1.8 times the expected rate and is likely a reflection of a skewed (higher) distribution of IRT among CF carriers [51,53]. It is recommended that all NBS-positive children (even those with two CFTR mutations) have a sweat test to rule out laboratory errors with screening.

Genetic analysis

Genetic analysis is performed as a second or third step (depending on the protocols used for neonatal screening) and in this case, there are no unambiguous criteria to define the panel of mutations to be investigated (search only for DeltaF508 or multiple mutations).
To date, more than 2000 mutations of the CFTR gene are known but for not all of them, their causal role in the disease has been established. Among these, the most widespread is DF508, with a variable relative frequency in different populations: it represents about 50% of mutations in southern Europe, while it is present in about 80-90% of Northern European countries.
The panel of other mutations covers a variable frequency depending on the population of belonging; on the other hand, some mutations are represented in specific populations (an example represented by T338I in the population of Sardinian origin or by R1162X, present in high frequency almost exclusively in Northeastern Italy), others are extremely rare. Recently it has been found a mutation S737F mutation has recently been found to be typical of patients originating in the Tuscany region [54].
Genetic analysis is performed as a second or third step (depending on the protocols used for neonatal screening) and in this case, there are no unambiguous criteria to define the panel of mutations to be investigated (search only for DeltaF508 or multiple mutations).
For all these reasons there is currently no ideal genetic test able to identify all mutations; the techniques used must meet the criteria of sensitivity, and reproducibility; for this

reason, standard commercial kits are available to overcome the increasing degree of complexity and extension of the genetic test.
Generally, each laboratory should use a panel of mutations most common in its area of use that allows a good identification rate (detection rate).
The presence of two mutations allows the diagnosis of the disease, while, in case of identification of a single mutation, the sweat test can discriminate, but not always, between carriers and patients with only one identifiable mutation.
The first step of genetic analysis (using techniques such as RDB - Reverse Dot Blot - or OLA - Oligonucleotide Ligation Assay) boasts a lower coverage but allows the search for known mutations (about thirty in Italy) and can identify about 75% of the most frequent mutations in the reference region of the laboratory and can be integrated into the protocol of neonatal screening as a second level.
Further genetic analysis identifies all exons and neighboring regions by scanning recognizes sequence variations and may subsequently lead to the sequencing of a specific region of the CFTR gene. The most widely used techniques for scanning are DGGE (Denaturing Gradient Gel Electrophoresis) and DHPLC (Denaturing High-Performance Liquid Chromatography).
Currently, gene scanning is not integrated into the protocols of neonatal screening, both for its technical complexity and because the identification of unknown variants (not necessarily disease-causing mutations) makes it difficult to interpret and correlate them with the phenotype. It is therefore to be reserved for targeted cases, of difficult diagnostic framing, when the sweat test is inconclusive, in the presence of a single mutation or no mutation in the first level genetic analysis, with persistent hypertyrosinemia [55].
The third level of investigation is aimed at discovering mutations not identified by the investigations of the first and second levels: these mutations are sometimes the result of large and complex changes in the sequence of the gene (genomic rearrangements) and the technique QMPSF (Quantitative Multiplex Polymerase Chain Reactions of Short Fluorescent Fragments) has been developed in recent years.
Whether to use a first or second or third level technique depends on the purpose for which the CF genetic test is performed: if it is to serve as a "screening", either screening of the disease at birth among all newborns, or carrier screening in couples who want to have children, a first-level technique is used, because it is faster and less expensive.
If a second or third level technique were used, more mutations could be identified (which are rarer mutations anyway), but it would be necessary to accept longer time and higher costs. In addiction, "variants" of unknown meaning instead of mutations may be diagnosed, with very confusing results for those who have performed the test.
For these reasons, the investigations of second and third level are reserved for particular situations, for example when there are uncertain diagnoses of cystic fibrosis disease or when the couple who want children have a very high risk of having children with CF, as in the case of a couple formed by a woman with CF and a person from the general population.
Another implication of CFTR mutation analysis is the ability to implement prenatal diagnosis. Prenatal diagnosis can be performed by chorionic villus sampling in the first trimester or by amniocentesis in the second or third trimester [56].
Such testing is usually performed in a family that has had a child previously affected by the condition or because of the identification of hyperechogenic fetal intestine during routine ultrasound [57].

Genotype-phenotype correlation

The correlation between CFTR genotype and disease phenotype is extremely variable and is best defined for pancreatic manifestations and less well defined for other manifestations, particularly pulmonary disease [25]. In general, patients who have two severe mutations (Class I-III) can expect the onset of suppurative lung disease and pancreatic insufficiency from childhood, whereas patients with at least one mild mutation (Class IV-VI) usually present with pancreatic sufficiency with late-onset suppurative lung disease. However, the outcome of individuals with the same CFTR mutations, even if they belong to the same family can be significantly different [24]. This suggests an influence of environmental factors (inhaled pollutants, infectious pathogens, and smoking) and perhaps secondary genetic factors that may act as modifiers of CFTR function [25, 58]. This suggests an influence of environmental factors (inhaled pollutants, infectious pathogens, and smoking) and perhaps secondary genetic factors that may act as modifiers of CFTR function [25, 59]. However, there are no regulatory CFTR genes that have been found to influence lung function. Genetic mutations for cytokines associated with pulmonary inflammation have been studied with no associations found [24, 59, 60].

Another variable to consider regarding the effect of CFTR mutations on phenotype is that CFTR mRNA splicing may differ between tissues, with some tissues such as the vas deferens being more sensitive to CFTR mutations than other tissues such as airway epithelium [46], there is thus a hierarchy of tissue responsiveness to defects in the functioning of CFTR.

The cystic fibrosis phenotype is a complex interplay of CFTR gene mutations, regulatory genes, chloride transport through CFTR and interaction with other ion channels, intracellular CFTR function, tissue expression of CFTR and tissue responsiveness to CFTR mutations in combination with environmental exposure to a range of possible agents.

There are many described cases in which clinical features, CFTR genotype, and electrophysiologic measurements are inconsistent with the diagnosis of CF. Warren et al described a patient in whom neonatal screening and mutation analysis suggested a diagnosis of cystic fibrosis, yet clinical manifestations and sweat testing were inconsistent with the diagnosis.[46] Direct sequencing of the patient's genomic DNA showed a compound heterozygosity condition for DF508 and DF508C, a polymorphism not associated with clinical disease. A report by Chmiel et al presented a case in which an asymptomatic baby girl (3 weeks of age) was given the diagnosis of CF-based solely on cord blood DNA analysis, positive for both DF508 and R117H mutations. [61] Despite any other presentation and a normal amount of chloride in sweat, she received pancreatic enzyme supplements. At 2 months of age, she was evaluated by sweat testing, difference in nasal potential (NPD), bronchoscopy, and bronchoalveolar lavage, all of which were negative for cystic fibrosis. This initial diagnosis of CF negatively affected the status emotional, employment, and financial status of the family.

These cases illustrate the potential pitfalls of using genetic testing as the sole diagnostic criterion for CF. A comprehensive evaluation should include a thorough clinical assessment and measurement of CFTR function (by sweat test or NPD) before making a diagnosis and imposing therapy [62]. At the other extreme are patients with clinical CF but without CFTR mutations, despite complete gene sequencing [28]. The diagnosis of CF was based on elevated chloride concentrations in sweat in the presence of CF-like pulmonary symptoms, but CFTR genetics and NPD results did not provide evidence of defective CFTR, and no molecular lesions were identified. [63] Taken together, these data suggest that factors other than CFTR dysfunction may cause a nonclassical phenotype.

Further studies on these patients may reveal additional pathways that contribute to CF phenotypes.

Nasal potential difference

Altered ion transport in CF patients at the level of respiratory epithelia can be studied in vivo by measuring the potential difference in the nasal mucosa [64]. This is a significantly more complex test than measuring electrolytes in sweat. The measurement of transepithelial potentials of the nasal mucosa consists of evaluating the potential difference established between an electrode placed on the nasal mucosa and one on the skin, employing an electrical potential detector (voltmeter). This
nasal potential difference (NPD), which is always negative, is strongly dependent on the movement of ions (chloride and sodium) that takes place at the level of the nasal epithelium and therefore on the degree of functioning of the CFTR protein, which regulates most of the movement of chloride and sodium. The protocol for NPD is well described and standardized but is only performed in specialized centers. CF patients have reduced chlorine transport with hyperabsorption of sodium, as measured by a more negative difference in nasal potential. In normal conditions NPD is around -20 millivolts (mV), in classical CF it ranges predominantly between -40 and -60 mV. In addition to the basal NPD measurement, other measurements are conducted after perfusing the mucosa with some substances that can change the starting NPD depending on the degree of functionality of CFTR: first, we perfuse with amiloride to see how much is corrected the basal resorption (by much in classical CF, because in this one is very increased the basal resorption); then with a chlorine-free solution, which normally stimulates chlorine secretion through CFTR (no or almost no correction in classic CF); then with isoproterenol, a substance that activates the CFTR chlorine channel (no or almost no activation in classic CF).

NPD can complement sweat testing and genetic mutation analysis. However, it can also produce indeterminate results, particularly in "borderline" cases.[65]
NPD measurement is technically challenging and requires experience for proper interpretation [66].
The presence of nasal inflammation, such as in allergic rhinitis or viral infection, can alter ion transport and give falsely negative results [67]. For patients in whom both sweat testing and genetic analysis are inconclusive, an abnormal nasal potential difference can be used as evidence of dysfunction of the CFTR.

Auxiliary tests

Other tests can predict CFTR function, such as measuring intestinal current (ICM). This involves measuring the electrical short-circuit current that is generated through a layer of intestinal mucosa. This test relies on ion transport and thus chlorine and sodium concentration at the surface of the intestinal epithelium (largely dependent on CFTR protein-channel function), which results in a potential difference from the deep layers of the wall. It is this potential difference that allows sophisticated, miniaturized devices to measure a passage of electrical current through the wall of the intestine. Unfortunately, this measurement, unlike NPD, cannot be obtained directly "in vivo" but can be obtained using a fragment of mucosa from the last portion of the rectum that is mounted alive ("ex vivo") in a special micro instrument called "Ussing chamber".
A 2-3 mm diameter fragment of rectal mucosa is removed by suction biopsy, inserted into the Ussing chamber and electrical measurements are made using different stimuli against the canal [67].

Another test that can assess CFTR function is pancreatic duct electrolyte secretion stimulation [67,68]. These tests are only available in some centers. Additional clinical information can be gathered by using tests to evaluate the sinuses (radiography or CT), evaluation of pancreatic exocrine function (e.g., fecal elastase, 3-day fecal fat analysis), and respiratory tract microbiology (sputum or bronchoalveolar lavage). Urogenital evaluation (semen analysis) may also be helpful in the diagnosis of CF.

Sweat testing

The association of electrolyte abnormalities in sweat with cystic fibrosis was established during the 1948 heat wave in New York City [2]. In 1956, Schwachman and Gahm used electrolyte analysis in sweat for diagnosis, but only after Gibson and Cooke described the method of sweat collection based on pilocarpine iontophoresis in 1959, it was accepted as a standardized test [3] and has nowadays remained the primary diagnostic test for the disease. Significant advances in the understanding of sweat electrolyte analysis as a measure of CFTR function and refinements in the use of the sweat test for the diagnosis of CF took place so far.

Physiology of the sweat gland

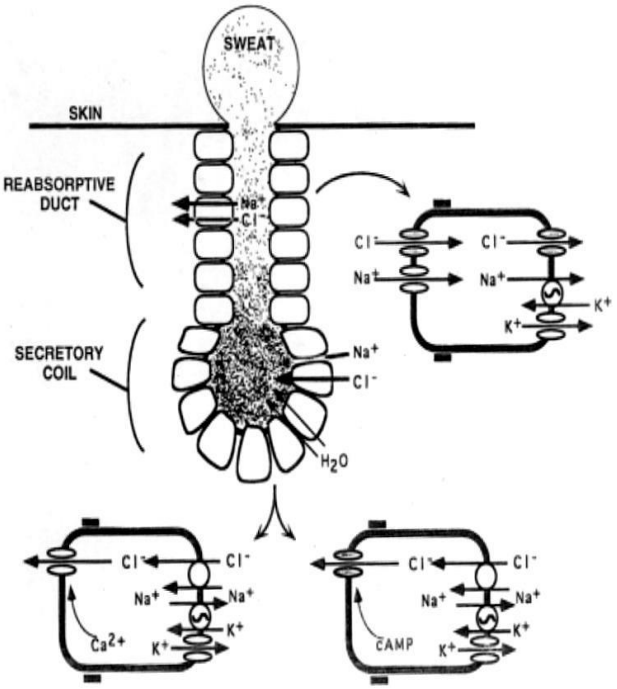


Figure.6. CFTR in the sweat gland.

The sweat gland is composed of two different regions: the secretory coil and the resorbable duct [69]. Primary sweat is contained in the secretory coil and is isotonic with serum. As isotonic secretions travel from the sweat gland acini through the water-impermeable conduit, sodium and chloride are absorbed resulting in hypotonic sweat. Sodium transport establishes ion concentrations and voltage gradients that drive passive chloride uptake. Chloride is transported from the channel lumen by CFTR and a distinct calcium-activated chloride channel (CaCC). Sodium is transported through the epithelial sodium channel, ENaC, which is also upregulated by CFTR through a mechanism that is not fully understood.

Sweat glands in patients with CF show no histologic abnormalities but have pronounced abnormalities in sodium chloride homeostasis because of defective CFTR function. The

absence of functioning CFTR is responsible for the inhibition of chloride reabsorption in the secretory duct of the sweat gland. Despite the pathways for sodium absorption, in the absence of a co-ion, sodium is also poorly reabsorbed. The consequences of this are:
1) the resulting sweat has a relatively high concentration of chloride and sodium compared to normal sweat
2) high chlorine/sodium ratio (> 1) is often seen in CF patients compared to the normal population
3) transepithelial potential difference between extracellular fluid and sweat at the opening of the sweat duct of the glands of patients with CF is approximately twice that present in sweat glands of healthy patients [4, 70,71].
Chloride and sodium values are usually greater than 60 mmol/L in patients with CF and can be as high as 120 mmol/L. This compares with values for normal subjects ranging from 10-50 mmol / L.
Guidelines for the appropriate performance of the sweat test procedure, which are complementary to each other, have been published by several organizations, including:
1) the National Committee for Clinical Laboratory Standards (NCCLS): sweat testing: Sample Collection and Quantitative Analysis.
2) Association of Clinical Biochemists: UK guidelines; and
3) Australian Association of Clinical Biochemists: The Australian guidelines for the performance of sweat testing for the diagnosis of CF [72, 16].
Sweat testing should be performed following these guidelines; alternative procedures are no longer acceptable for CF diagnosis.
Sweat collection today can be performed by 2 different methods:
- Gibson-Cooke method
- Conductivity method (or Wescor)
In both, the sweat test generally has three technical parts: sweat stimulation, collection, and analysis [68].

Sweat stimulation
The preferred site for sweat collection is the flexor surface of the forearm [16, 73]. Other sites used successfully include the upper arm, thigh, and calf. Localized sweating is produced by iontophoresis of the cholinergic drug, pilocarpine nitrate, in the selected area of the skin. Administration of aqueous solutions or Wescor gel discs containing pilocarpine nitrate at 2-5 g/L at the level of the positive electrode is recommended. [73, 16] The negative electrode may contain an equal or alternative dilute electrolyte solution (e.g., magnesium sulfate or potassium sulfate).13 The electrodes used are usually made of copper or stainless steel. A current of 0.5 mA is applied and gradually increased to a maximum of 4 mA [16]. With the applied current, the ions of positively charged pilocarpine move away from the positive electrode and enter the skin where they increase the intracellular calcium concentration and stimulate sweat production by opening the calcium-activated chloride channel [16]. The volume of sweat produced in response to muscarinic agonists is not altered in CF, allowing adequate collection of the same for biochemical analysis. Once 4 mA is reached, the current should be applied for no more than 5 minutes [16]. The electrodes are then removed, and the skin is cleaned with distilled water.
The possibility of urticaria or burns on the patient's skin after iontophoresis is rare (<1%) [68]. Skin burn may occur if the current is greater than 4 mA, if the bare metal of the electrode touches the skin, if the reagent interface is insufficiently moist, or if the electrode is damaged or oxidized [16]. Localized urticaria may occur if the patient reacts to

pilocarpine or electrical stimulation phenomenon [68]. In both circumstances, sweat should not be collected at the affected site. [16, 68].

Sweat collection

Immediately after stimulation, a pre-weighed gauze/filter paper or Wescor collection device is placed directly over the positive electrode site. Critical issues related to sweat collection include avoiding evaporation or contamination of the sample and ensuring an adequate sweat rate. At the end of the collection, the gauze/filter or Wescor collector is removed, and the weight or volume are determined, respectively.

A minimum rate of 1 g / m2 / min is required to ensure accurate results. Since the sweat velocity is related to the electrolyte concentration in the sweat, a minimum acceptable weight/volume is required.

The minimum acceptable weight/volume can be calculated using the formula shown in Figure 7.

Figure 7. Calculation of acceptable sample weight

$$\text{The average sweat rate } (g/m^2/min) = \frac{10000}{\text{area } (cm^2)} \times \frac{\text{weight } (mg/\mu L)}{1000} \times \frac{1}{\text{collection time } (min)}$$

$$= \frac{10 \times \text{weight } (mg/\mu L)}{\text{collection area } (cm^2) \times \text{time } (min)}$$

If the laboratory uses 2x2 inch gauze or filter paper, the minimum weight of sweat should be 75 mg collected in 30 minutes [16]. Using the Macroduct system, the electrodes and stimulation area are smaller, and the minimum acceptable sample is 15 μL collected in 30 minutes [68]. Therefore, the minimum acceptable volume/weight of sweat depends on the size of the electrode used, the type and size of collection material used, and the duration of sweat collection [97]. The Nanoduct system involves direct determination, at the stimulation site, of the concentration of NaCl equivalents (molecular concentration of sodium chloride) in sweat.

Sweat collection time should be no more than 30 minutes and no less than 20 minutes [68]. Insufficient samples should not be analyzed, and the entire test should be repeated.

The International Guidelines to date classify the above methods as follows:

- The Gibson-Cooke method is validated as a CONFIRMATION TEST, meaning a quantitative that takes into account the amount of sweat collected and in which the concentration of chlorine ions (or chlorine + sodium) in the sweat is determined.
- The conductimetric or Wescor method is validated as a SCREENING TEST, understanding a screening test as a qualitative test that may or may not consider the amount of sweat collected. The American Guidelines define this method of measurement as a 'non-selective measurement' of ions present in sweat since the conductivity of a solution depends on the concentration and mobility of all ions present.

The measurement of conductivity in sweat, continuing the American Guidelines, is not equivalent to the measurement of the concentration of chlorine ions (Gibson-Cooke method) because it is influenced by the presence of other ions. This method was ultimately

approved by the Cystic Fibrosis Foundation only outside of the Cystic Fibrosis Treatment Centers. Cystic Fibrosis Treatment Centers. Finally, our positive results must be confirmed with a confirmatory test.

Sweat analysis
Sweat analysis can be performed in two ways:
- indirect methods based on the colligative physicochemical properties of ions or solutes in sweat such as conductivity and osmolarity.
- direct measurement of chloride and sodium electrolytes.

1. Indirect measurement

On theoretical grounds, osmolarity and conductivity measurements can be expected to provide excellent discrimination between electrolyte concentrations in normal and CF sweat.
Sodium, potassium, and chloride, the predominant electrolyte constituents of sweat, are all increased in CF, and the incremental contribution of each would be additive by the colligative nature of the measurement [75]. The contribution of other solutes and electrolytes present in the sweat, in variable concentration and not specifically increased in CF, would predictably decrease the discriminating power of these indirect measurements [44].

A. Osmolality
Sweat osmolality reflects the total solute concentration of sweat measuring total cations and anions along with other solutes such as urea and amino acids.[44]
Thus, osmolality has little discriminatory ability compared to chloride in the distinction between CF patients and normal individuals and as such is not recommended for sweat analysis for CF diagnosis [75].

B. Conductivity
The US CF Foundation has approved the use of the Wescor Macroduct Sweat Chek to measure conductivity as a screening test. When evaluating sweat conductivity results, clinicians should be aware that sweat conductivity is approximately 15 mmol /L higher than chloride in sweat due to the presence of unmeasured anions measured such as lactate and bicarbonate [44, 74]. According to the US CF Foundation, sweat conductivity ≥50 mmol / L should be referred to an accredited CF care center for the measurement of sweat chloride by quantitative pilocarpine iontophoresis. [44, 47, 75]
Recently, a new conductivity analyzer, the Nanoduct system ("new system") has been developed for use primarily in the neonatal population as it requires only 3 μL of sweat [99]. However, the accuracy of the new system compared to the sweat test that assesses the actual amount of chlorine in discriminating between CF and normal individuals has yet to be studied. To date, current published international guidelines do not support the use of conductivity as a confirmatory test [76].

2. Direct measurement

A. Chloride
Chloride in sweat is the analyte most related to abnormal CFTR function and shows greater discrimination than sodium. Acceptable measurement techniques for measuring chloride are colorimetry, coulometry, or ion-selective electrode.

B. Sodium.
Acceptable measurement techniques for measuring sodium are flame photometry, atomic absorption spectrophotometry, or ion-selective electrode. Sodium in sweat is elevated in CF but is less discriminating than chloride for diagnosis and therefore should not be used alone for the diagnosis of CF [45].

Interpretation of results
Chloride concentration in sweat should be interpreted concerning the patient's clinical presentation, family history, age, and awareness that certain mutations in the CFTR gene are associated with borderline or negative sweat chloride concentration.

Reference Ranges
The reference ranges of chloride in sweat that are currently accepted for the diagnosis of CF are:
- <29 mmol/L: negative/normal.
- 30-59 mmol /L: borderline (could be CF).
- >60 mmol/L positive for CF.

Sweat electrolyte values normally increase from childhood to adulthood, and although rare, cases of healthy adults with sweat chloride concentrations above 60 mmol / L have been described. [45] Thus, caution should be exercised in the interpretation of sweat testing in older patients. Although most patients with CF have elevated sweat chloride concentrations, there are many reports of patients with clinical symptoms indicative of CF but with normal or borderline electrolyte values. These patients often maintain pancreatic sufficiency and have normal nutritional status, absent deferent ducts in males, and are diagnosed as CF subjects later in life [16, 55]. Some mutations are associated with a "normal" sweat test. Even borderline chloride values in sweat may represent a diagnostic dilemma. It should be noted that during childhood, a sweat chloride of 30 mmol/L or greater has a low probability of being truly normal and a sweat chloride level between 30-59 mmol/L is likely to be diagnostic for CF. Therefore, children with borderline sweat chloride values need to follow up, with a repeated sweat test and an extended analysis of the CFTR mutation.

Sweat electrolyte-phenotype correlation
The correlation between sweat electrolytes and genotype was initially explored by Witt and colleagues. Subsequently, with a better classification of various gene mutations, chloride values in sweat were correlated with certain groups of mutations [77].
No differences in sweat chloride values were found between patients carrying class I, class II, and class III mutations. The only statistical difference was seen in class IV and V mutations, which had a significantly lower mean sweat chloride level than patients homozygous for ΔF508 and closer to normal than class I, II, and III.
Some mutations associated with normal or borderline sweat electrolytes are R117H, D1152H, A455E, G551S, and 2789+ 5G. [77] An interesting phenotype, presenting with an elevated sweat chloride concentration in the absence of other CF symptoms, was described in a patient with a nonsense mutation, S1455X. On detailed clinical evaluation, this patient had normal lung function, normal bacterial flora on sputum examination, no manifestation of exocrine pancreatic disease, and serum IRT not indicative of CF. I CFTR mRNA transcripts bearing the S1455X mutation were normally processed and functional, suggesting therefore that the truncated C-terminal amino acid plays a key role only at the level of sweat glands.

Pitfalls in Sweat Testing

Most errors related to sweat testing are caused using an unreliable methodology, inadequate collection of sweat, technical errors, and occasionally misinterpretation of results. The technical aspects of performing a sweat test are challenging, and these errors occur most often in laboratories that do relatively few tests, usually not following published guidelines [45].

False positives in sweat testing

Approximately 98% of CF patients have sweat chloride concentrations greater than 60 mmol/L [77]. There are a variety of well-described, albeit rare, conditions associated with the elevation of sweat electrolytes [47, 78]. These conditions are generally easy to differentiate from CF and should not be considered true false positives. They include atopic eczema, untreated Addison's disease, ectodermal dysplasia, some types of glycogen storage disease, and untreated hypothyroidism. [11,17,78]

Sweat electrolytes measured within the first 24 hours after birth may also be transiently elevated. Up to 25% of normal infants show a chlorine concentration in sweat greater than 65 mmol/L on day 1, but it decreases rapidly on the second day after birth [20].

A sweat chloride concentration above 160 mmol / L is physiologically implausible and suggests laboratory error or Munchausen syndrome. Technical errors such as evaporation and contamination, except for failure to dilute the sample, tend to produce false-positive values [16].

False negatives in sweat testing

The noblest cause of a false-negative sweat test is edema [68, 78]. Edema is commonly seen in infants with hypoproteinemia, which may be secondary to exocrine pancreatic insufficiency, before diagnosis and treatment with enzyme pancreatic replacement.

The use of mineralocorticoids may also reduce electrolyte concentrations in sweat. From a technical point of view, the rate of sweating is important for accurate results because electrolyte concentration in sweat is related to the rate of sweat. At low sweat levels, the electrolyte concentration in sweat decreases, and the opportunity for evaporation of the sample increases. The average sweat velocity should exceed 1 g/m2/min. Insufficient samples may be due to several factors such as age, skin condition, hydration status, and collection system.

As a rule, sweat tests can be reliable if performed after 2 weeks of age in infants weighing more than 2 kg, and occasionally sweat testing can be attempted in term infants after 7 days of age. Collection systems vary; however, an error rate of 0.7% has been found when sweat is collected on gauze or filter paper compared with a failure rate of 6.1% associated with the use of Wescor Macroduct coils [47, 78].

A sweat test performed in a reliable laboratory is still a good measure of CFTR function in most cases. Mutation analysis informs us about the gene, but not about the gene product (mRNA or protein) or its function and is often a less useful diagnostic tool when the diagnosis is doubtful.

Therapy

Cystic fibrosis is usually identified after neonatal screening or during the first few years of life [78]. Individuals diagnosed after the age of 20 years usually have a mutation associated with residual CFTR function as Arg117His (also known as R117H) [79, 80]. These individuals may have mild respiratory symptoms in childhood and develop

bronchiectasis, pancreatitis, or present with infertility later in life. Diagnosis is made with a sweat test and DNA analysis [81,82].

Late-diagnosed individuals have good survival, reflecting the higher prevalence of mutations associated with residual function and a less severe phenotype [83,84]. The main goals of CF therapy are to promote clearance of secretions, control pulmonary infections, provide adequate nutrition, and prevent bowel obstruction. In the future, therapies that restore mutant CFTR processing or gene therapy may be the treatments of choice.

Treatment of pulmonary disease in cystic fibrosis is critical for clinical management [85,8]. Airway clearance is almost universally taught to the parents of infants diagnosed with cystic fibrosis and is encouraged throughout the life of the individual [86,87].

Oral antibiotics are used prophylactically in some countries to prevent S. aureus infection, despite concerns of an increased risk of P. aeruginosa infection [88]. In children, repeat infections are treated with oral antibiotics directed against S. aureus and H. influenzae. In case of a positive culture

of sputum for P aeruginosa or other Gram-negative microorganisms, antibiotic treatment with the goal of eradication will be required [88]. A regimen of oral ciprofloxacin and inhaled colistin for 3 months is effective for eradication of these bacteria [89]. Administration of inhaled tobramycin for 1 month may be equally effective in eradicating P. aeruginosa [90,91]. Both regimens are recommended as an eradication antibiotic therapy. Ensuring eradication of P. aeruginosa is an important priority in cystic fibrosis care, and many pediatric centers report a prevalence of chronic P aeruginosa infection of less than 10% in their clinics [92].

People with cystic fibrosis experience recurrent pulmonary exacerbations.

A range of therapies reduce the frequency of pulmonary exacerbations: Dornase alfa was the first treatment indicated to reduce exacerbations; subsequently, inhaled antibiotics, tobramycin, colistin and aztreonam, levofloxacin inhalation, hypertonic saline, mannitol, and oral azithromycin have also been shown to reduce exacerbations. These therapies are included in CF treatment guidelines worldwide [93,94] and generally result in a modest improvement in FEV1 of 3-5% and a substantial reduction in the frequency of pulmonary flare-ups.

Patients chronically infected with P. aeruginosa or other Gram-negative bacteria usually require treatment with intravenous antibiotics. This treatment usually consists of extended-acting penicillin, third-generation cephalosporin, or carbapenem in combination with an aminoglycoside or polymyxin [92]. Treatment is recommended for 14 days, although there is no solid evidence to support this duration of treatment [92]. Some people become extremely ill during these episodes and require support with nutrition and supplemental oxygen, and on some occasions may require noninvasive ventilatory support. Effective treatment of exacerbations is important, as approximately 25% of exacerbations do not resolve, and increased frequency of exacerbations is associated with a more pronounced decline in lung function, reduced quality of life, and poor overall survival [93,94].

Epithelial cells in the pancreatic and bile ducts are also affected by CFTR dysfunction [95], resulting in chronic obstructive pancreatitis in some patients. In contrast, individuals with class IV, V, or VI mutations have a normal pancreatic function at birth, although some will develop pancreatic insufficiency later in life [96,97]. Pancreatic insufficiency can be treated with replacement therapy by taking pancreatic enzymes [98,99].

The increased metabolism in affected patients is caused by a combination of factors including the underlying genetic mutation and the metabolic consequences of chronic infection [100].

Optimal nutrition management requires increased energy intake in addition to pancreatic enzyme replacement therapy [101]. Cystic fibrosis is also associated with biliary cirrhosis, although this occurs in less than 10% of patients.[102] Most commonly, people with cystic fibrosis have abnormally altered liver function tests. A small proportion of such patients develop cirrhosis or portal hypertension and, when this occurs, should be considered for liver transplantation.

The development of cystic fibrosis-related diabetes mellitus is a growing problem, occurring in more than 40% of adults, and is associated with survival risks, particularly in women [103]. Treatment is usually insulin-based; the setting of a high-energy diet is also particularly important [104]. Complications of cystic fibrosis-related diabetes are often evident and manifest with macrovascular and microvascular disease [104]. Intravenous aminoglycoside use and diabetes contribute to renal dysfunction in people with cystic fibrosis.

Osteopenia is common in affected patients due to pubertal delay, consequences of CFTR mutation, chronic inflammation, and inactivity [105]. The risk of osteopenia should be factored into the recommended diet and lifestyle, which should encourage good calcium intake and exercise with supplementation adequate vitamin D supplementation. If osteoporosis develops, therapy with

Bisphosphonates is recommended [106].

Renal dysfunction is a growing problem in patients with cystic fibrosis and is commonly caused using *intravenous aminoglycosides; in particular, gentamicin has been associated with acute and chronic* renal failure.

The progressive effects of respiratory tract infection and inflammation lead to extensive bronchiectasis and bronchiolitis obliterans, which inevitably result in respiratory failure. All patients with cystic fibrosis who develop respiratory failure should benefit from lung transplantation in case of a decline in lung function and frequent exacerbations [107].

Lung transplantation is increasingly successful with 60-70% 5-year survival [108]. Unfortunately, in countries where organ availability is less than the number of potential recipients, patients may die while waiting for a transplant.

Management of the end-stage of the disease is especially important for the individual, family, and friends. Whenever possible, an appropriate family or partner should be involved in the decision-making process about how the last period of life is managed [109].

Innovative Therapies

The recent development of drugs that correct the underlying defect in CFTR function has substantially improved the life expectancy of affected patients. Prevention of lung disease with therapies that correct CFTR function can be effectively undertaken as early as possible during diagnosis by neonatal screening.

Two different therapeutic approaches have been developed: the first is the use of small molecules that can modulate the CFTR protein and restore functional ion transport; the second approach is the use of molecular or gene therapy to correct the mutation, either at the RNA or DNA level and produce a normal CFTR protein that corrects the mutated CFTR dysfunction by bypassing it.

The first small molecule synthesized that is effective is ivacaftor [110].

Ivacaftor corrects CFTR-mediated chloride transport in most of the class III mutations, class IV mutations, and some other mutations that have CFTR residual function [111]. A significant efficacy of ivacaftor in class III mutations, particularly in patients with Gly551Asp Mutation (also known as G551D). Treatment with ivacaftor improved lung function (FEV1) by approximately 10%, reduced concentrations of chloride in sweat to

concentrations of approximately 60 mmol/L, improved patients' quality of life and reduced the frequency of pulmonary flare-ups.[122] This drug has also been tested in patients with class IV mutations, particularly those with the Arg117His mutation; in patients with this mutation, there is no significant effect on lung function, although ivacaftor reduced the concentration of sweat chloride by about 25 mmol/L.
The second pharmacological strategy targets patients who are homozygous for the DF508 mutation and is derived from a combination of a correcting drug that can restore CFTR trafficking and an enhancing drug that makes it functional. The combination of lumacaftor and ivacaftor improves FEV1 and reduces the frequency of pulmonary flare-ups [113]. In addition, treated patients show modest improvement in quality-of-life scores. Approximately 10% of patients reported chest oppression with associated bronchoconstriction. However, this effect seems to have stabilized after a few weeks of treatment. The improvement in FEV1 after treatment with lumacaftor and ivacaftor appears to be lower than that observed after treatment with ivacaftor in patients with Gly551Asp mutation (3% vs 10%); however, the effect on the frequency of pulmonary flare-ups was similar (30% reduction vs 40% reduction). Chloride concentrations in sweat showed a modest reduction, of approximately 9 mmol/L, after combination treatment with lumacaftor and ivacaftor. Overall, CFTR function can be treated with small molecules.
Another corrective drug such as VX-661 (Vertex Pharmaceuticals, Boston) has been used but was seen to interfere with the action of ivacaftor, thus reducing the functional effects on CFTR. An additional pharmacological approach was developed for class I mutations. Ataluren is a small molecule, designed by PTC Therapeutics, capable of making ribosomes less sensitive to premature stop codons [114]. This drug also indicates muscular dystrophies. In phase 2 studies, this drug showed electrophysiological correction of CFTR function and some improvement in FEV1 of CF patients.
Since the cloning of the CFTR gene, there is a great expectation of gene and molecular therapies. Some studies have shown that CFTR function can be restored in the nose of people with CF. Recently, the first major study on restoring CFTR function in the lung has been reported, which showed some effects on important clinical parameters of FEV1 and some measures on CT [115].
An oligonucleotide drug, PQR-010 (QR-010, ProQR, Leiden, The Netherlands), has been developed to repair the genetic defect in RNA and is currently in clinical trials.
Recently, experimental gene therapy 4D-710 consisting of an adeno-associated viral vector (AAV), called A101, which is used to transport a functional copy of the CFTR gene into cells. The CFTR gene is too large to be contained in the A101 viral vector, so the scientists have developed a smaller version - called micro CFTR - without sacrificing any of the "instructions" needed to generate a functioning CFTR protein. 4D-710 potentially targets many patients, regardless of the specific CFTR-damaging mutation.
One of the apparatuses most affected in patients with cystic fibrosis is the respiratory system, so the researchers thought of delivering their therapeutic construct to the cells via aerosol. No needles and IVs, just a nebulized solution of particles (some are working on other fronts of advanced therapies) that would allow the expression of CFTR in all epithelial cells of lung tissue.

Mass Spectrometry

Mass spectrometry is an indispensable analytical tool, considered the 'analytical gold standard' in chemistry, biochemistry, pharmacy, toxicology, medicine, and many fields of science.

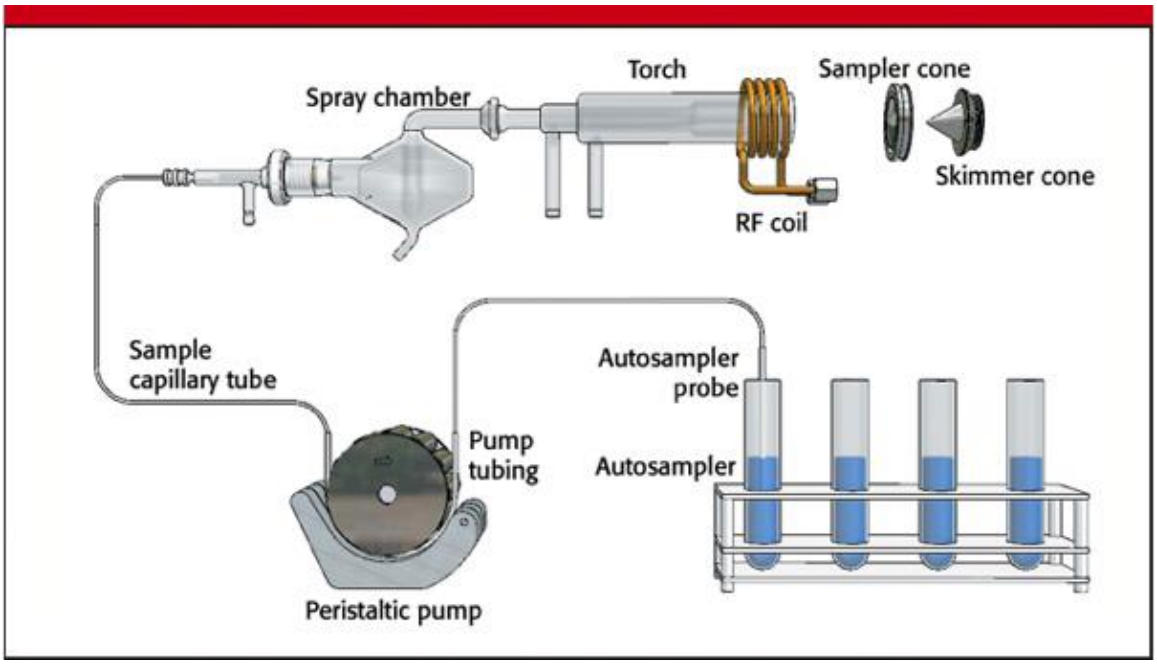
Mass spectrometry is used to analyze combinatorial databases, sequence biomolecules, to help explore single cells or objects from space. Everyone heavily relies on mass spectrometry for structural information of unknown compounds, in environmental and forensic analytics, for the quality control of drugs, foods, and polymers [116].
The basic principle of mass spectrometry is to generate ions from both inorganic and organic compounds, using any suitable method to separate ions based on their mass-to-charge (m/z) ratio and detect them qualitatively and quantitatively based on their respective m/z and abundance values.
The analyte can be ionized thermally, by electric fields, or by interaction with energetic electrons, ions, or photons at various wavelengths [117].
This technique is primarily due to the ingenuity and work of physicist J. J. Thomson (1847-1940), who is credited not only with the discovery of the electron but also with the measurement of the masses of stable isotopes of elements. In his mass spectrometer Thomson generated ions through electronic ionization he studied the trajectories under the influence of magnetic fields and/or electrical and measured the mass-to-charge ratio (m/z).
From the time of Thomson onward, many types of mass spectrometers have been designed, increasingly sophisticated, and using strategies and applications have flourished, including in the field of biomedicine [118].
Despite continuous technological advances, all mass spectrometers are structured according to a general scheme, involving an ion source, a mass analyzer, and a detector, the latter operating under high vacuum conditions.
In any case, the analysis in mass spectrometry involves a phase of sample preparation, which must precede the introduction of the same in the instrument, compatible with the platform used, specific for the type of analyte and matrix (an aqueous or organic phase), and compatible with the type of ionization used.
Only through proper preparation of the sample, it is possible to produce ions with high efficiency (high sensitivity) that, through a series of lenses, are conveyed to the analyzers, where they can be carefully selected based on their sensitivity. mass-to-charge ratio and detected.
The fundamental steps of an experiment in mass spectrometry are, therefore:
- ionization of the analytes: within the ion source all chemical species, among those present in the sample, can be ionized under the conditions in which Ionization of the analytes: within the ion source all chemical species, among those present in the sample, that can be ionized under the conditions in which the source itself operates are transformed into ions in the gas phase.
- separation of ions based on mass-to-charge ratio: ions in the gas phase are focused by a series of lenses and then separated in the analyzer(s) based on their mass-to-charge ratio, thanks to the action of electric fields and radio frequencies.
- selective detection of the various ions: the ions, after being separated in the analyzer(s), are selectively sent to the detector, where the ion signal of each of them is quantitatively converted into an electrical signal that can be recorded by the instrument management software.
Although mass spectrometry consumes analyte, it can be considered non-destructive, because the amount of analyte used is usually on the order of a few micro- or nano-grams. This makes mass spectrometry the method of choice when other analytical techniques fail due to poor sample availability.

Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) has as its peculiarity an inductively coupled plasma ionization source, which is particularly suitable for the ionization of inorganic species. In practice, this source operates through a plasma fed by argon in the presence of radiofrequency and operating under conditions of atmospheric pressure, which first performs the atomization of molecular species contained in the sample and then the subsequent ionization of the elementary species thus obtained.
The heart of the ICP source can be identified in the "plasma flashlight", which consists of three coaxial quartz tubes inserted along the central axis of a water-cooled radiofrequency coil. The plasma is fed by a flow of argon of about 12-20 L min 1, introduced into the flashlight through the intermediate tube, which is "ignited" by an electric discharge, produced by an electric arc, able to generate electrons and Ar+ ions. The electrons then interact with the electromagnetic field generated by the
radiofrequency produced by the induction coil and are accelerated reaching the energy necessary to feed the ionization of neutral argon to Ar+ through a mechanism of ionization similar to that of electronic ionization. The association of part of the electrons and Ar+ ions generated the high-temperature plasma rich in electrons and Ar+ ions, which are produced continuously, able to atomize the molecules and ionize the atoms so produced. Argon also performs other functions: it cools the walls of the flashlight, flows into the outermost quartz tube (auxiliary flow) and introduces the sample, previously nebulized in a special nebulizer, through the central tube (car flow).

Figure. 8. ICP-MS 7900 Agilent Technologies

Figure 9. Schematic of an ICP-MS sample introduction system



Both flow at approximately 1-2 l min 1. The ICP source reaches remarkably elevated temperatures, namely close to 10000 K in the induction zone near the coil, and about 8000 K in the center of the plasma, where the processes of evaporation and atomization of the sample take place.

As the plasma flows away from the coil, excitation of the neutral species occurs at about 7500 K followed by ionization in a zone well beyond the coil located at about 6500 K.

Ion transfer into the mass analyzer is achieved by differential pumping. A small portion of the plasma enters the first stage of pumping through a hole located in the center of the sampling cone, which is necessarily cooled with water to avoid rapid erosion of its surface by the hot plasma. The ions are then guided through the skimmer inlet by the application of a suitable potential difference and conveyed to the analyzer.

The ions are then guided through the skimmer inlet by the application of a suitable potential difference and conveyed to the analyzer, often a quadrupole analyzer, where they are separated based on the mass-to-charge ratio. Most neutral species, on the other hand, are pumped out by the supersonic expansion created in this region and ejected from the mass spectrometer.

RESEARCH PROJECT

The analysis of chloride in sweat, allowing us to evaluate the function of the transporter CFTR is essential for diagnosing CF.
The method developed by Gibson and Cooke remains the gold standard method for the collection of sweat, although the Wescor Macroduct system is a viable alternative, you are spreading rapidly. The analytical methods usually used for the assay of chloride ions in sweat samples collected with both techniques are not particularly effective, because they do not guarantee good accuracy and high sensitivity of the analysis. In fact, in most cases, it is about methods analytical developed for the analysis of other types of matrices and adapted to the sweat test a second time, but not suitable for guaranteeing adequate detection limits. Among these can be mentioned analytical methods based on colorimetry, coulometry, la flame photometry and conductivity, usually associated with the Wescor system, which are in any case provided for by the guidelines in force. They are generated by serum-based procedures, where there is often no specific calibration point capable of measuring low concentrations of chloride such as those normally found in subjects' sweat normal. To overcome this limit, the calibration curve of Cl- was trivially extrapolated to adapt it to the lower concentrations of chloride observed in "normal" sweat (≤ 29 mmol / L), with consequent distortions of the curve [16].
However, if we are interested in an early diagnosis and timely treatment of fibrosis cystic, we must have accurate and sensitive methods that can classify correctly the patient based on the reference ranges of chloride ion concentration.
Although this technique has only recently been introduced in the biomedical field [119], it is today
considered mature for this use as it has been commercially available since 1983 when it began its use for geological studies of metals and the analysis of isotopes.
The advantages of this technology are many:
- a low volume of material is required for the analysis.
- multiple evaluations can be carried out on the sample to improve accuracy.
- the inclusion of an internal standard (IS) is envisaged.
- the sensitivity range at the parts per trillion level.

The analysis of sweat chloride using ICP-MS was introduced in 2008 and currently, only a few laboratories use this approach, mainly due to the scarce availability of equipment of this type in hospital laboratories and the lack of operators with sufficient experience in the field of mass spectrometry.
From a practical point of view, the ICP-MS offers redundancy of information, because, although usually, the chloride ion is measured only in the form of 35Cl (atomic weight 35 g / mol), theoretically it is also possible to simultaneously dose 37Cl (37 g / mol), which it can be used to confirm the result with undoubted advantages in terms of quality of the analytical data. As with other analytical techniques, the assay of these ions in ICP-MS could potentially be affected by the interference of isobar ions present in the matrix or produced during the ionization process, with consequent overestimation of the concentration. Among these potential interferers can be polyatomic ions such as FO (35 and 37 g / mol), HAr (37 g / mol), HCl (37 g / mol), NeO (37 g / mol) are included. However, they do not represent a substantial problem in ICP-MS, because they can be easily differentiated from the ions of interest, by varying their speed of movement in the spectrometer due to the interaction with helium gas, which alters the kinetic energy in a manner proportional to their cross-section. Briefly, through this interaction the interferers, which have a distinct size than the analyte ion, reach the analyzer at separate times than the latter and can therefore be separated from it.

The objective of our work, given the problems listed above and the available technologies, is to develop and validate a sensitive and specific analytical method that can be used on a routine basis. In principle, one of the main difficulties can be attributed to the amount of chlorine within the sweat matrix.
Moreover, in the case of sweat samples, the collected matrix differs not only from one patient but between different age groups and probably between different ethnicities as well.
The composition of an infant's sweat is different from that of a child, and both are different, even in terms of quantity, from that of an adolescent or adult individual. In term infants, for example, chlorine content can be high in the first seven days of life, with extremely large peaks in the first hours of life. In addition, electrolytes in sweat may be elevated in underweight or dehydrated infants, whereas they are reduced in infants treated with corticosteroids or in the presence of edema.
Bearing in mind that all these variables can significantly affect the accuracy of the measurement, it is necessary to find an approach that can bypass the problems related to the variability of the composition of the matrix and the quantity of the sample, and certainly, the ICP-MS technology offers us this possibility.

Materials and methods

The procedure used in our work to analyze patient-derived samples who have undergone the test consists of two phases:

1. Pre-analytical phase, which consists in the preparation of the calibrators, the "quality controls" and sweat samples collected with the Gibson and Cooke technique.
2. Analysis of the samples, carried out by means of the ICP-MS.

Reagents and materials

NaCl, TraceCERT®-grade Chlorine Standard for IC in water, TraceCERT®-grade Gallium Standard for ICP, HNO_3 (69% w/w), and NH_4OH were from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was produced by a DIA® M deionizer from Quality Invents S.r.l. (Arluno, Italy), Argon ALPHAGAZ 1 grade (99.999% pure) was from Air Liquide (Milan, Italy), and Helium 5.6 purity grade (99.9996%) was from SOL (Monza, Italy). Three sweat quality controls with nominal concentrations of chlorine in the range 22.6–30.9 mM, 38.2–49.4 mM, and 78.8–102.4 mM were from LTA S.r.l (Milan, Italy); two ISE quality controls with nominal concentrations of 80 mM and 120 mM were from Roche Diagnostics GmbH (Basel, Switzerland). Whatman (Maidstone, UK) provided filter paper grade 40, 8 μm, used for sweat collection.

Instrumentation

The chlorine quantifications based on the off-line internal standard technique were performed by an Agilent Technologies 7900 ICP-MS (Santa Clara, CA, USA), equipped with an ASX-500 Series autosampler and a peristaltic pump for sample injection. The analyses were preceded by autotuning, which is an automated software routine that can optimize instrumental parameters to obtain the maximum level of sensitivity correlated with the maximum performance in terms of polyatomic interferences removal, and the acquisitions made use of the parameters reported in Table 2. System control and data

acquisition and processing were carried out by the MassHunter 4.2 Workstation® version C.01.02 software.

Table2. ICP-MS operative parameters

Parameter	Value
Carrier gas flow rate	0.89 L/min (Ar)
Aerosol dilution flow rate	0.28 L/min (Ar)
Plasma gas flow rate	15 L/min (Ar)
Collision gas flow rate	4.3E−3 L/min (He)
RF power	1600 W
Stabilization time	20 s
Peak pattern	3 points
Replicates	10
Integration time for ^{35}Cl	1 s
Integration time for ^{71}Ga	0.2 s
Sweeps per replicate	100
Peristaltic pump speed	0.1 rps

Study population

Sweat samples from 50 healthy volunteers, aged 20–65 years (36.84 ± 12.34), and two individuals with suspected CF were analyzed. Patient 1 was a 24-year-old male with a history of chronic rhinitis and recurrent wheezing with a wet cough from early childhood. Traditional sweat testing in the first year of life showed borderline values, i.e., 45 mM, 32 mM, and 36 mM when he was 1 month, 2 months, and 7 months old, respectively. When he was 10-years old, a computed tomography (CT) scan showed bronchiectasis in the middle and lower lobes and, more recently, genetic testing for CFTR mutations revealed a common heterozygous ΔF508 mutation (c.1521_1523delCTT, p. Phe508del). When he was 23 years old, a diagnosis of pansinusitis with nasal polyps was made by CT scan. Patient 2 was a 56-year-old woman with a history of chronic wet cough and recurrent fever, which started when she was fifty-five, after an acute respiratory infection. A CT scan performed 6 months after symptoms onset showed bronchiectasis in the middle lobe and multiple consolidations in the right upper lobe and lingula.
Patient 3 is a 63-year-old man with a history of acute pancreatitis at age 1-year, recurrent pulmonary infections, and altered fecal content with emission of greasy stools.
There are no analytical models in the literature that address the quantification of chlorine concentration even regarding the various age groups of subjects.

Sample collection

Sweat samples were collected by the pilocarpine iontophoresis test (QPIT) [12]. Briefly, after cleaning the skin of the forearm with deionized water and drying it with gauze, silver electrodes were placed, covered by a gauze soaked in 0.5% pilocarpine nitrate solution. Iontophoresis was performed by supplying a 4 mA current for 5 min After cleaning again the skin with deionized water, a pre-weighed filter paper sheet (4×3 cm, 95 g/m^2) was placed onto the iontophoresis skin area, covered with Parafilm M (Bemis Company, Inc., Neenah, WI, USA), and kept in position for 30 min The moist filter paper was removed from the skin and secured in a pre-weighed 50-mL tube with a tightly fitting lid, and weighed again. Sweat samples weighing 75 to 300 mg were stored for further analysis.

Preparation of calibrators and QCs

The NaCl stock solution was prepared at the concentration of 280.6 mM in deionized water. It was stable for at least 1 month when stored at room temperature in a polyethylene bottle. The IS stock solution containing Ga at the concentration of 28.7E−3 mM was prepared by adding 4 μL of TraceCERT® solution (1 mg/mL) to 1996 μL of deionized water. Such solution was stable for at least 1 month at room temperature, too. The calibration curve of Cl was built by six standard solutions, each of them prepared by parallel dilution with a deionized water solution of ammonium hydroxide, this latter having a final concentration of approximately 0.25% (w/w): 8.5E−3 mM (L1), 17.0E−3 mM (L2), 34.0E−3 mM (L3), 68.0E−3 mM (L4), 136.0E−3 mM (L5), 272.0E−3 mM (L6). Each calibrator contained IS at the concentration of 1.74E−5 mM. The calibrators were obtained according to a dilution procedure based on three consecutive steps. *Step 1* is as follows: 250 μL, 500 μL, 1000 μL, 2000 μL, 4000 μL, and 8000 μL of NaCl stock solution were all diluted with deionized water up to a final volume of 10 mL. *Step 2* is as follows: 200 μL of the solutions from *step 1*, 100 μL of the IS stock solution, and 3000 μL of deionized water were put into a 50-mL tube containing a 4 × 3 cm filter paper sheet, equivalent to that used for the sampling by Gibson and Cooke method, and the tube was then submitted to a 40-min stirring in a VDRL 711/D+ orbital stirrer (Asal, Cernusco Sul Naviglio, Italy), at 150 rpm. Then, a filter paper was squeezed by disposable plastic forceps and removed from the tube. *Step 3* is as follows: 200 μL of the solutions from *step 2* were added with 9800 μL of a freshly prepared ammonium hydroxide 0.25% (w/w), thus obtaining the abovementioned calibrators. Six quality controls were prepared according to *steps 2* and *3* of the preparation procedure used for calibrators. Three of them were prepared at the operative concentration levels in the range 27.4E−3 to 37.5E−3 mM (QC1), 46.3E−3 to 59.9E−3 mM (QC2), and 95.5E−3 to 124.1E−3 mM (QC3), from the LTA sweat quality controls with nominal concentrations of chlorine in the range 22.6–30.9 mM (QC1′), 38.2–49.4 mM (QC2′), and 78.8–102.4 mM (QC3′), respectively. By the same procedure, two more quality controls at the operative concentration levels of 97.0E−3 mM (ISE1) and 145.5E−3 mM (ISE2) were prepared from the Roche commercial quality controls with nominal concentrations of 80 mM and 120 mM, respectively, and one more low-level quality control, CS, was prepared at the concentration of 34.2E−3 mM, from a certificated chlorine solution with a nominal concentration of 28.2 mM.

Sample preparation

Sweat samples were prepared according to *steps 2* and *3* of the calibrators' preparation procedure, except for the amounts of IS stock solution and deionized water in *step 2*, which were proportional to the weight of collected sweat. In practice, considering a sweat density of 1 g/mL, a volume of IS stock solution and a volume of deionized water respectively corresponding to 50% and 1500% of the sweat volume were put into the 50-mL tube containing the moist filter (e.g., when 100 mg of sweat was collected, 50 μL of IS stock solution and 1500 μL of deionized water were necessary). A total of 1000 μL of the final solution was placed into the autosampler for the analysis.

Chloride quantification

As described above, samples were prepared using the same preparation procedure of calibrators and quality controls, submitting all of them to the same dilution factor, whose total value was 825. Hence, although the calibration curve was built by calibrators L1 ÷ L6 (8.5 ÷ 272.0E−3 mM), the concentration values attributed to them in the quantitative section of the MassHunter 4.2 Workstation® software were those of L1′ ÷ L6′ (7.0 ÷ 224.5 mM). This artifice avoided tedious calculations involving the sample dilution factors of both *steps 2* and *3* and allowed getting concentration values of chlorine immediately, in both samples and quality controls.
In case of Cl concentration greater than 40 mM, sweat crystallization test was performed as a confirmation test [119, 120].
Briefly, the test consisted of the observation by a polarized light optic microscope, of a droplet of sweat, placed on a slide and dried at room temperature. The test was considered negative when the obtained crystals showed a regular cuboid form and positive when exhibited a dendritic form or a fern pattern [120], with a variable score from 1 (+) through 4 (+) [121]:

- (+) crystals of geometric shape distributed uniformly over the entire field,
- (++) geometrically shaped crystals, in the shape of a cross or sword, scattered thickly over the entire field.
- (+++) like the previous one, with the addition of images of arborizations of branches of fir or fern, but only in the periphery, leaving the center of the preparation free.
- (++++) arborization images, such as fir branch or fern leaf, are evenly and uniformly distributed throughout the preparation, both in the periphery and in the center.

Genetic testing by next-generation sequencing was also conducted on positive patients.

Method validation

To ensure data reliability, reproducibility, and robustness, a detailed validation of the analytical procedure was performed, concerning selectivity, accuracy, precision, sensitivity, and stability, in compliance with EMA guidelines [122].
Selectivity is defined as the ability of an analytical method to measure and differentiate the analyte and the IS from other components in the sample, behaving as potential interfering compounds, which affect the accuracy of the measurements, mainly when measuring low levels. It was tested by evaluating the method performances with the main stable isotopes for Cl, i.e., ^{35}Cl and ^{37}Cl, and with some stable isotopes suitable as an IS, such as ^{69}Ga and ^{71}Ga, ^{45}Sc, ^{72}Ge, and ^{103}Rh [117].
Linearity of an analytical method is its capability to obtain results directly proportional to the concentrations of the analyte in the sample within a definite range, said field of validity.
This linearity must be verified by calculating the correlation coefficient of the calibration curve. Calibration curves carried out on different matrices or for standard solutions can be used after specifying the reasons for the test method. Generally, correlation coefficients are accepted if equal or greater than 0.99.

A significant non-linearity should be corrected by using functions of non-linear calibration or by restricting the range of concentrations in which it operates an experimental level.
Three to six replicates of four or more concentrations should be made. An acceptable method validation report should contain the following:

- correlation coefficient
- intercept
- residues
- graph of the experimental data and of the calculated curve.

It was evaluated by monitoring ^{35}Cl as an analyte and ^{71}Ga as an IS because they offered the best performances in terms of signal stability, signal-to-noise ratio, and precision of the measurements. The linearity of Cl, namely ^{35}Cl, was assessed within the calibration curve range, i.e., from 8.5E−3 mM through 272.0E−3 mM effective concentration.
Precision represents the closeness among repeated individual measurements of the analyte, and it is expressed as percent relative standard deviation (RSD%).
It can also be described as the level of agreement between independent measurements of the concentration of a particular analyte. A strict control of the whole analysis process, including the collection of the sample, its conservation, the preparation (extraction and purification) and finally instrumental analysis should be carried out.
Control materials for various concentration levels allow to evaluate the matrix effect; the concentration of the materials is chosen to cover the entire measurement range including a threshold or clinical decision value.
Precision should be evaluated for a minimum of three concentration levels (low, medium, high), with at least ten independent measurements for each level of concentration and is expressed as a percentage coefficient of variation:

$$\text{CV (coefficient of variation) \%} = \textit{SD of mean} * 100 / \textit{Mean}$$

Precision must be assessed both in the analytic session and between different analytic sessions as an evaluation of the accuracy over time. A recommendation is to use different operators, calibrators, reagents within an arc time that does not exceed thirty days in order to minimize the effects due to other variables in the experimental conditions.
Accuracy of an analytical method is the closeness of the experimental value to the nominal concentration of the analyte, expressed in percentage (accuracy %).
The accuracy depends on several factors: the accuracy of the choice of materials calibration, their concentration, and the choice of the matrix. The accuracy should be evaluated by analyzing for at least five replicated measurements in three different analytical runs for concentration, using at least three samples and a clinically relevant concentrations enough to cover the entire calibration range of the method.
Intra- and inter-day accuracy and precision were evaluated by five-replicate injection and three-replicate analyses on three different days, respectively, of QC1, QC2, QC3, CS, ISE1, and ISE2.
Limits of blank (LOB), *detection* (LOD), and *quantitation (*LOQ) of the analytical method were also achieved [23]. LOB is defined as the highest apparent analyte concentration expected to be found when replicates of a blank sample, containing no analyte, are evaluated. LOD is the lowest analyte concentration distinguishable from LOB, and LOQ is the lowest concentration at which the analyte can be reliably quantified, which usually is ≥ LOD. LOB and LOD were calculated by using the following equations: $LOB = \text{mean}_{blank} + 1.645(SD_{blank})$, $LOD = LOB + 1.645(SD_{\text{low concentration sample}})$, where

mean $_{blank}$ and SD $_{blank}$ were estimated by measuring 15 replicates of a blank sample and calculating the mean result and the standard deviation, respectively, and SD $_{low\ concentration\ sample}$ was assessed by measuring 15 replicates of lowest calibrator [117, 123, 124]. LOQ was assessed at a concentration level above that of LOD and with a reliable and accurate signal. Its concentration value was then assigned to the lowest level (7.0 mM) calibrator.
The *stability* study is an important part of the validation and is carried out to ensure correct management of all reagents and samples during the delicate analysis process. It involves the evaluation of all solutions, reagents, and products samples in the various phases of the experimental process:
- Sample collection (sampling)
- Short-term storage of samples
- Long-term storage of samples
- Freezing / thawing cycles
- Storage after processing / extraction (before analysis).

The parameters to be assessed should cover the normal times of the procedures sampling and analysis.
The essential conditions to evaluated are stability at different storage times and different temperatures, stability to light (direct and indirect), stability to repeated cycles of freezing and thawing. To ensure adequate sample stability, the weighed samples were stored for up to 72 h at refrigerator temperature (4 °C) before the analysis, as indicated in the CLSI guidelines [125]. However, experimental data in the literature demonstrated that chlorine can be measured within 5 days from the collection, without a significant affection on the reliability of results, irrespective of storage conditions [125]. Anyway, the stability of QC1, QC2, QC3, CS, ISE1, and ISE2 was evaluated by measuring the concentration of chlorine in freshly prepared solutions and after 1 and 7 days of storage at refrigerator temperature.

RESULTS

The analytical method

The analytical method was designed to be simple and time- and cost-effective, to make it suitable for routine chloride analysis. The preparation of both calibrators and quality controls by the same procedure used for samples, as well as the use of an internal standard, resulted crucial to achieving satisfactory results in the quantification of the samples; the high analytical sensitivity of the method allows analyzing samples containing less than 75 mg of sweat, which are often collected in newborns and infants.

Method validation

Selectivity

The main interferents in ICP-MS are ions having the same *m*/*z* of either the analyte or IS. According to the relative abundance of the natural isotopes, the monitored isotopes ^{35}Cl, ^{37}Cl, ^{69}Ga ^{71}Ga, ^{45}Sc, ^{72}Ge, and ^{103}Rh do not suffer from significant singly charged monoatomic interferences. Several polyatomic interfering compounds might affect the detection; those more frequently described [126] consist of precursors from different sources (Table 3) such as the sample matrix, solvents, plasma gases, and atmospheric gases. Agilent 7900 ICP-MS is equipped with a collision cell that, when filled with a non-reactive gas, such as helium, exerts kinetic energy discrimination (KED) and selectively attenuates the contributions of the polyatomic interferences [127]. Polyatomic ions are larger than both analyte and IS ions of the same mass and passing through the cell, they collide more frequently with inert gas molecules and emerge from the cell with lower kinetic energy for the ions of interest and can be selectively excluded from the ion beam by applying a suitable bias voltage at the cell exit. In contrast, the use of KED causes also significant analyte and IS sensitivity losses, which were not critical for our method. Also, doubly charged monoatomic ions could act as interferents (Table 3). Their cross-section usually is comparable with that of the analytes, but their greater masses provide them with higher kinetic energies, which allows them to pass through the cell and reach the mass analyzer [128]. Thus, $^{70}Zn^{2+}$ and $^{70}Ge^{2+}$ could potentially interfere with ^{35}Cl and comparative tests between ^{35}Cl and ^{37}Cl ions suggested measuring as an analyte since it provided a better sensitivity, signal stability, and reproducibility for ^{37}Cl. However, the lowest second ionization potentials of Zn and Ge, respectively 17.96 eV and 15.93 eV, are higher than the first ionization potential of the plasma-forming gas Ar (15.76 eV), which is the limiting value of the second ionization potential. This makes the formation of $^{70}Zn^{2+}$ and $^{70}Ge^{2+}$ quite improbable [129]. The potential interferents of ^{71}Ga, which was the most suitable IS, were $^{142}Ce^{2+}$ and $^{142}Nd^{2+}$, whose formation is justified by the lowest second ionization potentials of Ce and Nd, respectively 10.85 eV and 10.73 eV. Theoretically, the interference of these ions could be avoided by using the ICP-MS-MS technology [130], which, anyway, for our purpose is not strictly necessary, since the concentration of these two rare earth elements in the injected samples usually is insignificant and affects little the signal of the IS, as proved by the method accuracy.

Table 3. Monocharged polyatomic ions, which could interfere with the isotopes of interest in ICP-MS

Isotope	***m/z***	**Interferences**
^{35}Cl	35	$^{16}O^{18}O^{1}H^{+}$, $^{34}S^{1}H^{+}$
^{37}Cl	37	$^{36}Ar^{1}H^{+}$, $^{36}S^{1}H^{+}$
^{45}Sc	45	$^{12}C^{16}O_2{}^{1}H^{+}$, $^{28}Si^{16}O^{1}H^{+}$, $^{29}Si^{16}O^{+}$, $^{14}N_2{}^{16}O^{1}H^{+}$, $^{13}C^{16}O_2{}^{+}$
^{69}Ga	69	$^{35}Cl^{16}O^{18}O^{+}$, $^{35}Cl^{17}O_2{}^{+}$, $^{37}Cl^{16}O_2{}^{+}$, $^{36}Ar^{33}S^{+}$, $^{33}S^{18}O_2{}^{+}$, $^{34}S^{17}O^{18}O^{+}$, $^{36}S^{16}O^{17}O^{+}$, $^{33}S^{36}S^{+}$
^{71}Ga	71	$^{35}Cl^{18}O2^{+}$, $^{37}Cl^{16}O^{18}O^{+}$, $^{37}Cl^{17}O_2{}^{+}$, $^{36}Ar^{35}Cl^{+}$, $^{36}S^{17}O^{18}O^{+}$, $^{38}Ar^{33}S^{+}$
^{72}Ge	72	$^{36}Ar_2{}^{+}$, $^{37}Cl^{17}O^{18}O^{+}$, $^{35}Cl^{37}Cl^{+}$, $^{36}S^{18}O_2{}^{+}$, $^{36}S_2{}^{+}$, $^{36}Ar^{36}S^{+}$, $^{56}Fe^{16}O^{+}$, $^{40}Ar^{16}O_2{}^{+}$, $^{40}Ca^{16}O_2{}^{+}$, $^{40}Ar^{32}S^{+}$
^{103}Rh	103	$^{40}Ar^{63}Cu^{+}$

Table 4. Doubly charged monoatomic ions, which could interfere with the isotopes of interest in ICP-MS

Isotope	***m/z***	**Interferences**
^{35}Cl	35	$^{70}Zn^{2+}$, $^{70}Ge^{2+}$
^{37}Cl	37	$^{74}Ge^{2+}$, $^{74}Se^{2+}$
^{45}Sc	45	$^{90}Zr^{2+}$
^{69}Ga	69	$^{138}Ba^{2+}$, $^{138}La^{2+}$, $^{138}Ce^{2+}$
^{71}Ga	71	$^{142}Ce^{2+}$, $^{142}Nd^{2+}$
^{72}Ge	72	$^{144}Nd^{2+}$, $^{144}Sm^{2+}$
^{103}Rh	103	$^{206}Pb^{2+}$

Linearity

The calibration curve used to quantify chloride in the samples showed good linearity in the range 7.1–224.5 mM, corresponding to concentrations in the range 8.5E−3 to 272.0E−3 mM effectively injected into the ICP-MS instrument, with a reproducible slope and a correlation coefficient (R) always greater than 0.999. The linear regression with no weighing provided a curve equation $y = mx + q$, with $m = 0.0187 \pm 0.0014$ and $q = 0.0689 \pm 0.0337$.

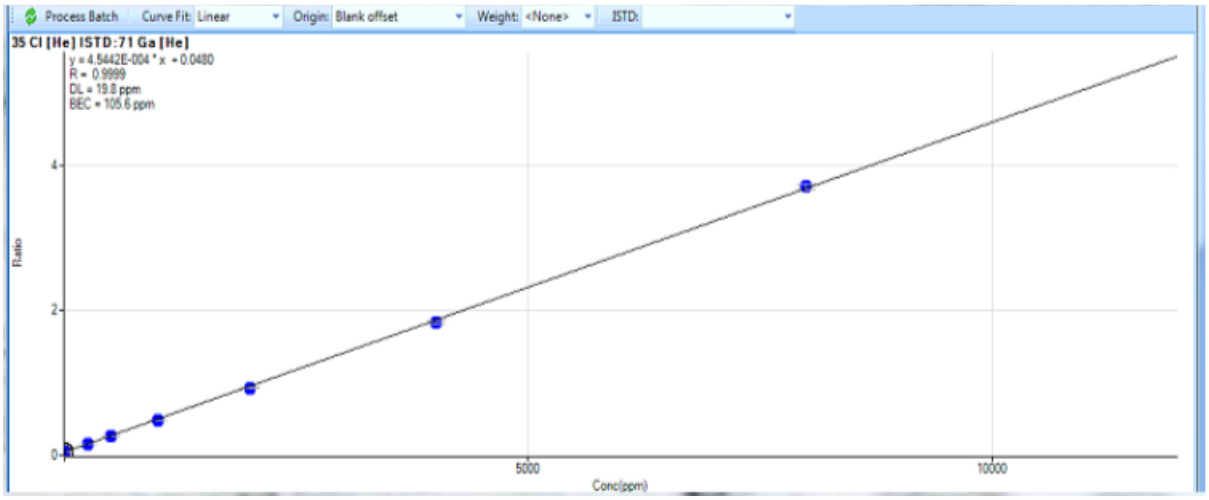


Fig.10 *Cl- calibration curve obtained in our laboratory with ICP-MS*

Accuracy and precision

As shown in Table 5, the intra- and inter-day precision and accuracy were satisfactory. Precision exhibited an RSD always < 6%, while accuracy was in the range of 99.7–103.8%. Accuracy was evaluated just for CS and ISE quality controls, as a certificate of analysis of QC quality control provided concentration ranges instead of nominal concentration values.

Table 5. *Accuracy and precision in the assessment of chlorine*

	Nominal concentration (mM)	**Mean concentration (mM)**	**RSD (%)**	**Accuracy (%)**
Intra-day variation				
QC1	22.6–30.9	29.4	1.4	
QC2	38.2–49.4	48.4	0.5	
QC3	78.8–102.4	97.2	1.6	
CS	28.2	27.9	1.2	99.8
ISE1	80	80.7	4.2	103.7
ISE2	120	119.6	3.9	99.7
Inter-day variation				
QC1	22.6–30.9	30.8	1.5	
QC2	38.2–49.4	48.1	0.6	
QC3	78.8–102.4	95.4	1.4	
CS	28.2	28.6	3.9	102.1
ISE1	80	83.1	6.0	103.8
ISE2	120	120.1	4.2	100.1

LOB, LOD, and LOQ

Limit of blank, limit of detection, and limit of quantitation of the analytical method were 2.1 mM, 3.2 mM, and 7.0 mM, respectively. Interestingly, the real concentrations injected into the mass spectrometer were 825 folds lower, corresponding to 3.9E−3 mM for LOD and 8.5E−3 mM for LOQ.

Stability

No significant difference in the concentration of chlorine was observed by comparing QC1, QC2, QC3, CS, ISE1, and ISE2 to themselves with separate times of storage (freshly prepared, day 1, day 7), suggesting that quality controls were stable for at least up to 7 days at refrigerator temperature.

Quantification of clinical samples

The mean chloride concentration among the 50 healthy volunteers was 15.7 ± 7.4 mM, (25–75th percentile 10.1–19.3 mM, range 2.8–37.4 mM); in contrast, two patients with suspected cystic fibrosis had respectively a sweat chlorine value of 65.6 mM (patient 1) and 81.2 mM (patient 2). These results were further confirmed by the crystallization test, with a score of 4 (+) being assigned to both patients.
For patient 1, further genetic testing by next-generation sequencing on CFTR gene showed a D1152H heterozygous mutation (c.3454G>C, p.Asp1152His) [131, 132].
The concentration of chlorine in healthy subjects is shown in table 6, where this value is been reported both in ppm (acronym for "parts per million", which indicates the quantity in grams of the test substance present in one million grams of solution or total mixture), and in μM (μmol / liter). In addition to the chlorine concentration other significant data, including the average concentration and standard deviation of the concentration, the age of the subjects, the average age and the standard deviation of age are reported in the table. The data showed in this table have been compared with those showed in table 7, illustrated later in this report, that figure out the values concerning the three subjects affected by cystic fibrosis taken in consideration.
It can be observed, as previously assumed, that the amount of chlorine undergoes variations also in reference to age.

Table 6. D*ata of healthy subjects not suffering from cystic fibrosis.*

Volunteers	Age	Mean age	Standard Deviation age	PPM	μM Cl	Standard Deviation μM Cl
1	28			460,941	12.9	
2	22			981,895	27.5	
3	30			356,566	10.1	
4	65			561,021	15.7	

5	54			578,327	16.2	
6	28			394,267	11.0	
7	30			520,088	14.6	
8	20			372,762	10.4	
9	35			361,965	10.1	
10	44			292,661	8.2	
11	65			278,975	7.8	
12	29			681,134	19.1	
13	65			539,693	15.1	
14	27			360,105	10.1	
15	50			860,226	24,1	
16	53			890,621	25.0	
17	30			571,740	16.0	
18	21			100,865	2.8	
19	57			657,293	18.4	
20	48			242,231	6.8	
21	36			299,711	8,4	
22	43			287,052	8.0	
23	29			374,191	10.5	
24	28			400,152	11.2	
25	32			844,631	23.6	
26	31			639,923	17.9	
27	30			530,611	14.9	
28	25			465,573	13.0	
29	26			360,733	10.1	
30	55			364,804	10.2	
31	43			292,414	8.2	
32	41			1064,404	29.8	
33	39			1336,470	37.4	
34	24			392,769	11.0	
35	39			278,175	7,8	
36	38			618,954	17.3	
37	33			370,411	10.4	
38	28			995,819	27.9	
39	54			505,937	14.2	
40	39			16,871	17.0	
41	28			17,670	18.0	
42	23			20,030	20.0	
43	55			12,043	12.0	
44	41			9,865	10.0	
45	37			29,364	29.0	
46	31			13,529	14.0	
47	29			920,616	25.8	
48	37			778,612	21.8	
49	24			946,139	26.5	
50	23			653,989	18.3	
		36.8	12.3			15.7

Table 7. *The table shows three cases of patients with abnormal sweat Cl values compared to normal physiological condition that surely need to be further investigated to have a diagnosis.*

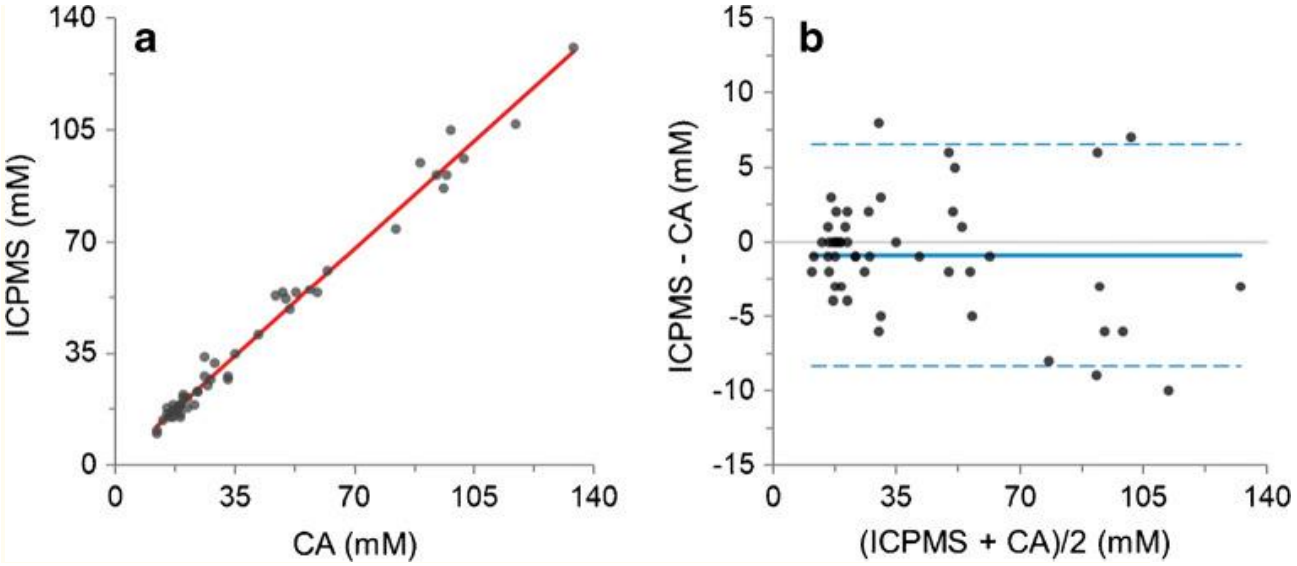
	Description	Cl value in sweat
Patient 1	24-year-old male with a history of chronic rhinitis and recurrent wheezing with wet cough since early childhood. Sweat tests carried out in the first years of life have shown borderline values.	65.6 mM
Patient 2	56-year-old female with a chronic wet cough and recurrent fever, which began at the age of 55 after an acute respiratory infection	81.2 mM
Patient 3	63-year-old male with a history of acute pancreatitis at age of 1 year and relapsing bronchitis.	48 mM

Cross-validation

To cross-validate the method [122], 50 serum samples from 30- to 50-day-old newborns with chloride concentration values in the range of 10 ÷ 131 mM, were assayed with either our ICP-MS method or the Chloride Analyser (Model 926S Mk II Chloride Analysers, Sherwood Scientific Ltd., Cambridge, UK), the latter method usually used for cystic

fibrosis newborn screening in Tuscany (Italy), and their results were compared with each other. The normality of data distribution was assessed using Kolmogorov-Smirnov and Shapiro-Wilk tests. Mean chloride concentrations measured with ICP-MS and Chloride Analyser (CA) were 40.12 ± 30.89 (median 26, range 10–131) mM and 41.00 ± 31.87 (median 26, range 12–134) mM, respectively. The difference was not statistically significant ($P = 0.096$, Wilcoxon's signed-rank test). The intra-class correlation coefficient (ICC) for chloride concentration was 0.996 (95% confidence interval [CI] = 0.993 ÷ 0.998, $P < 0.0001$). The linear regression line represents the relationship between ICP-MS and Chloride Analyser (CA), shown in Fig. 1a, exhibited an equation with a slope of 0.9626 (95% confidence interval [CI] = 0.9297 ÷ 0.9954, $P < 0.0001$), an intercept of 0.6548 (95% confidence interval [CI] = − 1.044 ÷ 2.354, $P = 0.4423$), and a correlation coefficient (R) of 0.993. The Bland-Altman plot, reported in Fig. Fig.1b,1b, confirmed the analogous concentration levels for CA and ICP-MS since bias had a value of − 0.9 (95% confidence interval [CI] = − 1.96 ÷ 0.20), with lower and upper limit of agreement of − 8.3 (95% confidence interval [CI] = − 10.18 ÷ − 6.47) and 6.6 (95% confidence interval [CI] = 4.71 ÷ 8.42), respectively [133]. Results are fully in compliance with EMA guidelines [122], which, for study samples, require a difference between the two values obtained by the different assays within 20% of the mean for at least 67% of the repeats, since 96% of the obtained pairs of values were within 20% of the mean.

Figure 10. *Comparison between ICP-MS and Chloride Analyser (CA) in the assay of chloride: linear regression analysis provides a regression line with a slope of 0.9626 (0.9297 to 0.9954), an intercept of 0.6548 (− 1.044 to 2.354), and a correlation coefficient (R) of 0.993 (a), while Bland-Altman plot exhibits a bias of − 0.9 units and 95% limits of agreement of − 8.3 and 6.6 (b)*



DISCUSSION

A sensitive, accurate and precise sweat Cl method was successfully developed and validated utilizing the ICP-MS technology. Although other work groups have previously reported the successful use of ICP-MS for sweat Cl determination, our method novelty is the ability to selectively determine the quantity of chlorine in the sweat. This validates the superiority of this analytical method by providing a significant improvement in the accuracy of the method demonstrated by reduced CV%, bias and 95% confidence intervals. Furthermore, the analytical range is improved over all currently listed methods all guidelines for sweat testing.

Based on the results of this study, we considered the ICP-MS method as a reference method for sweat Cl analysis.

Ge and Sc were selected from the Agilent ICP-MS IS mix as well as the IS for this test due to their proximity to Cl in terms of molecular weight. Although both were sufficiently reliable, the absence of an IS during sample preparation is not considered good laboratory practice in a clinical biochemical laboratory. It was decided to include Ga as the off-line IS; this was added directly to the sample prior to the addition of the diluent. Ga was also chosen as it is similar to Cl in molecular weight but is not present in the on-line IS mix.

The inclusion of the off-line IS may reflect any loss of sample or any dilution/evaporation that may occur during the preparation, storage or analysis phase of each sample providing a more accurate result. Without the addition of an off-line IS, any analyte loss that has occurred prior to the analysis of the sample, would not be compensated for, and therefore produce erroneous results.

A systematic error for the recovery of Ga was noted. This may be due to several factors. Polyatomic ion formation may have occurred during sample aspiration causing a loss in Ga, matrix composition causing partial suppression of the Ga IS or during the preparation of the working IS.

The working IS was prepared once, to a concentration of 28.7E−3 mM and utilized though out the project. Any dilution to the working IS would project a constantly reduced recovery%.

Any dilution to the working IS would project a constantly reduced recovery%.

Despite this reduced IS recovery% it was still within the allowable ±5% recovery limits of the IS. This ICP-MS method can provide the required reference point for all current sweat Cl quantitation method calibrations.

CONCLUSIONS

Inductively coupled plasma mass spectrometry (ICP-MS) is increasingly used in the clinical laboratory, mainly for assessments on a routine basis of oligo-elements and isotopes of biomedical interest [34–36]. Its versatility, as well as its sensitivity, selectivity, and accuracy, can be advantageously utilized also for sweat chloride assay, providing a significant advancement over the commonly used ion-selective procedures, whose analytical performances at low concentrations can be quite critical in terms of quantification accuracy. This is the case of coulometry-based assay (namely Chloride Analysers, used in the newborn screening laboratory of Tuscany) that was chosen as the reference method to cross-validate our ICP-MS-based method. The results, which in compliance with the CLSI guidelines were obtained with sweat samples ≥ 75 mg, confirmed the suitability of the ICP-MS method as a diagnostic tool. The good correlation

between the two techniques confirms that the recommended reference values are suitable also for the ICP-MS assay, which was adopted for the clinical routine of the University Hospital of Pisa. Its improved analytical performances above the approved quantitative methods for Cl, which derive from serum-based methods whose Cl concentration curve is extrapolated to fit the lower Cl concentrations detected in sweat [116], will probably allow a better definition of the reference values of sweat chloride, especially in adolescents and adults. The increased analytical sensitivity, and the possibility to improve method sensitivity even further by reducing the sample dilution, could allow reliable quantification of samples < 75 mg, potentially influencing the future guidelines concerning the collection methods. Moreover, the accurate quantification by ICP-MS as the sweat chloride concentration may reveal useful to improve the prediction of survival of patients with unclassified CFTR genotype [137]. As far as the price of a single determination is concerned, considering the workload of our instrumentation and personnel, we estimated it around 9 euros that at the best of our knowledge is equivalent, if not lower, to those of the routine diagnosis of cystic fibrosis. We must emphasize that the attainment of reference results by ICP-MS is subjected to the availability of highly trained personnel, with a good knowledge of the technique and the ability to solve the frequent problems that inevitably occur with these instruments.

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