UNIVERSIDADE ESTADUAL DE CAMPINAS
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Indicações clínicas, variações com a idade e proporção entre os eletrólitos do teste de suor para o diagnóstico de fibrose cística

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Clinical indications, variations with age and proportion between sweat test electrolytes for the diagnosis of cystic fibrosis

Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciências, na área de concentração em Saúde da Criança e do Adolescente

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Este exemplar corresponde a versão final da tese defendida pela aluna Alethéa Guimarães Faria, orientada pelo Prof. Dr. José Dirceu Ribeiro e coorientada pelo Prof. Dr. Fernando Augusto de Lima Marson

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RESUMO

Introdução: Apesar da busca constante de um biomarcador único de alta especificidade e sensibilidade, o teste do suor (TS), desde sua implantação e padronização, há 69 anos, por Gibson & Cooke em 1959, continua sendo o padrão ouro para o diagnóstico de fibrose cística (FC). Acredita-se que graus variados de disfunção da proteína CFTR de acordo com o comprometimento molecular causado pelas mutações no gene *CFTR* sejam a as principais determinantes da concentração dos níveis de cloreto de suor. Além disso, a concentração de eletrólitos no TS pode variar de acordo com a variabilidade individual, idade, sexo e fatores ambientais. Apesar dos avanços no campo da biologia molecular com mais de 2.000 mutações identificadas do *CFTR*, a associação das mutações com os resultados do TS ainda não está bem esclarecida. **Objetivo:** Apresentar e discutir três estudos relacionados ao TS em três situações: (1) qualidade do exame do TS pela proporção entre os íons cloreto e sódio no exame; (2) variação no valor do cloreto no suor de acordo com a idade; (3) análise descritiva do TS e associação do mesmo com indicações, dados epidemiológicos, mutações do *CFTR,* assim como, a correlação entre as concentrações de cloreto e sódio. **Métodos:** Os estudos foram desenvolvidos por modelos retrospectivos e descritivos com variáveis obtidas do banco de dados do Centro de Referência em FC da Unicamp dos últimos 30 anos. A inclusão dos indivíduos foi baseada nos dados clínicos relacionados a indicação do exame de TS e para aqueles nos quais se conhecia as mutações do *CFTR*. **Resultados:** Foram obtidas e analisadas 5.721 amostras do TS. Estudo 1: considerando a qualidade do TS pela diferença entre os níveis de cloro e sódio, o exame foi realizado corretamente em 5.023/5.692 (88,2%) das amostras, e em 669/5.692 (11,8%), incorretamente. As amostras fora7m agrupadas de acordo com os níveis de cloreto no TS (mEq/L): (i) < 30: 3.651/5.692 (64,1%); (ii) ≥ 30 a < 40: 652/5.692 (11,5%); (iii) ≥ 40 a < 60: 673/5.692 (11,8%); (iv) ≥ 60: 716/5.692 (12,6%). Estudo 2: houve variação do cloreto no suor com a idade, sendo que nos pacientes com FC, os níveis de cloreto no TS

aumentaram no primeiro ano de vida e, a partir do segundo ano de vida, reduziram gradualmente. Estudo 3: a associação do TS com dados epidemiológicos mostrou que os sintomas digestivos apresentaram maior razão de prevalência para o diagnóstico de FC, assim como, associação entre indivíduos mais jovens e valores mais elevados de cloreto no suor. A indicação de TS devido a sintomas respiratórios foi maior nos pacientes com idade entre seis meses e 18 anos. O sexo, idade e mutações do *CFTR* alteram os valores do TS. **Conclusões:** Os resultados do TS mostraram variabilidade dependente da idade, sexo, razão para a indicação do exame, mutações do *CFTR* e peso da amostra. A concentração de sódio no suor está correlacionada com os níveis de cloreto e pode ser usada como parâmetro de qualidade no TS.

Palavras chave: cloreto no suor; diagnóstico; fibrose cística; teste do suor

ABSTRACT

Introduction: Despite the constant search for a unique biomarker of high specificity and sensitivity, sweat test (ST) since its implantation and standardization 69 years ago by Gibson & Cooke in 1959, continued the standard for the diagnosis of cystic fibrosis (CF). It is believed that varying degrees of CFTR protein dysfunction according to the molecular impairment caused by mutations of CFTR gene are the main determinants of the concentration of sweat chloride levels. In addition, the concentration of ST electrolytes may vary according to individual variability, age, gender, and environmental factors. Despite advances in the field of molecular biology with more than 2,000 CFTR mutations identified, an association of mutations with ST results is still unclear. **Objective:** To present and discuss three studies related to ST in three situations: (1) quality of ST examination by proportion of chloride and sodium ions in the exam; (2) change in the value of chloride in sweat according to age; (3) descriptive analysis of ST and its association with indications, epidemiological data, CFTR mutations, as well as a correlation between chloride and sodium concentrations. **Methods**: The studies were developed by retrospective and descriptive models with variables obtained from the database of the Reference Center in CF from Unicamp, including the data from the last 30 years. The subjects inclusion was based on the clinical data related to the ST exam indication and for those in whom the *CFTR* mutations were known. **Results**: We analyzed 5,721 ST samples. Study 1: considering the ST quality by the difference between chloride and sodium levels, the test was performed correctly in 5,023/5,692 (88.2%) of the samples, and in 669/5,692 (11.8%), incorrectly. The samples were clustered according to the levels of chloride (mEq/L) in ST: (i) < 30: 3,651/5,692 (64.1%); (ii) ≥ 30 at < 40: 652/5,692 (11.5%); (iii) ≥ 40 at < 60: 673/5,692 (11.8%); (iv) ≥ 60: 716/5,692 (12.6%). Study 2: there was variation of the chloride in the sweat with the age. In the group of patients with CF, the chloride levels in the ST increased in the first year of life

and, from the second year of life, gradually reduced. Study 3: the association of ST with epidemiological data showed that digestive symptoms had a higher prevalence rate for the diagnosis of CF, as well as, association between younger individuals and higher values of chloride in sweat. The indication of ST due to respiratory symptoms was higher in patients aged between six months and 18 years. It was observed that sex, age and *CFTR* mutations alter ST values. **Conclusions**: The ST variability showed values depending on age, sex, indication to perform of the test, *CFTR* mutations and sample weight. The concentration of sodium in sweat is correlated with chloride levels and can be used as a quality parameter in ST.

Key words: cystic fibrosis; diagnosis; sweat chloride; sweat test

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AMPc – Adenosina monofosfatase cíclica

ATPase – Adenosina trifosfatase

Ca^{2+} – Cálcio

CFF – Cystic fibrosis foundation

CFTR – Cystic Fibrosis Transmembrane Conductance Regulator

CFTR – Cystic fibrosis transmembrane regulator

Cl^- – Cloreto

CLSI – National Committe for Clinical Laboratory Standards

DPN – Diferença de potencial nasal

ENaC – Canal epitelial eletrocondutivo de sódio

FC – Fibrose cística

FCM – Faculdade de Ciências Médicas

mEq/L – Miliequivalente por litro

NaCl – Cloreto de sódio

NBD – Domínio de ligação de nucleotídeos

p.Phe508del – Deleção da Fenilanina na posição 508

R – Domínio regulatório

TMD – Domínio transmembrana

TS – Teste do suor

Unicamp – Universidade Estadual de Campinas

SUMÁRIO

1. Introdução

Pesquisas históricas nos levam a crer que os sintomas característicos da fibrose cística (FC) foram reconhecidos e associados a mortalidade nas sociedades primitivas do leste europeu antes mesmo da idade média. O critério diagnóstico de suor salgado era visto como premonição de doença, emagrecimento e morte (Quinton, 1999). Daquela época, até os dias de hoje, o gosto salgado na pele pode ser utilizado como valor clínico significativo para suspeita diagnóstica de FC (Camargos *et al.*, 2015).

Registros do século XVII relatam condições relacionadas à esteatorreia, presumindo, o aparecimento de insuficiência pancreática, que ocorre na maioria dos pacientes com FC (Busch, 1986). No século XIX, alguns registros, realizaram associações entre esteatorreia, complicações meconiais e lesões pancreáticas (Quinton, 1999).

Em 1905, o patologista Landsteiner, estudou em recém-nascidos, a presença de íleo meconial, que, décadas depois passou a ser considerado patognomônico da FC. Nesta ocasião foi realizada a primeira descrição histológica da FC. O pâncreas dos lactentes mostrou aumento do tecido conjuntivo intralobular e interlobular, infiltração celular e ductos dilatados. Landsteiner, sugeriu que a falta de secreção pancreática causava o espessamento do mecônio. Esse achado foi considerado pioneiro em descrever que mudanças microscópicas no pâncreas podem interferir na composição de mecônio e, assim, resultar em obstrução intestinal (Disponível em: *www.cfmedicine.com* Landsteiner, 1905).

Em 1919, Passini descreveu a doença pancreática em um neonato desnutrido, com dois meses de idade. A análise histológica pancreática foi típica da descrita posteriormente em indivíduos com FC. Este autor também descreveu outros lactentes com: alterações císticas no pâncreas, redução das ilhotas de Langerhans, alargamento dos ductos pancreáticos e áreas de necrose no parênquima. Segundo os relatos, todos os pacientes morreram de broncopneumonia e, presumivelmente, tinham FC (Disponível em: *www.cfmedicine.com*).

Em 1936, Guido Fanconi descreveu uma síndrome celíaca com alterações pancreáticas que eram distintas da doença celíaca. O autor, possivelmente, foi o primeiro a se referir a doença como *Fibromatosi Cistica* com bronquiectasia (Disponível em: *www.cfmedicine.com;* Quinton, 1999).

No século XX, ocorreram as primeiras observações que associaram a doença pulmonar com diarreia crônica e função pancreática anormal (Garrod e Hurtley, 1912; Disponível em: *www.cfmedicine.com*). Porém, a primeira descrição clínica e anatomopatológica detalhada da FC foi realizada pela Drª. Dorothy Andersen, em 1938, que avaliou as características histológicas do pâncreas em necropsias de 49 crianças com obstrução intestinal e/ou complicações respiratórias. O tamanho dos cistos, no pâncreas, variou em cada caso, mas os cistos grandes não foram frequentemente observados nos bebês mais novos. Dos 49 casos, 45 pacientes apresentaram, na microscopia, secreção espessa no acino e células acinares achatadas (Andersen, 1938).

Em 1945, Sydney Farber, chefe do departamento de patologia do hospital infantil de Boston, reconheceu a FC como uma desordem generalizada que afeta numerosos órgãos, além do pâncreas, e introduziu o termo *Mucovicidose*. Farber acreditava que o dano do trato respiratório dependia da obstrução das vias aéreas por muco espesso, falha na lubrificação do epitélio ciliado e infecção secundária por *Staphylococcus aureus (*Farber, 1945).

O tratamento da FC, na década de 40, concentrava-se, principalmente, nos aspectos da nutrição. Acreditava-se que a desnutrição proteica calórica e a deficiência de vitamina A, causavam danos na estrutura do epitélio respiratório (entre eles: metaplasia escamosa) que contribuíam para a gravidade da FC por causar infecções respiratórias que eram impossíveis de se tratar (Disponível em: *www.cfmedicine.com*).

O pediatra Paul Di Sant Agnese, em 1948, observou que muitos lactentes que apresentavam prostração em altas temperaturas tinham FC. Foi observado que o suor dessas

crianças apresentava excesso de íons sódio e cloreto (duas a cinco vezes acima do valor do indivíduo saudável). A alteração persistia, em pacientes com FC, mesmo em baixas temperaturas (Disponível em:*www.cfmedicine.com;* Di Stan Agnese *et al.*, 1953; Davis, 2006).

Webb e colaboradores (1956) criaram um teste para estimar os eletrólitos no suor. Os pacientes eram colocados em uma bolsa de plástico amarrada até o pescoço e eram cobertos por três cobertores. O suor era coletado em papel filtro alocado na parte dorsal do tórax do paciente e, posteriormente, os níveis eram dosados (Webb *et al.*, 1956).

Um dos grandes marcos na história do diagnóstico da FC aconteceu em 1959, quando Gibson e Cooke, desenvolveram o teste do suor (TS) pela técnica de iontoforese por pilocarpina (Gibson e Cooke, 1959). A partir de então, a concentração elevada de íons cloreto e sódio, no suor, foi proposta como teste diagnóstico para a FC. O TS permanece, até os dias atuais, como a principal ferramenta para o diagnóstico da FC em todo o mundo (Davis, 2006; Collie *et al., 2014).*

Após a implantação e padronização do TS, tanto os pacientes com insuficiência pancreática e alguns com suficiência pancreática, puderam ser diagnosticados. Desde então, a técnica quantitativa de iontoforese por pilocarpina é o "padrão ouro" para o diagnóstico de FC, sendo realizado nos mesmos moldes, conforme originalmente descrito (Mattar *et al.*, 2010).

Em 1983, os ductos sudoríparos foram estudados por Paul Quinton, que identificou o defeito do transporte do íon cloreto como base da FC. Nesse mesmo período, foi observado que a reabsorção aumentada de sódio era comum no epitélio das vias aéreas na FC (Knowles *et al.*, 1983; Boucher *et al.*, 1986; Davis, 2006; Mattar *et al.*, 2010).

Outro grande marco para o entendimento da FC ocorreu em 1989, quando foi identificado o gene que causa a doença – *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) (Kerem e Rommens, 1989).

O gene *CFTR* possui 250 Kilobases e codifica uma proteína também denominada CFTR, que funciona principalmente como canal do íon cloreto e é regulada pela adenosina monofosfatase cíclica (AMPc), presente na superfície apical, de células epiteliais (Quinton, 2007).

A partir desse momento, descobriu-se que o principal papel da CFTR era regular o transporte iônico no epitélio de numerosos órgãos como; as vias aéreas, intestino, ductos sudoríparos e pancreáticos (Kerem e Rommens, 1989; Kunzelmann, 2001).

Atualmente são conhecidas mais de 2.000 mutações de *CFTR*, divididas em sete classes, de acordo com a gravidade e uso na medicina de precisão, que causam: (i) defeitos na síntese da CFTR, resultando na ausência de produção [IA e IB (Marson *et al*., 2016; ou 1 e 7 por De Boeck e Amaral, 2016); (ii) bloqueio no processamento da CFTR; (iii) bloqueio na ativação e regulação da CFTR pelo AMPc, apesar da produção e transporte adequado da CFTR; (iv) prejuízo na função da CFTR, com redução do transporte do íon cloreto; (v) redução nos níveis de CFTR funcional, que é transportada para a membrana plasmática; (vi) defeito na estabilidade da CFTR (Marson *et al*., 2016; De Boeck e Amaral, 2016) (**Figura 1**).

Mutações de classes I a III do *CFTR* são as mais frequentes e associadas com insuficiência pancreática, enquanto pacientes com mutações mais raras, de classes IV a VI, geralmente não costumam ter insuficiência pancreática, e como ainda existe função residual da CFTR, os níveis de íons cloreto podem ser limítrofes ou normais. A mutação com maior prevalência é pertencente a classe II e causa a deleção da fenilalanina na posição 508 de *CFTR* (p.Phe508del) (Coutinho *et al.*, 2013; Marson *et al.*, 2013).

Traditional classification	Class I		Class II	Class III	Class IV	Class V	Class VI
Proposed classification	Class IA	Class IB	Class II	Class III	Class IV	Class V	Class VI
De Boeck and Amaral's classification	Class VII	Class I	Class II	Class III	Class IV	Class V	Class VI
CFTR defect	No mRNA	No protein	No traffic	Impaired gating	Decreased conductance	Less protein	Less stable
Mutation examples	Dele2,3(21 kb), 1717-1G→A	Gly542X, Trp1282X	Phe508del, Asn1303Lys, Ala561Glu	Gly551Asp, Ser549Arg, Gly1349Asp	Arg117His, Arg334Trp, Ala455Glu	3272-26A→G, 3849+10 kg C→T	c. 120del123, rPhe580del
Corrective therapy	Unrescuable	Rescue synthesis	Rescue traffic	Restore channel activity	Restore channel activity	Correct splicing	Promote stability
Drugs (approved)	Bypass therapies (no)	Read-through compounds (no)	Correctors (yes)	Potentiators (yes)	Potentiators (no)	Antisense oligonucleotides, correctors, potentiators? (no)	Stabilisers (no)
Clinical features (global aspect)	More-severe disease				Less-severe disease		

Figura 1 – Sistema de classificação das mutações do *CFTR* (Marson *et al.*, 2016).

Até a identificação do gene *CFTR*, o diagnóstico da FC era baseado em critérios clínicos, associados à análise dos eletrólitos no suor. A descoberta do gene e a implementação de técnicas laboratoriais para detectar mutações de *CFTR* expandiu o diagnóstico da FC, possibilitando o conhecimento de formas leves e "atípicas" da doença (Mattar *et al.*, 2010). Nas últimas décadas, numerosos conhecimentos na fisiopatologia, diagnóstico e manejo da FC, permitiram melhora na qualidade e expectativa de vida. Apesar disso, o desafio tem sido entender como o defeito no transporte de íons resulta em deterioração dos órgãos afetados e como isso pode ser corrigido em nível molecular pela medicina de precisão (Marson *et al.*, 2017).

Em pacientes com FC, a maior relevância clínica, ocorre por alterações no: (i) trato digestivo (pâncreas, fígado e intestinos), má nutrição e desnutrição; (ii) sistema respiratório (morbidade/mortalidade principalmente associada a doença pulmonar); (iii) glândulas sudoríparas (importância no diagnóstico) (Quinton, 2007).

Quando o diagnóstico é realizado precocemente, o acompanhamento e manejo da deterioração da estrutura e função dos órgãos, favorece no melhor prognóstico.

1.1. A proteína CFTR – estrutura molecular

A CFTR é uma proteína de membrana, grande e complexa, cuja principal função é conduzir íons pela membrana plasmática (Sheppard e Welsh, 1999). A CFTR consiste de 1.480 aminoácidos que formam dois conjuntos de seis regiões que atravessam a membrana plasmática, entre essas duas regiões, existem dois domínios de ligação de nucleotídeos, que flanqueiam um domínio citoplasmático – domínio regulatório (Quinton, 2007) (**Figura 2**).

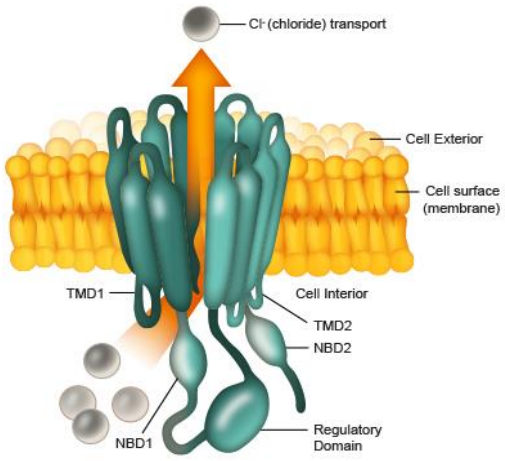

Figura 2 – A proteína CFTR é fixada na membrana apical por 12 domínios transmembrana (dois conjuntos com seis regiões – TMD1 e TMD2) que são ligados ao domínio regulatório (R) por dois domínios intracelulares de ligação a nucleotídeos (NBD1 e NBD2) (Quinton, 2007).

A mutação p.Phe508del ocorre no primeiro domínio de ligação de nucleotídeos (NBD1), e outras mutações, aparecem ao longo de *CFTR*, acarretando em alterações quantitativas e qualitativas na proteína CFTR. Assim, a proteína CFTR normal é um canal de condução de íons e também de regulação de outros componentes e mecanismos de transportes (Kunzelmann, 2001; Quinton, 2007).

1.2. A proteína CFTR – função molecular

Na membrana apical de vários órgãos, a proteína CFTR interage com um grande número de proteínas e atua, principalmente, em um vasto número de processos: (i) transporte de cloro, sódio, glutationa, ATP e bicarbonato; (ii) regulação de volume e secreção de muco; (iii) acidificação endossomal; (iv) diferenciação e proliferação celular; (v) efeitos anti-inflamatórios, fagocítico, apresentação de antígeno e defesa antimicrobiana; (vi) absorção de glicose; (vii) antagonismos de modelos pro-inflamatórios (Kunzelmann, 2001; Kunzelman *et al,* 2017).

A função da proteína CFTR pode ser mensurada na glândula sudorípara, epitélio nasal, trato digestório (biópsia retal) e glândula salivar. Na glândula sudorípara, a primeira metade do túbulo secreta fluido isotônico e a segunda, o ducto reabsortivo, absorve a solução hipertônica do fluido secretado (Quinton e Prompt, 1978; Quinton, 2007).

Entre os mecanismos de homeostase, a transpiração é necessária para o resfriamento do corpo. Para isso ocorrer, nervos simpáticos liberam acetilcolina em sinapses neuro-glandulares. Este processo é mediado por aumento do Cálcio (Ca^{2+}) intracelular e ativação de canais de íons cloreto. Uma vez que, o potencial elétrico da membrana apical da célula secretora, é mais negativo no interior do que no lúmen, o potencial eletroquímico sobre o íon cloreto, negativamente carregado, o leva do interior da célula para o lúmen, pela presença da CFTR "aberta" – ativa. A carga negativa do íon cloreto, no lúmen, cria um gradiente elétrico favorável para o movimento do sódio pela junção estreita do lúmen, mantendo a neutralidade elétrica (Kidd e Thorn, 2000; Quinton 2007). O acúmulo de NaCl e aumento de volume de fluido no lúmen, em proporções isotônicas, forçam o fluido secretado na metade restante do túbulo e na superfície da pele para evaporação. Esse mecanismo mediado pelo Ca^{2+} não é afetado na FC (Bijman e Quinton, 1984).

Para a reabsorção de íon cloreto, a proteína CFTR é expressa abundantemente, em condições normais, na membrana luminal da segunda metade do túbulo da glândula sudorípara

– ducto reabsortivo, que move o íon cloreto, na direção oposta, ao fluxo de saída. Durante a reabsorção, o íon sódio entra passivamente na célula do ducto do lúmen pelo canal epitelial eletrocondutivo de sódio (EnaC) presente na membrana apical (Prompt e Quinton 1978; Kidd e Thorn, 2000). Dessa forma, do lúmen para o interior da célula, o gradiente de íon sódio é mantido pelo transporte ativo via sódio, potássio e ATPase presentes na membrana basolateral. Simultaneamente, com o transporte de carga positiva pela célula, ocorre a formação de um gradiente eletroquímico positivo, que é suficiente para induzir o transporte do íon cloreto, do lúmen para o interior da célula, via CFTR (Quinton, 2007).

Na FC, o transporte de fluido secretivo e o absortivo apresentam deficiência, sendo que o mecanismo de reabsorção falha devido à falta ou deficiência qualitativa ou quantitativa da CFTR (Quinton, 1983; Reddy e Quinton, 1992).

Pacientes com FC apresentam, normalmente, concentração de íon cloreto no suor superior a 60 mEq/L. Dessa forma, se o canal CFTR está ausente ou inativo, o íon cloreto não é transportado do lúmen para o interior da célula, e concomitantemente, a absorção do sódio é impedida. Desse modo, na FC, os íons sódio e cloreto não são reabsorvidos do ducto e o suor hipertônico ocorre na superfície da pele do paciente, mesmo na presença de canais de sódio sem alteração qualitativa e/ou quantitativa (Ballestero *et al.*, 2006)

1.3. Avaliação da função da proteína CFTR, nas glândulas sudoríparas, pelo teste do suor.

As primeiras técnicas para induzir a sudorese envolviam estresse térmico e o paciente era deixado numa sala quente por uma a duas horas (Mauer *et al.*, 1956). No século XX foi descrito um método simples de estresse térmico em que o paciente usava uma bolsa plástica que abrangia o corpo do paciente e era amarrada até o pescoço, sendo o paciente, posteriormente, coberto por uma manta por aproximadamente 60 a 90 minutos. Embora

houvesse risco ao paciente, este método, foi utilizado durante anos (Mish *et al.*, 1958) (**Figura 3**).

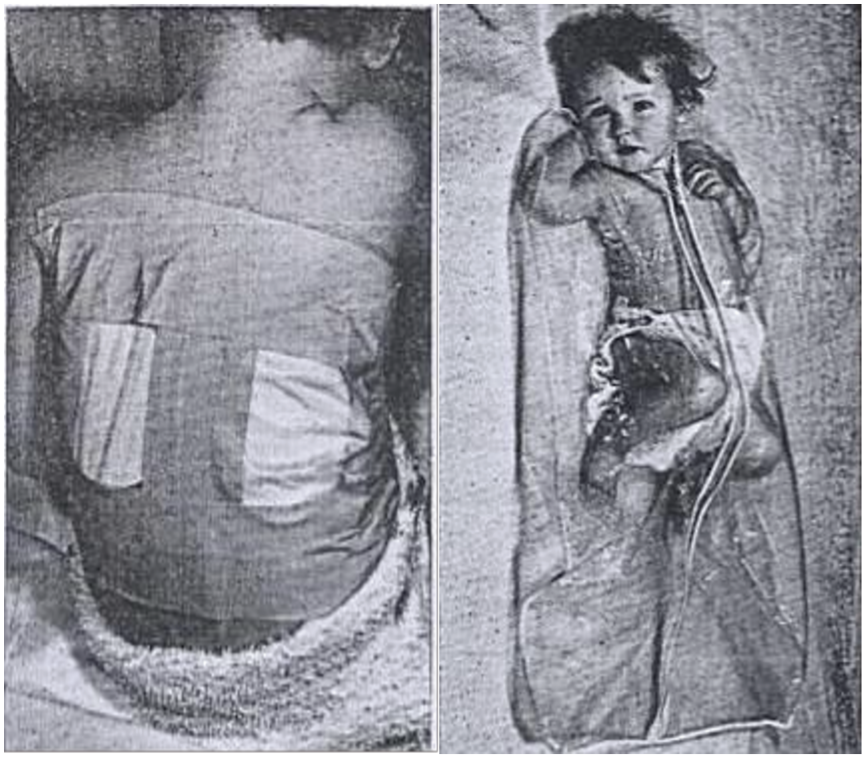

Figura 3 – Realização do teste do suor em paciente envolto em plástico (Retirado de *www.cfmedicine.com*)

Em virtude dos riscos que a técnica de estresse térmico causava, estratégias alternativas foram implantadas, como: (i) pintura dos dedos com nitrato de prata e cromato de potássio (**Figura 4**); (ii) uso de papel filtro embebido em nitrato de prata e colocado entre os dedos; (iii) atadura com película plástica usada na palma da mão (Shwachman *et al.*, 1956; Webb *et al.*, 1957; Gluck, 1959; Collie *et al.*, 2014; Disponível em: *www.cfmedicine.com*).

Embora, o teste com nitrato de prata fosse seguro e simples se comparado com o de estresse térmico, sua validação não foi possível, e a técnica foi substituída pela análise quantitativa do íon cloreto por iontoforese com pilocarpina (Collie *et al.*, 2014).

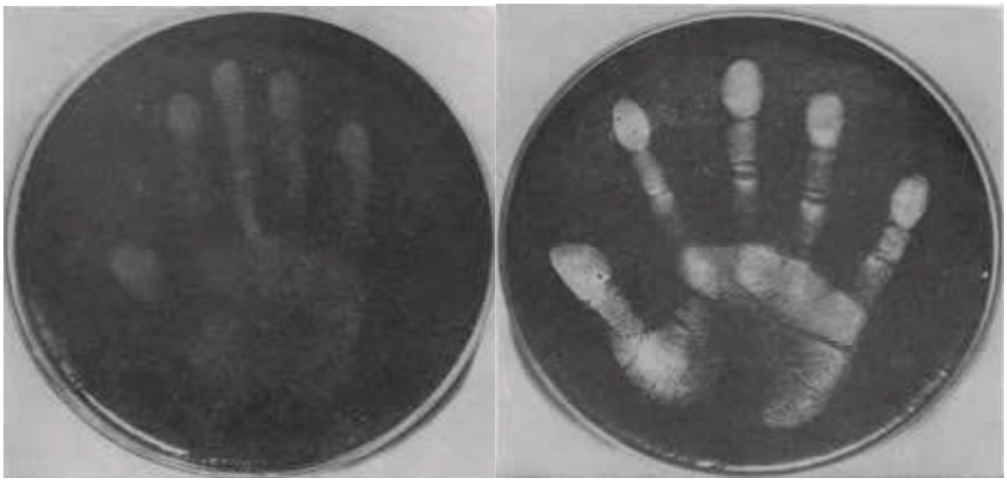

Figura 4 – Impressão palmar de uma criança sem FC (esquerda) e de uma com FC (direita) (retirado de *www.cfmedicine.com*).

Em 1959, Gibson e Cooke publicaram o método que se tornou um marco histórico no diagnóstico de FC, constituído de três etapas: estimulação, coleta e dosagem do íon cloreto do suor obtido pela sudorese. O estímulo era feito no antebraço do paciente com a aplicação tópica de um parasimpatomimético – pilocarpina – alcaloide que age nos receptores colinérgicos imitando o neurotransmissor acetilcolina. A iontoforese estimula os receptores muscarínicos nas glândulas de suor induzindo a sudorese (Quinton, 2007)

No método de Gibson e Cooke, o suor era coletado por uma gaze ou papel filtro. O suor é precipitado do material coletado e analisado por cloridrometria e fotometria de chama, respectivamente, para se obter a concentração dos íons cloreto e sódio.

A realização do TS pela técnica de Gibson e Cooke apresentou dificuldades no início, pois a exposição aos eletrodos causava queimaduras e bolhas no local de contato, decorrente, muitas vezes, da inexperiência dos técnicos na coleta do suor e pelo excesso de tempo da estimulação elétrica (David, 1990). Além disso, até os dias atuais, outros dispositivos para estimular sudorese suor têm sido propostos, utilizando diferentes valores de correntes e tipos de ondas para evitar efeitos danosos pelo mau uso da técnica proposta por Gibson e Cooke (Gomez *et al.,* 2014).

Outra dificuldade no TS inclui a presença de valores elevados do íon cloreto decorrente da baixa quantidade de suor coletada ou evaporação da amostra. Apesar dos problemas descritos, a técnica de Gibson e Cooke, permaneceu e permanece como método reconhecido de coleta do suor (Collie *et al.,* 2014).

Em 1983, foi desenvolvido o sistema Wescor Macroduct® (Wescor, Inc, Utah, USA) para coleta do suor (Webster, 1983). O sistema reproduz o método de Gibson e Cooke, sendo o suor coletado, para dentro de uma espiral de plástico, após a estimulação por iontoforese por pilocarpina (Mattar *et al.*, 2010). A popularidade do teste se deve a automação da estimulação do suor e de sua fácil aplicabilidade.

O sistema de coleta de suor Wescor Macroduct® foi reconhecido pela Fundação Americana de FC em 1990. Atualmente, ambos os testes (TS clássico e Wescor Macroduct®) são recomendados para o diagnóstico de FC tendo resultados equivalentes (Hammond *et al.*, 1994; CLSI, 2009; Mattar *et al.,* 2010; Collie *et al.*, 2014). A diferença entre os sistemas é a amostra matriz. Na técnica de Gibson e Cooke, a amostra, é diluída devido à necessidade de eluição da amostra. Já no sistema Wescor Macroduct®, a amostra, é coletada diretamente na espiral e é analisada de maneira pura (Collie *et al.*, 2014). Dessa forma, a técnica de coleta influência o método de análise.

Nas últimas décadas, com o desenvolvimento de g*uidelines*, houve uma padronização no método de coleta de suor, na iontoforese com pilocarpina e nos métodos para a quantificação dos íons no suor, principalmente do íon cloreto. Algumas dessas variações incluem qual item será analisado, como será analisado e quais os intervalos de referência que serão usados para interpretar os resultados (Mattar *et al.,* 2010; Collie *et al.*, 2014).

A padronização da coleta, análise e registro do TS foi realizada pelo *National Committe for Clinical Laboratory Standards* (CLSI) em 1994. A partir desse momento houve refinamento do método, desenvolvimento de *guidelines* em outros países e atualização do *guideline* da CLSI.

A *Cystic Fibrosis Foundation* (CFF), em 2007, publicou um *guideline* próprio baseado no *guideline* da CLSI (LeGrys *et al.*, 2009).

O teste quantitativo de iontoforese por pilocarpina (TS clássico) é uma técnica trabalhosa que requer a determinação de peso exato do suor na balança analítica e é preciso cuidado para evitar a evaporação da amostra. A amostra do suor é submetida a análise bioquímica dos eletrólitos e um mínimo de 75 mg de suor é necessária para o resultado acurado (LeGrys *et al.*, 2007; Mattar *et al.*, 2010).

Os *guidelines* avaliam a inclusão dos níveis do íon sódio e do método analítico para quantificar o íon cloreto. O íon sódio no suor tem sido usado, principalmente, como controle de qualidade para o TS em alguns centros de diagnóstico e pesquisa (Farrel *et al.*, 2008; Traeger *et al.*, 2014). Além disso, alguns estudos propõe o uso da razão dos níveis dos íons sódio e cloreto no suor para diagnosticar a FC, em casos de resultados limítrofes, no TS. Porém, não há consenso sobre essa informação (*Multi Disciplinary Working Group*, 2003; Mishra *et al.,* 2008; Collie *et al.,* 2014). O íon sódio no suor é menos discriminatório do que o íon cloreto. Por esta razão, alguns autores sugerem dosar apenas o íon cloreto no TS (CLSI, 2009; Collie *et al.,* 2014).

Embora haja o reconhecimento que na FC o déficit no transporte esteja relacionado com o íon cloreto e que o íon sódio tem menor poder discriminatório, alguns laboratórios continuam a analisar o íon sódio (Mirsha *et al.*, 2007; Collie *et al.*, 2014). Por essa razão, a última versão do CLSI de 2009, não incluiu a análise do íon sódio no TS, enquanto outros *guidelines*, continuam a ressaltar a importância de sua quantificação (Collie *et al.*, 2014).

Além da FC, várias doenças (dermatite atópica, hipogamaglobulinemia, glicogenose tipo 1, mucopolissacaridose tipo 1, diabetes insipidus nefrogênico, pseudohipoaldosteronismo, doença celíaca, insuficiência adrenal e hipotireoidismo), se não tratados, podem elevar a

concentração do íon cloreto no suor, porém apresentam fenotípico clínico característico (Mattar *et al.*, 2010).

Resultados falsos positivos no TS ocorrem na desnutrição, desidratação, má condição da pele (eczema ou exantema) e por erros metodológicos e técnicos, como a evaporação da amostra. Resultados falsos negativos estão relacionados à presença de edema, uso de mineralocorticoides, coleta e análise de quantidade insuficiente de suor e problemas técnicos. Além disso, na interpretação do TS, deve-se considerar que algumas mutações do *CFTR* podem induzir a valores limítrofes ou normais de íons cloreto (Beauchamp *et al.*, 2005; Matar *et al*, 2010).

O intervalo de referência para o TS foi originalmente descrito por Gibson e Cooke (1959) (normal ≤ 39 mEq/L; *borderline* = 40 a 59 mEq/L; FC ≥ 60 mEq/L). O intervalo é adequado para a maioria dos casos (Mirsha *et al.*, 2008). No entanto, com o reconhecimento do espectro clínico de doenças mediadas pela CFTR e o advento da triagem neonatal, acentuou-se a necessidade de intervalos de referências mais sensíveis. Dessa forma, intervalos de referência para as idades de cinco a 55 anos foram publicados em 2008 e, intervalos de referência para bebês de cinco a seis semanas de idade foram publicados em 2009. Ainda há uma lacuna para pacientes de seis semanas a cinco anos de idade (Beauchamp *et al.*, 2005; Collie *et al.*, 2014). Assim, resultados do TS intermediários (30 a 59 mEq/L para bebês e 40 a 59 mEq/L além da infância) podem desafiar o diagnóstico da doença (Collie *et al.*, 2014; Farrell *et al*, 2017).

Recentemente, o *guideline* da CFF, definiu alguns critérios para o diagnóstico de FC. A FC é diagnosticada quando um paciente tem duas condições mutuas: triagem neonatal positiva, clínica da doença (sinais e/ou sintomas e/ou história familiar de FC), evidência da disfunção da proteína CFTR e/ou presença de duas mutações de *CFTR*. Caso um dos elementos do fenotípico clínico seja característico da FC, o TS deve ser realizado. (Farrell *et al.*, 2017)

Pacientes com valor de íon cloreto maior que 30 mEq/L e menor que 60 mEq/L devem ser submetidos a análise genética de *CFTR* e/ou outros métodos de análise funcional da CFTR (Farrell *et al.*, 2017).

O ideal é que pacientes com FC tenham o TS e análise genética realizados, principalmente, quando o TS apresenta resultados limítrofes (30-59mEq/L). Raramente indivíduos com TS inferior a 30 mEq/L terão FC, a não ser que, ocorra diagnóstico por métodos diferenciais (genético e da função da CFTR) (Farrell *et al.*, 2017).

Apesar da publicação dos *guidelines* para padronizar o TS, vários relatórios indicam que a consistência e confiabilidade do TS é baixa em numerosos laboratórios, descrevendo falta de adesão aos *guidelines*, baixo controle de qualidade e número de testes realizados anualmente, bem como, inconsistências no volume mínimo de suor a ser utuilizado para quantificar o íon cloreto (LeGrys *et al.*, 2001; LeGrys *et al.*, 2009; Liu *et al.*, 2010; Servidoni *et al.,* 2017).

Embora o TS seja o padrão ouro para o diagnóstico da FC, algumas dificuldades podem ser realçadas: (i) pequenas quantidades de suor inviabilizam o TS; (ii) falta de equipe treinada e de conhecimento dos protocolos para se executar corretamente a realização e interpretação do TS; (iii) falta de equipamentos adequados para a indução de sudorese e quantificação iônica. Devido a essas dificuldades, a busca de outras técnicas, tem sido estimulada para maximizar o diagnóstico de FC. Além disso, deve-se considerar a viabilidade no TS, por exemplo, a taxa de suor é menor no sexo feminino quando comparado ao masculino em virtude de diferenças constitutivas. No sexo masculino ocorre menor número de glândulas sudoríparas ativas, com maior taxa de suor por glândula (Behm *et al.*, 1987), e esse fator deve ser avaliado no TS e por outras ferramentas que quantificam a função da CFTR.

Na medicina de precisão, a correção dos defeitos da CFTR, para restaurar o transporte de íons é uma abordagem terapêutica promissora em indivíduos com FC, exigindo

biomarcadores para avaliar a função da CFTR. Os métodos mais usados para avaliar a função de CFTR são o TS e a diferença de potencial nasal (DPN).

As drogas moduladoras da CFTR [por exemplo, Ivacaftor (Kalydeco®) e a combinação de Ivacaftor e Lumacaftor (Orkambi®)] podem potencializar o transporte do íon cloreto. O uso com sucesso dessas drogas foi descrito em 11 mutações de *CFTR* que englobam ~60% dos pacientes com FC (Accurso *et al.*, 2014; Collaco *et al.*, 2016). No entanto, os resultados ainda estão longe de acarretar na total correção da CFTR e na ausência de fenótipos clínicos da doença.

Expandir o uso de corretores, potencializadores e moduladores da CFTR para todos os pacientes com FC representa um desafio por numerosos motivos como: (i) 40% dos pacientes com FC possuem mutações de *CFTR* de baixa frequência populacional; (ii) são necessários biomarcadores precisos para medir a função CFTR, uma vez que, a medicina de precisão pode não fornecer melhora clínica equivalente para todas as mutações de *CFTR*; (iii) como realizar estudos clínicos para mutações de *CFTR* com poucos pacientes com determinada mutação? (Accurso *et al.,* 2014; Collaco *et al*., 2016).

A avaliação da função pulmonar é importante em ensaios clínicos de medicina de precisão, uma vez que, a doença pulmonar é a principal causa de morbidade e mortalidade na FC. No entanto, a variabilidade da função pulmonar entre pacientes da mesma idade e com o mesmo genótipo *CFTR* é elevada, dificuldade a análise dos desfechos ao uso da medicina de precisão (Collaco *et al.*, 2016).

Estudos têm demonstrado a correlação entre as concentrações de eletrólitos no TS e a presença de diferentes classes de mutações do *CFTR* (McKone *et al.,* 2003; Bonadia *et al.,* 2014). No entanto, os achados são pontuais e outros estudos devem ser realizados.

A função da CFTR, após o uso da medicina de precisão, em estudos clínicos, tem sido avaliada de modo direto ou indireto pela função pulmonar, TS, tomografia computadorizada de

tórax e questionário de qualidade de vida. Os marcadores avaliados são motivos de críticas (Collaco *et al.*, 2016) e a melhoria no desfecho fenotípico é limitada.

1.4. Outros métodos para medir a função da CFTR

1.4.1. Diferença bioelétrica na glândula sudorípara – evaporimetria

Glândulas sudoríparas secretam suor via estímulo adrenérgico e colinérgico. O estímulo via colinérgica é responsável pelo controle da temperatura intrínseca e possui atividade normal nos indivíduos com ou sem FC. O estímulo via beta adrenérgica é mediado pelo AMPc e estimula a secreção de suor proporcionalmente a atividade da proteína CFTR (Sato e Sato, 1984; Behm *et al.*, 1987; Reddy e Quinton, 1992).

A evaporimetria quantifica a taxa de suor na pele pelo evaporímetro e está sendo estudada para avaliar a função de CFTR no diagnóstico, prognóstico e resposta na medicina de precisão na FC (Quinton *et al.*, 2012).

Em 1980, Sato e Sato, investigaram a secreção da glândula sudorípara na FC e observaram que, diferentemente de indivíduos saudáveis, na FC não havia sudorese induzida pelo estímulo beta adrenérgico, mas havia sudorese normal, pelo colinérgico. Além disso, a sudorese via beta adrenérgica em indivíduos heterozigotos para mutações de *CFTR*, era de ~50% quando comparado aos indivíduos sem mutações de *CFTR* (Behm *et al.*, 1987).

Quinton e colaboradores (2012) avaliaram a sensibilidade, especificidade e confiabilidade da evaporimetria considerando a atividade da função secretora da CFTR nas glândulas sudoríparas de três grupos: (i) indivíduos saudáveis; (ii) pais de pacientes com FC (portadores de uma mutação de *CFTR*); (iii) pacientes com FC. Dessa forma, foi descrito que a secreção colinérgica é igual nos três grupos para o sexo masculino e feminino. Em contrapartida, a secreção de suor por estímulo via beta adrenérgica foi diferente entre os três grupos avaliados, sendo maior nos saudáveis, intermediária nos indivíduos com uma mutação

de *CFTR* e baixa nos pacientes com FC (**Figura 5**). Dessa forma, a evaporimetria pode avaliar, *in vivo*, na FC, três aplicações: (i) diagnóstico de FC; (ii) presença de heterozigose; (iii) eficácia da medicina de precisão.

A – Homens B - Mulheres

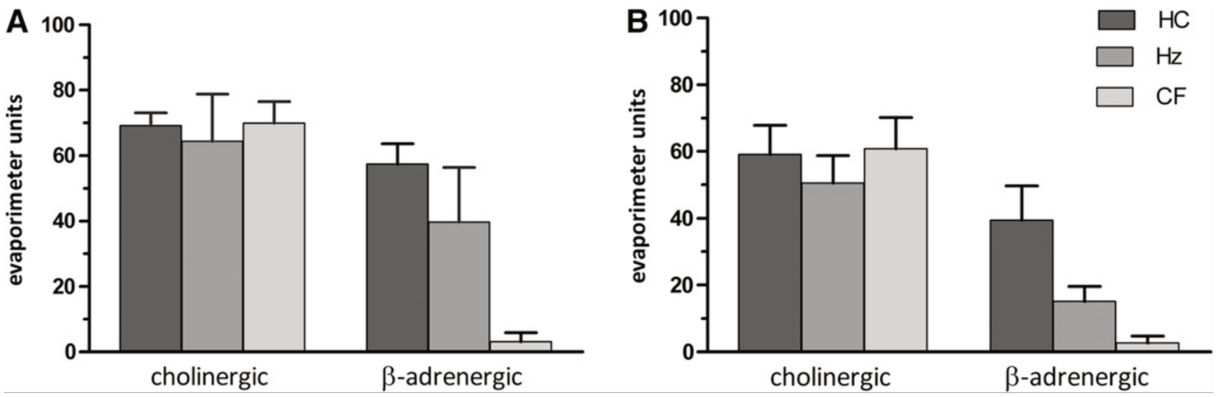

Figura 5 – resposta secretora de suor ao estímulo beta adrenérgico e colinérgico em indivíduos saudáveis (HC = *healthy Control*), heterezigotos (Hz) e pacientes com FC (CF, *cystic fibrosis*) considerando o sexo (A = homens e B = mulheres) (Retirado: Quinton *et al.*, 2012).

Uma limitação do estudo de Quinton e colaboradores (2012) foi a não inclusão de bebês e crianças pequenas devido a necessidade de estímulo subcutâneo com subsequentes injeções. Porém, Salinas e colaboradores (2017), realizaram, posteriormente, o primeiro estudo para avaliar a sudorese pelo estímulo beta adrenérgico em crianças pré-escolares. No estudo, crianças de quatro a seis anos, com teste de triagem neonatal positivo para FC foram avaliadas e o protocolo de evaporimetria foi modificado para a aplicação de uma única injeção. A avaliação de secreção beta adrenérgica foi segura e confiável em crianças pré-escolares e como foi utilizado uma única injeção por criança, não foi possível potencializar a resposta beta adrenérgica pelo estímulo pré-colinérgico. No entanto, não houve diferença no pico da resposta beta adrenérgica, sugerindo que a estimulação pré-colinérgica pode ser desnecessária para estimular a sudorese na evaporimetria e, portanto, uma única injeção pode ser o suficiente em crianças menores.

Na evaporimetria, a atividade da CFTR é detecta em quantidades mínimas de suor. Na sonda teste é realizada a injeção intradérmica de carbacol, que estimula inicialmente a sudorese via colinérgica, em seguida, essa via é inibida pela atropina, e por fim, é realizada a aplicação do *beta coktail* composto por isoproterenol, aminofilina e atropina, que estimula a CFTR. Na sonda controle é realizada a injeção de atropina no início do exame (Behm *et al.*, 1987; Quinton *et al.,* 2012).

Comparando a fase colinérgica e beta adrenérgica, na FC, não ocorre sudorese após o estímulo com o *beta coktail*, indivíduos com uma mutação de *CFTR* apresentam ~50% de sudorese na fase beta adrenérgica quando comparada a colinérgica e indivíduos sem mutação do *CFTR* possuem sudorese semelhante em ambas as fases (Quinton, 2012).

A evaporimetria tem potencial como teste de diagnóstico e, possivelmente, como biomarcador da CFTR em ensaios clínicos. Além disso, como o potencial elétrico do ducto de suor reflete diretamente a função da CFTR, esta técnica avalia o impacto da medicina de precisão em ensaios clínicos que incluem a correção do defeito na condutância do íon cloreto. Embora a técnica seja promissora, algumas limitações devem ser citadas e incluem: (i) necessidade de padronização de protocolos para administração do teste e interpretação dos resultados; (ii) falta de equipamentos; (iii) falta de profissionais especializados.

1.4.2. Diferença de potencial nasal (DPN)

A DPN mede a função da CFTR no epitélio nasal e foi desenvolvida nos últimos 30 anos (Hug *et al.*, 2011). A DPN pose ser utilizada como teste de diagnóstico para FC desde que foi aceita como procedimento complementar ao TS, principalmente, em indivíduos com valores de íons cloreto no suor entre 30 e 60 mEq/L (Farrel *et al.,* 2017).

A DPN mede *in vivo* a diferença de potencial entre o lado interno e externo da célula epitelial da mucosa nasal, o qual é correlacionado à diferença de potencial da mucosa brônquica

(Knowls *et al.*, 1983; Davies *et al.*, 2005). Na FC, os defeitos de CFTR resultam no potencial bioelétrico alterado dos ductos sudoríparos, aproximadamente, 10 vezes mais negativo que o normal (Quinton, 1983). O mesmo comportamento ocorre no epitélio das vias aéreas e é reproduzido na mucosa da cavidade nasal na FC (Knowls *et al.*, 1983).

Nos indivíduos saudáveis, o potencial basal, é mantido pelo balanceamento da absorção do íon sódio e transporte do íon cloreto, resultando em um controle da quantidade de líquido na superfície da via aérea e seu conteúdo iônico (Schüler *et al.*, 2004). O valor encontrado relaciona-se ao transporte de íons pela membrana celular, em especial os íons sódio e cloreto, sendo o lado mucoso mais negativo em relação ao interior da célula. Valores normais se situam em torno de -20 mV. Valores de DPN de -30 mV ou mais negativos são aceitos como diagnóstico de FC (Wilschanski *et al.*, 2006). Dessa forma, a DPN pode diferenciar pacientes com FC dos saudáveis, heterozigotos para mutações de *CFTR,* pacientes com outras doenças respiratórias e entre pacientes com FC e diferentes classes de mutações de *CFTR* (Ng *et al.,* 2015; Delmarco *et al.*, 1997; Rosenstein e Cutting, 1998; Procianoy, 2011). A DPN é importante em casos de dúvida de diagnósitco por demonstrar uma função CFTR normal, excluindo o diagnósitco de FC (Wilschanski *et al.*, 2001; Middleton e Hose, 2010; Ng *et al.*, 2015; Farrell *et al.,* 2017).

Os achados na DPN na FC incluem a despolarização maior do potencial em resposta à perfusão do epitélio com solução contendo amiloride – droga bloqueadora do canal de sódio, e ausência ou menor hiperpolarização do potencial em resposta ao estímulo da secreção do íon cloreto pelo uso de potencializadores do AMPc, como o isoproterenol (Middleton e House 2010; Procianoy, 2011).

Em 2006, a *European Cystic Fibrosis Society* enfatizou a importância da DPN como ferramenta para o diagnóstico de FC (De Boeck *et al.*, 2006). A confirmação contrasta com o consenso da CFF de 2008 (Farrell *et al.*, 2008), que aceitou a DPN como teste complementar

devido à falta de validação, valores de referência e protocolos padronizados para medir a DPN (Farrell *et al.*, 2008). Os valores de referência da DPN precisam ser definidos e devem considerar idade, sexo, classes de mutações de *CFTR*, e respostas entre as duas narinas.

1.4.3. Eletrofisiologia em biópsia retal

A CFTR é expressa em elevada quantidade no epitélio intestinal, na membrana luminal dos enterócitos, principalmente no reto, sendo acessível para estudo (Mall *et al.*, 2000; Servidoni *et al.*, 2013). A medida bioelétrica do epitélio intestinal do reto é um método de alta especificidade e sensibilidade, minimamente invasivo e é promissora como ferramenta de diagnóstico em pacientes com formas atípicas de FC (Cymberknoh *et al.*, 2013).

Nos últimos 20 anos, micro câmeras de Ussing foram desenvolvidas para estudar o defeito no transporte de íons dos tecidos intestinais na FC (Mall *et al.*, 2004). Desde então, técnicas de eletrofisiologia foram desenvolvidas e refinadas para o diagnóstico de FC, na presença de sintomas leves ou subclínicos, e resultados de TS ambíguos ou limítrofes, ou para pacientes com mutações de *CFTR* desconhecidas ou raras (Sloane e Rowe, 2010; Derichs *et al.*, 2010; Cymberknoh *et al.*, 2013; Servidoni *et al.*, 2013). Além disso, a medida biolétrica do epitélio intestinal foi aplicada em estudos de genótipo-fenótipo para avaliar a influência de genes modificadores no epitélio intestinal nativo (Hirtz *et al.*, 2004).

Na FC, no intestino, a atividade iônica não é associada ao efeito de inflamação, hemorragia ou infecção, como nas vias aéreas, e a análise da função de CFTR é promissora (Hug *et al.*, 2011; Servidoni *et al.*, 2013). Cymberknoh e colaboradores (2013) avaliaram a biópsia retal em três grupos: pacientes com FC, saudáveis e indivíduos com suspeita da doença. Foi observado que a biópsia retal é um método seguro e que pode ser utilizado no diagnóstico na presença de quadros atípicos da doença, principalmente, em bebês e crianças.

Derichs e colaboradores (2010) estabeleceram valores de referência para a biópsia retal e demonstraram que o método é confiavel para o diagnóstico de FC para toda faixa etária e fenótipos de FC, mesmo na presença de TS com resultado duvidoso e/ou mutações de *CFTR* com função residual. Outros estudos exploraram a possibilidade de expansão de técnicas de eletrofisiologia para a validação da medicina de precisão. Uma das propostas envolve o uso de organóides intestinais – que utiliza epitélio cultivado – ao invés de biópsia. A técnica permite a correlação das mutações de *CFTR* com a respectiva função da CFTR, bem como, a análise do efeito de novas drogas no canal CFTR (Dekkers *et al.*, 2013; Servidoni *et al.*, 2013).

1.4.4. Glândula salivar

A composição de saliva está alterada em numerosas condições fisiológicas e patológicas. Por esse motivo, a saliva pode ser ferramenta útil no diagnóstico de algumas doenças (Pfaffe *et al.*, 2011). Além disso, a CFTR e os canais de sódio desempenham papel importante nos ductos das glândulas salivares, similarmente ao das glândulas sudoríparas, como demonstrado em modelo animal p.Phe508del/p.Phe508del (Catalan *et al.*, 2010).

A saliva é produzida por quatro glândulas: parótida (secreção serosa), oral (secreção mucosa), submandibular e sublingual (secreção serosa e mucosa). A secreção primária no ácino das glândulas tem concentração iônica similar ao fluido intersticial e plasmático. Ao longo dos ductos, a absorção de íons e secreção e/ou movimento passivo em direções opostas reduz os níveis dos íons sódio e cloreto, e ocorre aumento do íon potássio e bicarbonato (Gonçalves *et al.*, 2013).

Os parâmetros bioquímicos da saliva na FC e os métodos de análise de concentração iônica foram avaliados em alguns estudos (Fritz *et al.*, 1972; Blomfield *et al.*, 1973; Kollberg *et al.*, 1982; Jimenez-Reyes e Sanches-Aguirre, 1982; Gonçalves *et al.*, 2013). O perfil das

concentrações dos íons sódio e cloreto na saliva, sugere que esses indicadores podem ser uma ferramenta para o diagnóstico de FC (Pfaffe *et al.*, 2011; Gonçalves *et al.*, 2013).

Os estudos que avaliam o efeito da FC no funcionamento das glândulas salivares têm resultados conflitantes, no entanto, demonstram que na FC ocorre alteração no fluxo da saliva (Blomfield *et al.*, 1973; Catalán *et al.*, 2011). Porém, as alterações podem estra relacionadas aos componentes químicos usados para estimular o fluxo da saliva e, que podem alterar o volume, pH e parâmetros bioquímicos (Gonçalves *et al.*, 2013).

Além disso, na literatura, a hiposalivação foi associada a numerosas causas, incluindo: medicações, doenças autoimunes, desordens endócrinas, doenças genéticas (por exemplo a FC), desnutrição e infecções (Pedersen *et al.*, 2002). Existem alterações conflitantes na mudança do fluxo de saliva na FC, porém, o número de pacientes avaliados é pequeno, o que inviabiliza a conclusão sobre a saliva como método de diagnóstico.

As limitações dos estudos da função da CFTR na glândula salivar incluem: (i) diferentes métodos para coletar a saliva; (ii) uso de diferentes ferramentas para quantificar os íons na saliva; (iii) populações pequenas. Desse modo, são necessários estudos com maior número de indivíduos, com a comparação entre diferentes métodos para quantificar os íons do conteúdo da saliva, juntamente a realização do TS, na FC.

1.5. O gene *CFTR* versus a proteína CFTR

A medicina de precisão é uma realidade na FC, e dessa forma, é importante identificar as mutações do *CFTR*. A triagem genética pode ser realizada de diferentes maneiras, porém, inicialmente se aconselha a análise da mutação p.Phe508del, seguido de um painel padrão de mutações do *CFTR* que contém as mutações mais comuns (~80 a 85% das mutações). Quando duas mutações de *CFTR* não são identificadas e o diagnóstico de FC é quase certo (TS com cloreto acima de 60 mEq/L) ou altamente provável (com quadro clínico sugestivo), o segundo

passo é o sequenciamento completo do *CFTR* e avaliação de deleções e/ou inserções (Marson *et al.*, 2013; De boeck *et al.*, 2017).

A análise das mutações mais frequentes do *CFTR* nem sempre é a resposta para os dilemas no diagnóstico da FC. Por outro lado, a análise completa das mutações pode revelar que o potencial patogênico de muitas mutações ainda é incerto.

O encontro da mutação p.Phe508del, é frequente e ocorre em 50 a 90% dos alelos de pacientes com FC, dependendo da população estudada. Na maioria dos países, seis a oito mutações têm frequência de aproximadamente 1% e as outras mutações são muito mais raras (Sosnay *et al.*, 2013; De Boeck *et al.*, 2017).

Diferentes mutações do *CFTR*, causam diferentes alterações na função da CFTR propiciando em um espectro de distúrbios, que nem sempre podem ser diferenciados pelas ferramentas de diagnóstico existentes e/ou disponíveis. Os fatores que sustentam a falta de precisão das ferramentas para avaliar a CFTR podem estar relacionados com o genótipo do indivíduo (influência de mutações do *CFTR* e variabilidade biológica) ou limitações no próprio teste (Collie *et al.*, 2014).

Embora, a busca de marcadores da função da CFTR tem sido estimulada e constitua um promissor campo de pesquisa (Esteves *et al.*, 2018), dificilmente teremos um biomarcador tão sensível, específico e valorizado pela comunidade científica, como tem sido o TS, nas últimas décadas, para avaliar a função da CFTR. Apesar da importância do TS, a análise das mutações se consolida, cada vez mais, como o único marcador de certeza para o diagnóstico de FC.

2. Justificativa

No nosso centro de referência em FC, o TS é realizado desde 1970, pela técnica de Gibson & Cooke. Porém, há três décadas as variáveis clínicas, valores de eletrólitos e indicações para a realização do TS, dos indivíduos que fizeram o TS, foram registradas e propiciou na coleta de dados para a realização da tese.

No Brasil, ainda existem limitações para o diagnóstico da FC. Em muitos centros faltam equipamentos e/ou métodos adequados para realização do TS. Além disso, os centros que realizam o TS, nem sempre, seguem as diretrizes internacionalmente aceitas (Servidoni *et al.*, 2017).

Em locais onde o TS é realizado com os métodos corretamente aplicados, uma série de informações podem ser obtidas sobre a compreensão da função da CFTR que propiciam na melhor avaliação clínica e laboratorial para a equipe local e de outros centros. O conhecimento dos desfechos entre a análise das variáveis demográficas, clínicas e laboratoriais, e o TS, permitem a avaliação do entendimento dos diferentes fenótipos da FC, variação do valor do íon cloreto com a idade, diferença entre os sexos e dados epidemiológicos relacionados a solicitação do TS e propiciam em ações de saúde para a comunidade dos centros de referência em FC.

3. Objetivo

3.1. Objetivo geral

Apresentar e discutir três estudos relacionados ao TS para o diagnóstico de FC relacionados a:

(i) qualidade do TS;

(ii) variabilidade dos níveis do íon cloreto no suor conforme a idade;

(iii) associação dos resultados do TS com dados demográficos, clínicos e laboratoriais.

3.2. Objetivos específicos

- Artigo 1 – Verificar a qualidade do TS pela proporção entre os íons sódio e cloreto;
- Artigo 2 – Avaliar como o íon cloreto no suor varia com a idade na população estudada e comparar com os dados da literatura;
- Artigo 3 – Realizar análise descritiva do TS associando os resultados com variáveis demográficas, clínicas e laboratoriais, incluindo mutações do *CFTR*, razões para indicação do TS, assim como, correlação entre as concentrações dos íons sódio e cloreto.

4. Método

4.1. Desenho dos estudos

Realizou-se a análise de dados de três estudos (artigos 1, 2 e 3) caracterizados como desenho retrospectivo, utilizando o banco de dados do TS contendo o peso do suor (mg), valores dos íons sódio e cloreto, indicação para o exame do TS, sexo dos indivíduos e outras variáveis obtidas nos prontuários de pacientes com FC, do Centro de Referência em FC do Hospital Universitário da Unicamp. Os exames do TS foram realizados entre 1986 e 2016, totalizando 30 anos de monitoramento.

4.2. Seleção dos participantes

Foram obtidos os resultados de 5.721 amostras de TS que fizeram a análise de eletrólitos. Os TS foram realizados pela técnica de Gibson e Cooke, no laboratório de Gastroenterologia Pediátrica e no setor de Gastroenterologia da Universidade Estadual de Campinas (Unicamp).

4.3. Critérios de inclusão

Foram considerados para os três estudos: nome, idade do paciente no momento do exame, sexo, idade do sujeito, indicação do TS (sintoma respiratório, digestivo, nutricional), mutações de *CFTR,* valores do tripsinogênio imunorreativo feito pela triagem neonatal, peso da amostra de suor e níveis dos íons sódio e cloreto no TS (mEq/L).

4.4. Critérios de exclusão

Foram excluídas amostras seguindo os seguintes critérios: peso no suor abaixo de 75 mg ou acima de 400 mg; níveis do íon cloreto inferior a 10 mEq/L ou superior a 160 mEq/L;

níveis do íon sódio inferior a 10 mEq/L ou superior a 150 mEq/L e/ou ausência de dados descritivos (peso do suor e concentração do íon sódio).

4.5. Descrição da amostra

Nos três estudos os pacientes foram divididos de acordo com a idade em três grupos: (i) do nascimento até < seis meses; (ii) ≥ seis meses e < 18 anos; (iii) ≥ 18 anos.

Os níveis do íon cloreto foram usados nos grupos de amostras de acordo com diagnóstico de FC, sendo cloreto (mEq/L): (i) < 30; (ii) ≥ 30 e < 60; (iii) ≥ 60 (grupo de pacientes com FC).

4.6. Identificação das mutações de *CFTR*

Nos três estudos, as mutações do *CFTR* foram analisadas pela reação em cadeia da polimerase para a mutação p.Phe508del e digestão enzimática para G542X, R1162X, R553X, G551D e N1303K. Outras mutações do *CFTR* foram identificadas por sequenciamento ou pela técnica SALSA MLPA (*Multiplex Ligation – dependente probe Amplification*) Kit P091-CI CFTR - MCR Holland: S4X, 2183A>G, 1717G>A, I618T com MegaBace1000® (GE Healthcare Biosciences, Pittsburgh, USA) e ABI 3500® (Applied Biosystems-Thermo Fisher Scientific, São Paulo, Brasil).

Considerando os genótipos do *CFTR*, os pacientes foram divididos em três grupos:

(i) duas mutações identificadas pertencentes as classes I, II e/ou III;

(ii) uma mutação identificada pertencente a classe I, II ou III;

(iii) nenhuma mutação identificada pertencente as classes I, II e/ou III. Outras mutações identificadas do *CFTR* pertencentes as classes IV, V e/ou VI não foram incluídas na análise estatística. A classificação das mutações do *CFTR* foi feita de acordo com a literatura (Marson *et al.*, 2016).

4.7. Análise estatística

Foram analisados todos os exames solicitados para um mesmo paciente, mesmo quando um paciente realizou mais de um exame. Além disso, os estudos abordaram o TS propriamente dito e não a prevalência de FC na casuística estudada.

Utilizou-se análise descritiva com número de observações, média, desvio padrão, mediana, mínimo e máximo para variáveis contínuas. O intervalo de confiança de 95% foi calculado para as proporções. Para variáveis categóricas os dados estão apresentados pela frequência e porcentagem.

A análise estatística foi realizada no *software* SPSS (St*atistical Package for Social Sciences*) versão 21.0. A comparação entre as variáveis com distribuição categórica foi realizada pelos testes de χ^2 e Exato de Fisher, dependendo da distribuição dos dados. Na análise para variáveis com distribuição numérica foram utilizados os testes Exato de Fisher e análise de variância de uma via, e quando necessário, foram aplicados os testes não paramétricos correlatos, Mann-Whitney e Kruskal-Wallis. Para as análises realizadas foi adotado alpha de 0,05. O poder da amostra foi superior a 80%.

O teste de regressão linear e o teste de correlação de Spearman foram utilizados, quando necessário.

5. **Resultados**

Esta tese foi escrita de acordo com o modelo alternativo, conforme as normas do curso de Pós-graduação em Saúde da Criança e do Adolescente da Faculdade de Ciências Médicas (FCM) – Unicamp, desta forma, os resultados foram apresentados em artigos publicados pela doutoranda durante o período do seu doutorado.

5.1. ARTIGO 1 – ARTIGO ORIGINAL

Faria AG, Marson FAL, Gomez CCS, Ribeiro MAGO, Morais LB, Servidoni MF, Bertuzzo CS, Sakano E, Goto M, Paschoal IA, Pereira MC, Hessel G, Levy CE, Toro AA, Peixoto AO, Simões MC, Lomazi EA, Nogueira RJ, Ribeiro AF, Ribeiro JD. Quality of sweat test (ST) based on the proportion of sweat sodium (Na) and sweat chloride (Cl) as diagnostic parameter of cystic fibrosis: are we on the right way? Diagn Pathol. 2016;11(1):103.

A autorização da Editora para inclusão do artigo na tese, encontra-se no anexo 1.

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Quality of sweat test (ST) based on the proportion of sweat sodium (Na) and sweat chloride (Cl) as diagnostic parameter of cystic fibrosis: are we on the right way?

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Abstract

Background: To assess the quality of sweat test (ST) based on the proportion of sweat sodium and sweat chloride as diagnostic parameter of cystic fibrosis (CF).

Methods: A retrospective study of 5,721 sweat samples and subsequent descriptive analysis were carried out. The test was considered "of good quality" (correct) when: (i) sweat chloride was lower than 60 mEq/L, and sweat sodium was higher than sweat chloride; (ii) sweat chloride was higher than 60 mEq/L, and sweat sodium was lower than sweat chloride.

Results: The study included 5,692/5,721 sweat samples of ST which had been requested due to clinical presentations compatible with CF and/or neonatal screenings with altered immunoreactive trypsinogen values. Considering the proportion of sweat sodium and sweat chloride as ST quality parameter, the test was performed correctly in 5,023/5,692 (88.2 %) sweat samples. The sweat chloride test results were grouped into four reference ranges for chloride (i) chloride < 30 mEq/L: 3,651/5,692 (64.1 %); (ii) chloride ≥ 30 mEq/L to < 40 mEq/L: 652/5,692 (11.5 %); (iii) ≥ 40 mEq/L to < 60 mEq/L: 673/5,692 (11.8 %); (iv) ≥ 60 mEq/L: 716/5,692 (12.6 %). In the comparative analysis, there was no association between ST quality and: (i) symptoms to indicate a ST [respiratory ($p = 0.084$), digestive ($p = 0.753$), nutritional ($p = 0.824$), and others ($p = 0.136$)], (ii) sweat weight ($p = 0.416$). However, there was a positive association with: (i) gender, (ii) results of ST ($p < 0.001$), (iii) chloride/sodium ratio ($p < 0.001$), (iv) subject's age at the time of ST [grouped according to category ($p < 0.001$) and numerical order ($p < 0.001$)]. For the subset of 169 patients with CF and two *CFTR* mutations Class I, II and/or III, in comparative analysis, there was a positive association with: (i) sweat chloride/sodium ratio ($p < 0.001$), (ii) sweat chloride values ($p = 0.047$), (iii) subject's age at the time of the ST grouped by numerical order ($p = 0.001$).

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Conclusions: Considering that the quality of ST can be assessed by levels of sweat sodium and sweat chloride, an increasing number of low-quality tests could be observed in our sweat samples. The quality of the test was associated with important factors, such as gender, CF diagnosis, and subjects' age.

Keywords: Cystic fibrosis, Diagnosis, Electrolytes, Sweat

Background

The sweat test (ST) is considered the gold standard for the diagnosis of cystic fibrosis (CF) [1]. Around half a century has passed since its description; however, questions remain about its reproducibility and reliability, especially in cases of borderline sweat results [2]. There are only a few quality parameters to perform ST. In addition, the role of sweat sodium as quality marker for ST is unknown.

Increased chloride values observed in ST are due to mutations in the *CFTR* gene (Cystic Fibrosis Transmembrane Conductance Regulator), which encodes a protein with the same name [3]. A conclusive diagnosis of CF can be made with the identification of two mutations in the *CFTR* gene [4, 5]. It is not always possible to conduct genetic tests and perform complete *CFTR* gene sequencing for all patients, due to high costs and/or technical limitations. And yet, ST has been widely used as a tool for the diagnosis of CF for over 50 years.

Although ST has high sensitivity and specificity, it may produce inconsistent results. Analysis of ST alone may be insufficient to diagnose CF. Therefore; additional tests should be performed, such as nasal potential difference measurement, assessment of CFTR function in rectal biopsies, and evaporimetry [6–9]. Patients with CF usually have low sodium conductance, and consequently, elevated sodium concentration in sweat. This is because the activity of the epithelial sodium channel depends on the activity of the CFTR protein [10]. In CF diagnosis, sodium has a poor discriminatory power in comparison with chloride, even with an existing correlation between their levels in sweat [1, 11, 12]. Current protocols do not recommend the use of concentrations of sweat sodium as a diagnostic parameter for CF and/or as a quality marker to perform exams [12]. Although not used for CF diagnosis, concentrations of sweat sodium are analyzed by some laboratories, and used as an internal quality control procedure, since concentrations of chloride and sodium tend to be similar [1, 2, 11]. The use of chloride/sodium ratio has been proposed in order to screen patients with CF and borderline values in the ST. However, there is no consensus on this quality parameter [2].

This study aimed to verify the quality of ST based on the levels of sweat sodium and sweat chloride measured in ST of subjects with and without CF, performed in a referral center for a period of approximately 30 years with the same sweat dosage method.

Methods

A retrospective study of 5,721 sweat samples and ST descriptive analysis were carried out. The ST was considered "of good quality" (correct) when: (i) sweat chloride was lower than 60 mEq/L, and sweat sodium was higher than sweat chloride in subjects without CF; (ii) sweat chloride was higher than 60 mEq/L, and sweat sodium was lower than sweat chloride in patients with CF (Fig. 1).

The concentrations of chloride and sodium in sweat were determined by chloridrometry and flame photometry, respectively. The collection of sweat was performed by the classical Gibson & Cooke method [13].

The study was approved by the Ethics Committee from University of Campinas (Unicamp) (# 474326). The variables were collected from records of ST performed in the laboratories of the center for Gastroenterology Services and Pediatric Gastroenterology at the University Hospital of the Unicamp.

The patients' medical records included: name, age at time of the examination, gender, medical record number, indications for ST (pulmonary, digestive, nutritional and/or others), family history of CF, weight of collected sweat sample, concentrations of sweat chloride and sweat sodium, and the chloride/sodium ratio in the sweat samples. Tests with sweat weight lower than 75 mg were excluded.

Patients were grouped into three categories according to age: (i) birth to < six months; (ii) ≥ 6 months to <18 years; (iii) ≥ 18 years [2]. The concentration of sweat chloride was used to group the sweat samples according to the CF diagnosis, as follows: (i) chloride < than 30 mEq/L; (ii) chloride ≥ 30 mEq/L to < 40 mEq/L; (iii) chloride ≥ 40 mEq/L to < 60 mEq/L; (iv) ≥ 60 mEq/L (positive test for CF) [14].

All test ordered for the same patient were analyzed, even when a patient had undergone more than one test. The study approached ST and not the result of the prevalence of CF in the samples.

For a subset of 169 patients with CF, the *CFTR* mutation screening was positive for two *CFTR* mutation Class I, II and/or III. The samples with two *CFTR* mutation Class I, II and/or III were analyzed individually. Mutations of *CFTR* were analyzed by polymerase chain reaction (PCR) (F508del) followed by enzymatic digestion (G542X, R1162X, R553X, G551D and N1303K) – [PCR/restriction fragment length polymorphism (RFLP)].

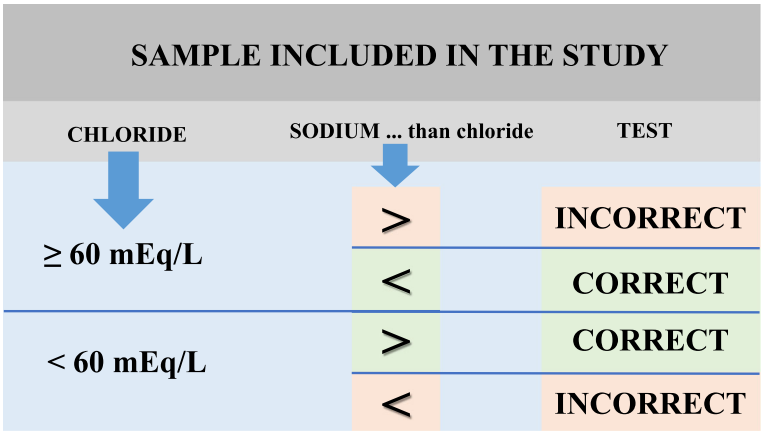

Fig. 1 Sweat test quality by the chloride sweat and sodium sweat (proposed criteria)

Other mutations in the *CFTR* gene could be identified by sequencing or use of the SALSA MLPA method (Multiplex Ligation-dependent Probe Amplification) Kit P091-C1 CFTR-MRC-Holland S4X, 2183A > G, 1717-G > A, I618T with MegaBace1000® (GE Healthcare Biosciences, Pittsburgh, USA) and ABI 3500 (Applied Biosystems - Thermo Fisher Scientific, São Paulo/SP, Brazil).

Descriptive statistics including numbers of observations, means, standard deviations, medians, minimums and maximums were used to summarize continuous variables. Confidence intervals (95 %) were calculated for proportions. Categorical data was presented as tables of frequency counts and associated percentages.

Statistical analysis was performed using SPSS software (Statistical Package for Social Sciences) version 22.0 (SPSS Inc., Chicago, IL, USA) [15]. The comparison between variables with categorical distribution was carried out by Test χ^2 (Pearson and Likelihood ratio) and Fisher's exact test, depending on the data distribution. For the analysis of variables with numerical distribution, Fisher's exact test and one-way analysis of variance were used. Non-parametric statistical test, such as Mann-Whitney and Kruskal-Wallis, were applied when necessary. The graphics and identification of difference between the groups obtained by Kruskal-Wallis test were performed in MedCalc® for Windows, version 16.1 (MedCalc® Software, Ostend, Belgium). $\alpha = 0.05$ was set for all analyses. The GPower software version 3.1.9.2 (Moorenstraße, Düsseldorf, Germany) [16, 17] was used to calculate the power of the sample adopting power value above 80 %.

Results

The study included 5,721 sweat samples of ST, which had been requested due to clinical presentation compatible with CF and/or neonatal screening with altered immunoreactive trypsinogen values. Of these sweat samples, 29 (0.51 %) were excluded: (i) 23 showed sweat weight lower than 75 mg; (ii) four had no indication about sweat weight; (iii) one lacked laboratory data; (iv) one had no sodium value. Thus, 5,692 sweat samples were included in this study. The gender of 17 subjects (0.3 %) could not be determined, as the tests had been carried out with the newborn's mother's name, after newborn screening. So, 3,023 sweat samples collected from males (53.3 %) and 2,652 (46.7 %) sweat samples collected from females were included and analyzed.

The mean age of the subjects was 12.12 ± 17.84 years; and median 4 (ranging from 0 to 85.58) years. In 146 (2.6 %) sweat samples, there was no record to confirm the exact age at the time of the examination. The following frequency was obtained for each age group: (i) ≤ 0 to 6 months: 634/5,611 (11.33 %) samples; (ii) > 6 months to ≤ 18 years: 3,897/5,611 (69.5 %) samples; (iii) > 18 years: 1,080/5,611 (19.2 %) samples.

The mean sweat chloride concentration was 32.45 ± 27.67 mEq/L, median 22.30 (ranging from 1 to 213.10) mEq/L. The mean sweat sodium level was 36.45 ± 21.56 mEq/L, median 29.5 (ranging from 6.30 to 154.70) mEq/L. Clinical indications for having the sweat test performed included (i) breathing symptoms: 2,920/3,791 (77 %); (ii) digestive symptoms: 464/3,791 (12.2 %); (iii) nutritional symptoms: 435/3,791 (11.5 %); (iv) others: 467/3,791 (12.3 %). The initial medical request for ST of 1,901/5,692 (33.4 %) sweat samples could not be obtained. The sweat chloride/sodium ratio showed a mean level of 0.821 ± 0.250; median of 0.799 (ranging from 0.06 to 2.51).

Considering the quality of the ST based on the proportion of sweat chloride and sweat sodium, the test was performed correctly in 5,023/5,692 (88.2 %) samples, and incorrectly in 669/5,692 (11.8 %).

The sweat samples were grouped into four reference ranges for chloride and their respective interpretative

comments: (i) chloride < 30 mEq/L: 3,651/5,692 (64.1 %); (ii) chloride ≥ 30 mEq/L to < 40 mEq/L: 652/5,692 (11.5 %); (iii) chloride ≥ 40 mEq/L to < 60 mEq/L: 673/5,692 (11.8 %); (iv) chloride ≥ 60 mEq/L: 716/5,692 (12.6 %).

In comparative analysis, there was no association between the quality of ST and: (i) symptoms to request the test [breathing ($p = 0.084$), digestive ($p = 0.753$), nutritional ($p = 0.824$) and others ($p = 0.136$)], and (ii) sweat weight ($p = 0.416$). At the same time, there was a positive association with: (i) gender ($p = 0.001$), (ii) result of ST ($p < 0.001$), (iii) sweat chloride/sodium ratio ($p < 0.001$), and (iv) subject's age at the time of the ST [grouped by category ($p < 0.001$) and numerical order ($p < 0.001$)] (Table 1 and Fig. 2).

For the subset of 169 patients with CF and two *CFTR* mutations Class I, II and/or III, in comparative analysis. There was no association between the quality of ST and: (i) gender ($p = 1$); (ii) subject's age at the time of the ST grouped by category ($p = 0.128$); (iii) symptoms to request the test [breathing, digestive, nutritional and others – all patients with CF showed corrected data ($p > 0.05$)], (iv) sweat weight ($p = 0.191$), (v) sweat sodium values ($p = 0.151$). At the same time, there was a positive association with: (i) sweat chloride/sodium ratio ($p < 0.001$), (ii) sweat chloride values ($p = 0.047$), (iii) subject's age at the time of the ST grouped by numerical order ($p = 0.001$) (Table 2).

The frequency of *CFTR* mutations Class I, II and/or III is showed in the Table 3.

Discussion

Patients with two identified mutations in the *CFTR* gene do not usually show normal sweat values in their tests. CF patients show proportionately elevated values for both sodium and chloride electrolytes; with a difference between them, that does not usually exceed 15 mEq/L. In CF, sweat sodium concentration is usually lower than sweat chloride concentration, and the opposite relationship is observed in individuals without CF [18].

In this study, this parameter was used to assess the quality of ST. When comparing the quality of ST with gender, it was observed that the numbers of correct tests were greater in males than females. A possible explanation is the fact that women produce lower sweat volume due to the constitution of their sweat glands. Men have fewer active sweat glands, but higher sweating rate per gland. Women show lower cholinergic and β-adrenergic sweat secretion rates than men [8, 9].

The comparison between the quality and results of ST for CF diagnosis showed a higher number of incorrect tests in the chloride concentration range of 40 to 60 mEq/L, known as borderline range for the ST, as determined by Gibson and Cooke [14]. Some studies approach the need to assess the test results with age-related reference intervals [1, 2, 11, 12, 19]. Patients with clinical CF and chloride levels in ST between 30 and 59 mEq/L may have two mutations in the *CFTR* gene [12, 20].

Sweat chloride reference value between 30 and 59 mEq/L is associated with borderline range, depending on the individual's age, and it may possibly include individuals with Transmembrane Conductance Regulator Related Metabolic Syndrome. It is estimated that 8 to 15 % of subjects in this group may receive delayed diagnosis of CF and the initial clinical presentation of CF may be confused with other respiratory diseases [21, 22].

Table 1 Comparison between the quality of the sweat test based on the concentrations of sweat chloride and sweat sodium (proposed criteria) and the gender and age of subjects examined, as well as the results of the sweat test in view of the sweat chloride concentration obtained in the exam

Variable	Group	Quality of Sweat Test by Proposed Criteria			*p*-value	$OR^{correct}$	95 % CI	$OR^{incorrect}$	95 % CI
		Correct	Incorrect	Total					
Gender	Male	2707	316	3023	0.001	1.311	1.115 to 1.541	0.763	0.649 to 0.897
	Female	2300	352	2652		1	-	1	-
	Total	5007	668	5675					
Result of diagnosis of cystic fibrosis	<30 mEq/L	3462	189	3651	< 0.001	5.633	4.711 to 6.734	0.178	0.148 to 0.212
	≥ 30 to < 40 mEq/L	538	114	652		0.584	0.468 to 0.728	1.712	1.374 to 2.135
	≥ 40 to < 60 mEq/L	471	202	673		0.239	0.198 to 0.289	4.180	3.456 to 5.057
	≥ 60 mEq/L	552	164	716		0.380	0.312 to 0.463	2.630	2.160 to 3.203
	Total	5023	669	5692					
Subject's age	0 to 6 months	598	36	634	< 0.001	2.312	1.634 to 3.27	0.433	0.306 to 0.612
	> 6 months to ≤ 18 years	3591	306	3897		2.883	2.44 to 3.405	0.347	0.294 to 0.410
	> 18 years	778	302	1080		0.210	0.177 to 0.25	4.755	4.001 to 5.650
	Total	4967	644	5611					

OR odds ratio, *CI* confidence interval, *%* percentage, *mEq/L* milliequivalents per liter. Alpha = 0.05

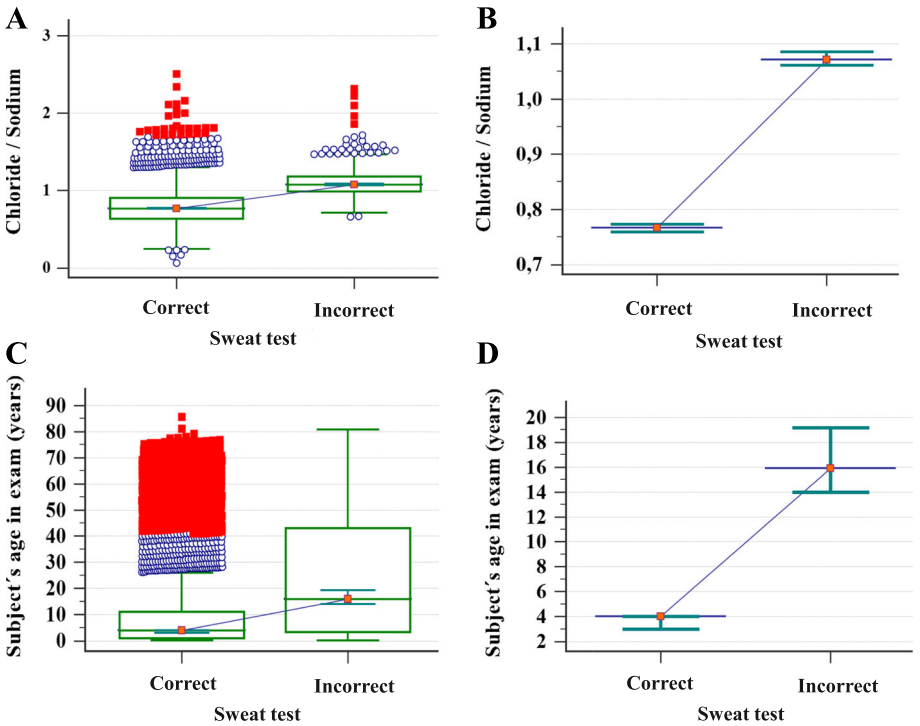

Fig. 2 **a** and **b** Association between quality of sweat test (proposed criteria) and sweat chloride/sodium ratio. Correct: $N = 5,023$; mean = 0.784 ± 0.234; median = 0.766; range from 0.06 to 2.51; Incorrect: $N = 669$; mean = 1.102 ± 0.184; median = 1.071; range from 0.66 to 2.31. $p < 0.001$. **c** and **d** Association between quality of the sweat test and the subject's age at the time of the examination. Correct: $N = 4912$; mean = 10.545 ± 16.523; median = 4; range from 0 to 85.58; Incorrect: $N = 634$; mean = 24.370 ± 22.366; median = 15.92; range 0 to 80.83. $p < 0.001$. Statistical analysis was performed by Mann-Whitney test. α = 0.05

According to the Cystic Fibrosis Foundation, CF is likely to be diagnosed when chloride concentration is greater than or equal to 60 mEq/L in two-sample ST. For infants up to six months of age, CF is very unlikely to be diagnosed when the chloride concentration is equal to or less than 29 mEq/L; as well as for individuals older than six months of age, when chloride concentration is equal to or less than 39 mEq/L. In our study, a greater number of incorrect tests were observed in the age group over 18 years. It appears that sweat chloride peaked in adults over 18 years of age, suggesting that the borderline value of 60 mEq/L to diagnose CF may not be sensitive for all age groups [2]. During the first 24 h after birth, sweat electrolyte values may be transiently elevated in normal infants, followed rapid decline of electrolytes in the first days of life. Moreover, it can be difficult to obtain adequate amount of sweat during the first weeks after birth, especially in preterm infants [23].

The concentration of electrolytes in the sweat increases with age and healthy adults may have chloride levels above 60 mEq/L [24, 25]. Furthermore, at the time of interpretation of ST, it should be considered that some rare *CFTR* gene may be related to borderline or negative values ST [18, 25].

Table 2 Comparison between the quality of the sweat test based on the concentrations of sweat chloride and sweat sodium (proposed criteria) and age of subjects examined, as well as the results of the sweat test in view of the sweat chloride concentration obtained in the exam. All subjects had cystic fibrosis and two *CFTR* mutations Class I, II and/or III

Variable	Group	Number	Mean ± SD	Median	Minimum	Maximum	*p*-value
Sweat chloride/sodium ratio	Correct	155	1.33 ± 0.24	1.27	1	2.11	<0.001
	Incorrect	4	0.94 ± 0.03	0.93	0.9	0.97	
Sweat chloride values	Correct	155	112.48 ± 19.70	111.61	63.70	159.20	0.047
	Incorrect	4	93.34 ± 13.52	94.18	79.61	105.40	
Subject's age	Correct	133	3.47 ± 5.53	1	0	37.33	0.001
	Incorrect	2	24.13 ± 8.66	24.12	18	30.25	

N number of patients, *SD* standard deviation, *CFTR Cystic fibrosis transmembrane regulator*. The statistical analysis was performed by the Mann-Whitney test. Alpha = 0.05

Table 3 Distribution of patients with cystic fibrosis considering the genotype for mutations in the CFTR gene and classes of identified mutations

Genotype	Number	Percent	Group of patients
F508del/F508del	88[a]	52.1	Patients with two Class I, II and/or III
F508del/G542X	22	13	
F508del/N1303K	8[b]	4.7	
F508del/R1162X	8	4.7	
F508del/R553X	5	3	
F508del/1584-18672pbA > G	4	2.4	
F508del/c.1717-1G > A	3	1.8	
F508del/R1066C	4	2.4	
3120 + 1G > A/R1066C	3	1.8	
F508del/2183AA > G	1	0.6	
F508del/ 6b-16 exon duplication	2	1.2	
F508del/G85E	2	1.2	
F508del/S549R (T > G)	2	1.2	
F508del/S4X	3[c]	1.8	
G542X/2183AA > G	1	0.6	
G542X/R1162X	2	1.2	
R1162X/R1162X	4	2.4	
F508del/1812-1G > A	4	2.4	
2183AA > G/2183AA > G	2	1.2	
3120 + 1G > A/3120 + 1G > A	1	0.6	

[a] 4 patients with cystic fibrosis and normal sweat chloride values were excluded (sweat chloride values: 13.10 mEq/L; 21,90 mEq/L; 35.70 mEq/L; 55.30 mEq/L); [b] 1 patient with cystic fibrosis and normal sweat chloride values in 5 sweat tests was excluded (sweat chloride values: 21.60 mEq/L; 23.44 mEq/L; 24.40 mEq/L; 29.50 mEq/L; 47 mEq/L); [c] 1 patient with cystic fibrosis and normal sweat chloride value was excluded (sweat chloride value: 52.40 mEq/L); N, Sample size; *CFTR*, *Cystic fibrosis transmembrane regulator*

In addition to CF, some diseases may cause increased concentrations of sweat chloride, and most diseases can be differentiated based on clinical presentations. Some examples include: atopic dermatitis, hypogammaglobulinemia, glycogen storage disease type I, mucopolysaccharidosis type I, nephrogenic diabetes insipidus, pseudohypoaldosteronism, celiac disease, adrenal insufficiency, and untreated hypothyroidism. False positive result may occur in case of malnutrition, dehydration, skin conditions (eczema or rash) and ST technical errors during induction, collection and measure of chloride and sodium concentrations [18, 25].

False negative result is related to the presence of edema, use of mineralocorticoid, collection and analysis of insufficient amount of sweat, and other technical problems [25, 26]. Sweat sample was collected by experienced personnel in accordance with international guidelines and internal quality control procedures, in order to minimize possible methodological errors and misdiagnosis, as some symptoms may resemble those of CF.

There were two limitations to this study. First, this study did not include controls: all subjects were referred to ST due to their clinical manifestations, positive newborn screening results for CF, or family history. Second, it was not possible to confirm a diagnosis of CF for all patients using a genetic study. However, the data including *CFTR* mutations was included in the present manuscript and showed similar results as the first analysis for all exams performed.

Conclusions

It is assumed that quality of the ST can be assessed by concentrations of sweat sodium and sweat chloride; however, our study showed a great number of poor quality sweat tests. The quality of the tests was associated with some important factors, such as gender, CF diagnostic results, and age of subjects. Although ST is considered the gold standard for the diagnosis of CF, it has limitations and may produce both false positive and false negative results. Constant efforts should be targeted to understand ST results and seek quality markers to perform the tests, in order to allow accurate screening of patients. The objective is to make identification of mutations in the *CFTR* gene possible for all patients and/or to make it a regular screening method for patients suspected of having CF. The *CFTR* mutation screening can

only be achieved with minimized costs and improved technical resources, which enable complete *CFTR* gene sequencing, and include all mutations with their respective classes and types. Thus, considering the proportion adopted in this study, the quantification and use of sweat sodium is still needed in ST. Special attention should be paid to borderline range for the diagnosis of CF, where a greater chance of errors could be observed.

Abbreviations
CF: CYSTic fibrosis; CFTR: Cystic fibrosis transmembrane conductance regulator; SPSS: Statistical package for social sciences; ST: Sweat test; Unicamp: University of Campinas

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Availability of data and materials
The data and materials achieved in the manuscript were available at Laboratory of Pulmonary Physiology, Center for Pediatrics Investigation, Faculty of Medical Sciences, University of Campinas.

Authors' contributions
AGF, FALM, JDR: made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; been involved in drafting the manuscript or revising it critically for important intellectual content; given final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CCSG, MAGOR, LBM, MFS, CSB, ES, MG, IAP, MCP, GH, CEL, AADCT, AOP, MCRS, EAL, RJNN, AFR: made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. All authors read and approved the final manuscript.

Competing interest
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was approved by the Ethics Committee from University of Campinas (#474326).

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5.2. ARTIGO 2 – ARTIGO ORIGINAL

Faria AG, Marson FAL, Ribeiro JD. The correlation between age and sweat chloride levels in sweat tests. Rev Port Pneumol. 2016;23(4):227-230.

A autorização da Editora para inclusão do artigo na tese, encontra-se no anexo 2.

The correlation between age and sweat chloride levels in sweat tests

Dear Editor,

Although the sweat test (ST) is considered the gold standard for the diagnosis of cystic fibrosis (CF), it still remains the center of great interest as well as debate over its execution and interpretation.[1]

We read carefully the important article by Traeger and colleagues.[2] The study showed that in 13,775 ST [313 (2.3%) patients with CF], sweat chloride concentrations decrease in the first year of life, increase in the second year until the age of 18, and decrease slowly after age 18.

We assessed 5196 ST [671 (12.91%) patients with CF] in our university referral center. Unlike the study conducted by Traeger and colleagues, we assessed chloride levels by age, considering the reference values for the ST[3,4] (Fig. 1).

The sweat was collected following Gibson and Cooke's traditional method (1959) and the sweat chloride concentration was determined by chloridometry and the sweat sodium concentration by flame photometry. The sweat chloride levels were used to group the samples according to the CF diagnosis: (i) chloride < 30 mEq/L; (ii) chloride ≥ 30 mEq/L to <40 mEq/L; (iii) chloride ≥ 40 mEq/L to <60 mEq/L; (iv) chloride ≥ 60 mEq/L (group of patients with CF). The ST data was obtained from the medical records of the Pediatric Gastroenterology Laboratory and the Gastroenterology Center at the University Hospital.

The patients were divided into three age groups: (i) from birth to <six months; (ii) ≥six months to <18 years; (iii) ≥18 years.

Statistical analysis was carried out with linear regression test and Spearman's rank correlation test by the MedCalc software version 16.4.3. Significance level was set at 0.05 for all analyses. The power of the sample was greater than 80%.

The study was approved by the Ethics Committee of the University of Campinas (#474326).

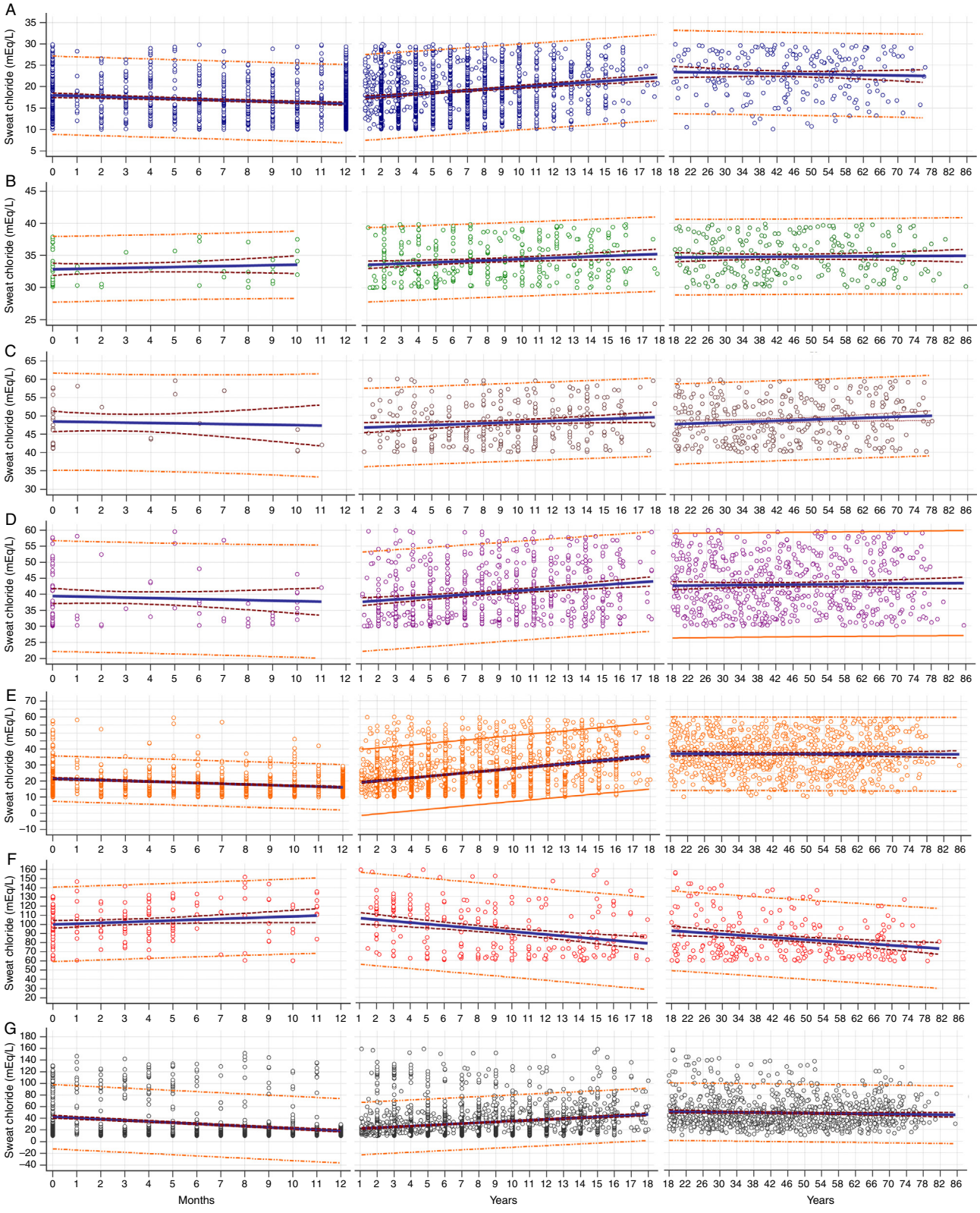
As observed in Fig. 1G, the results from our total population agree with those reported by Traeguer and colleagues.[2] The same correlation was observed for the groups of sweat chloride levels in mEq/L: (i) chloride < 30 mEq/L; (ii) chloride ≥ 30 mEq/L to <40 mEq/L; (iii) chloride ≥ 40 mEq/L to <60 mEq/L; (iv) chloride ≥ 30 mEq/L to <60 mEq/L; (v) chloride < 60 mEq/L (Fig. 1A–E).

In contrast, as shown in Fig. 1F, CF patients show increased sweat chloride levels in the first year of life. These levels gradually reduced after the second year or life. This was not evidenced by Traeguer and colleagues,[2] possibly due to the effect of sample dispersion of CF patients within the total sample.

It is important to note that sweat chloride levels tend to be lower among adults compared to children. Another important factor is the evidence that sweat chloride levels have intra- and inter-individual variability, even in patients with the same genotype in the Cystic Fibrosis Transmembrane Regulator (*CFTR*) gene.[5]

Such alterations should be further studied by measuring amounts of sweat chloride in CF patients and healthy individuals on a long-term basis.

One hypothesis that may explain decreased amounts of sweat chloride in sweat with increasing age is related to changes in the stability of the CFTR protein in healthy individuals and CF patients with borderline sweat chloride levels. On the other hand, for all subjects, reduced sweat chloride levels after 18 years of age may be a consequence of the aging process. Aging is a natural response, which causes physiological changes, including alterations in other chloride regulating channels and the action of modifier genes.


A
B
C
D
E
F
G
Sweat chloride (mEq/L)
Months
Years
Years

Therefore, studies should also be made on the factors that interfere with sweat chloride levels in different ages among healthy individuals and CF patients.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of University of Campinas (#474326).

Authors' contributions

AGF, FALM, JDR made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; were involved in drafting the manuscript and revising it critically for important intellectual content; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work by ensuring that questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved.

AFR made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data.

Conflicts of interest

All the authors declare that they have no conflicts of interest.

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Figure 1 Correlation between levels of sweat chloride and age of subjects undertaking sweat tests, considering possibilities of groups by chloride concentrations. (A) Chloride < 30 mEq/L: (age: $\leq$12 months) $N = 1178$; Spearman's coefficient rank correlation (rho) = -0.159, 95%CI = -0.214 to -0.103; $p < 0.001$. Coefficient of determination $R^2 = 0.025$; $y = 17.9905 + (-)0.1643x$; $p < 0.001$. (Age: >1 year to $\leq$18 years) $N = 1827$; rho = 0.192, 95%CI = 0.148–0.236; $p < 0.001$. $R^2 = 0.039$; $y = 17.1432 + 0.2749x$; $p < 0.001$. (Age: >18 years) $N = 230$; rho = -0.048, 95%CI = -0.176 to 0.082; $p = 0.469$. $R^2 = 0.002$; $y = 23.7087 + (-)0.01622x$; $p = 0.538$. (B) Chloride $\geq$ 30 mEq/L to <40 mEq/L: (age: $\leq$12 months) $N = 52$; rho = 0.097, 95%CI = -0.181 to 0.360; $p = 0.495$. $R^2 = 0.013$; $y = 32.8243 + 0.07205x$; $p = 0.427$. (Age: >1 year to $\leq$18 years) $N = 340$; rho = 0.151, 95%CI = 0.045–0.253; $p = 0.005$. $R^2 = 0.021$; $y = 33.4949 + 0.09905x$; $p = 0.007$. (Age: >18 years) $N = 244$; rho = 0.026, 95%CI = -0.100 to 0.151; $p = 0.692$. $R^2 < 0.001$; $y = 34.6330 + 0.003532x$; $p = 0.758$. (C) Chloride $\geq$ 40 mEq/L to <60 mEq/L: (Age: $\leq$12 months) $N = 33$; rho = 0.025, 95%CI = -0.321 to 0.365; $p = 0.892$. $R^2 < 0.001$; $y = 48.4653 + (-)0.09778x$; $p = 0.748$. (Age: >1 year to $\leq$18 years) $N = 278$; rho = 0.134, 95%CI = 0.016–0.248; $p = 0.026$. $R^2 = 0.018$; $y = 46.4328 + 0.1699x$; $p = 0.026$. (Age: >18 years) $N = 343$; rho = 0.117, 95%CI = 0.011–0.220; $p = 0.031$. $R^2 = 0.014$; $y = 46.9588 + 0.03910x$; $p = 0.031$. (D) Chloride $\geq$ 30 mEq/L to <60 mEq/L: (Age: $\leq$12 months) $N = 85$; rho = -0.049, 95%CI = -0.259 to 0.166; $p = 0.656$. $R^2 = 0.005$; $y = 39.4291 + (-)0.1577x$; $p = 0.5224$. (Age: >1 year to $\leq$18 years) $N = 618$; rho = 0.210, 95%CI = 0.134–0.285; $p < 0.001$. $R^2 = 0.042$; $y = 37.2303 + 0.3772x$; $p < 0.001$. (Age: >18 years) $N = 587$; rho = 0.017, 95%CI = -0.064 to 0.098; $p = 0.678$. $R^2 < 0.001$; $y = 42.3785 + 0.01182x$; $p = 0.565$. (E) Chloride < 60 mEq/L: (Age: $\leq$12 months) $N = 1263$; rho = -0.246, 95%CI = -0.297 to -0.193; $p < 0.001$. $R^2 = 0.078$; $y = 21.5579 + (-)0.4503x$; $p < 0.001$. (Age: >1 year to $\leq$18 years) $N = 2445$; rho = 0.336, 95%CI = 0.300–0.370; $p < 0.001$. $R^2 = 0.127$; $y = 17.9823 + 0.968.9x$; $p < 0.001$. (Age: >18 years) $N = 817$; rho = -0.01, 95%CI = -0.078 to 0.0586; $p = 0.775$. $R^2 < 0.001$; $y = 37.6153 + (-)0.007263x$; $p = 0.776$. (F) Chloride $\geq$ 60 mEq/L: (Age: $\leq$12 months) $N = 179$; rho = 0.134, 95%CI = -0.013 to 0.275; $p = 0.073$. $R^2 = 0.021$; $y = 99.9960 + 0.8686x$; $p = 0.058$. (Age: >1 year to $\leq$18 years) $N = 238$; rho = -0.287, 95%CI = -0.400 to -0.166; $p < 0.001$. $R^2 = 0.085$; $y = 107.6871 + (-)1.6094x$; $p < 0.001$. (Age: >18 years) $N = 254$; rho = -0.190, 95%CI = -0.306 to -0.068; $p = 0.002$. $R^2 = 0.056$; y= 98.6663 + (−)0.3083x; $p < 0.001$. (G) All samples: (Age: $\leq$12 months) $N = 1442$; rho = -0.352, 95%CI = -0.396 to -0.306; $p < 0.001$. $R^2 = 0.1032$; $y = 42.6550 + (-)2.0145x$; $p < 0.001$. (Age: >1 year to $\leq$18 years) $N = 2683$; rho = 0.348, 95%CI = 0.315–0.381; $p < 0.001$. $R^2 = 0.066$; $y = 20.8087 + 1.4323x$; $p < 0.001$. (Age: >18 years) $N = 1071$; rho = -0.052, 95%CI = -0.111 to 0.008; $p = 0.09$. $R^2 = 0.003$; $y = 52.1251 + (-)0.07962x$; $p = 0.090$. $\alpha = 0.05$. Statistical analysis was made with linear regression test and Spearman's rank correlation test.

Gonçalves de Oliveira Ribeiro, Maria de Fátima Servidoni, Mônica Corso Pereira; Roberto José Negrão Nogueira.

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Survival analysis of patients with non-small cell lung cancer treated by surgery with curative intent

Dear Editor,

Lung cancer is the leading cause of cancer deaths in the entire world, representing 19.4%–27% of all deaths from cancer.[1] Surgical resection is considered the treatment standard for early stage disease (stage I and II) and for some cases in IIIA stage.[1] Accordingly to the TNM system, 5-years survival for IA, IB, IIA, IIB, IIIA, IIIB and IV stage disease, is about 73%, 58%, 46%, 36%, 24%, 9% and 13% respectively.[2]

The purpose of this study is to describe a population of patients submitted to curative intent surgery for lung cancer and analyze the survival.

Data was collected retrospectively from the clinical process of patients with NSCLC who had indergone pulmonary resection surgery with curative intent who were being followed at the Pneumological Oncology Service of a University Hospital. Patients who had not had lymphadenectomy were excluded.

After initial treatment was completed, patients were followed every 3 months with a complete physical examination, blood analyses and chest X-ray for 5 years, every 6 months with computed tomography (CT) for 2 years and then annually.

A descriptive analysis of the variables of 102 patients undergoing pulmonary resection surgery was carried out. This included patients who were operated on between 1 January 2008 and 31 December 2012 and were followed until 31 December 2015.

The comparison of the distribution of variables was made using adjustment tests (Binomial and Chi-square). Kaplan–Meier survival analysis was used to determine mean survival time and mortality rate according to the disease stage and comparison of these by log-rank test. The association between pathologic stage and survival was assessed using Fisher's exact test. The analysis was performed in the SPSS, version 23, and the statistical tests analyzed at the significance level of 5%.

The study was conducted with a group of 102 patients with an average age of 63.69 ± 9.31; 68.6% were men. The distribution of tumor location is preferentially peripheral (72.5%), appearing in the lobar bronchus (15.7%) or main bronchus (11.8%) with less frequency ($p < 0.001$). Most of the patients had Adenocarcinomas (61.8%) or squamous tumors (26.5%). Most of the surgeries performed were lobectomies (81.4%), followed by pneumonectomy (14.7%), wedge excision (2.9%) and segmentectomy (1%). 26.5% cases were in pathological stage IA, 24.5% in IB, 21.6% in IIA, 9.8% in IIB and 17.6% in IIIA ($p = 0.064$).

Of the 102 cases analyzed, 41 died during the study period (40.2%). The estimated mortality rate in the population should be between 30.7% and 47.7%. The median overall survival time was 65.61 ± 3.56 months and the 5-year survival (60 months) was 46.20 ± 1.93 months. There was a statistically significant difference in mean survival time depending on the disease stage ($p < 0.001$) [Fig. 1]. The tendency is that patients with stage IA have a better prognosis, with a statistically significant difference between this and any of the other stages. Table 1 presents the mean survival time observed at the 5-year follow-up, and the respective estimated mean survival time in the population, in relation to the disease stage. In the legend, the *p*-values adjusted for multiple comparisons of the survival time between pairs of stages evaluated are presented.

5.3. ARTIGO 3 – ARTIGO ORIGINAL

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Thirty Years of Sweat Chloride Testing at One Referral Center

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Objective: To conduct a descriptive analysis of the sweat test (ST), associating ST results with epidemiological data, *CFTR* (cystic fibrosis transmembrane conductance regulator) mutations and reasons to indicate the ST, as well as correlating sweat sodium and sweat chloride concentrations in subjects.

Methods: Retrospective survey and descriptive analysis of 5,721 ST at a university referral center.

Results: The inclusion of the subjects was based on clinical data related with cystic fibrosis (CF) phenotype. The samples were grouped by (i) sweat chloride concentrations (mEq/L): <30: 3,249/5,277 (61.6%); ≥30 to <60: 1,326/5,277 (25.1%); ≥60: 702/5,277 (13.3%) and (ii) age: (Group A—GA) 0 to <6 months; (Group B—GB) ≥6 months to <18 years; (Group C—GC) ≥18 years. Digestive symptoms showed higher prevalence ratio for the CF diagnosis as well as association between younger age and higher values of sweat chloride, sweat sodium, and chloride/sodium ratio. The indication of ST due to respiratory symptoms was higher in GB and associated with greater age, lower values of sweat chloride, sweat sodium, and chloride/sodium ratio. There was higher prevalence of ST with sweat chloride levels <30 mEq/L in GB, ≥60 mEq/L in GC, and with borderline level in GB. There was positive correlation between sweat sodium and sweat chloride. Sweat chloride/sweat sodium and sweat sodium–sweat chloride indexes showed association with sex, reason for ST indication, and *CFTR* mutations. Sex alters some values presented in the ST. The number of ST/year performed before and after the newborn screening implementation was the same; however, we observed a higher number of borderlines values. A wide spectrum of *CFTR* mutation was found. Severe *CFTR* mutations and F508del/F508del genotype were associated with highest probability of ST chloride levels ≥60 mEq/L, and the absence of *CFTR* mutations identified was associated with borderline ST and respiratory symptoms.

Conclusions: ST data showed wide variability dependent on age, sex, reason for examination indication, *CFTR* mutations, and weight of the collected sweat sample. Sweat sodium concentration is directly correlated with sweat chloride levels and it could be used as a quality parameter.

Keywords: cystic fibrosis, diagnosis, sweat chloride, sweat sodium, sweat test

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INTRODUCTION

Cystic fibrosis (CF) (OMIM: #219700) is a chronic disease that leads to variability of genotypic and phenotypic expression. The CF diagnosis is based on neonatal screening findings and/or phenotypic manifestations, family history, and higher chloride ion (Cl^-) concentration in sweat, in addition to two mutations in the *CFTR* gene (cystic fibrosis transmembrane conductance regulator) (OMIM: *602421) (1). If mutations in the *CFTR* gene and/or altered CFTR protein functions cannot be detected by any method, a definitive CF diagnosis cannot be made.

The function and/or presence of the CFTR protein has been demonstrated in the sweat glands by measurement of ion concentrations in sweat [sweat test (ST) and evaporimeter] (2–5), nasal epithelium (nasal potential difference) (6), salivary gland (ions in saliva) (7), and in the digestive tract (presence and function of the CFTR protein in rectal biopsy) (8, 9). Although *CFTR* gene mutations are the most important and appropriate markers for CF diagnosis, the analysis of ion chloride concentration in sweat is still considered the gold standard for the diagnosis, as well as the simplest method used to assess functional properties of the CFTR protein. In 1938, Dr. Dorothy Hansine Andersen pointed out that the high concentration of salt in sweat of patients with CF was almost accepted as pathognomonic for CF (10).

Over the past 65 years, there have been advances in the implementation and interpretation of the ST (3). Sweat chloride concentrations ≥60 mEq/L in at least two tests performed at different collection times are considered the gold standard for CF diagnosis. Individuals with borderline values ranging from 30 to 59 mEq/L require evaluation of the mutations in the *CFTR* gene (11).

The application of appropriate methods to perform ST is essential for accurate CF diagnosis. Therefore, referral centers should follow internal procedures which are in line with the guidelines provided by the cystic fibrosis foundation (CFF) (12). One of the ST collection methods approved by the CFF was developed by Gibson and Cooke (2). The quantitative pilocarpine iontophoresis ST determines the weight of sweat collected with analytical balance. Special care should be taken to avoid evaporation of the sample. This procedure is likely to fail unless it is carried out by experienced and trained personnel (13).

Currently, sweat chloride concentration dosage has also been useful to demonstrate the function of the CFTR protein after the administration of correctors, potentiators, or stabilizers drugs by personalized/precision medicine (14). Therefore, the future role of ST should include the successful monitoring of personalized medicine therapy.

The aim of this study was to conduct a descriptive analysis of the ST, associating ST results with epidemiological data, *CFTR* mutations screening, and reasons to indicate the test (respiratory, digestive, or nutritional symptoms), as well as correlating concentrations of sweat sodium and sweat chloride in subjects.

Abbreviations: CF, cystic fibrosis; CFF, cystic fibrosis foundation; CFTR, cystic fibrosis transmembrane conductance regulator; Cl^-, chloride ion; IRT, immunoreactive trypsinogen; mEq/L, milliequivalents per liter; MLPA, multiplex ligation-dependent probe amplification; OMIM, online Mendelian inheritance in man; ST, sweat test.

MATERIALS AND METHODS

A retrospective study between 1986 and 2016, characterizing 30 years of monitoring, was conducted on 5,721 sweat samples at a CF reference center.

The sweat was collected following the traditional Gibson–Cooke method (2), and concentrations of sweat chloride and sweat sodium were determined by chloridometry and flame photometry, respectively.

This study was carried out in accordance with the recommendations of Ethics Committee of the University of Campinas (Protocol no. 474326), with written informed consent from the institution in accordance with the Declaration of Helsinki. The ST and subjects' clinical data were obtained from medical records of the Pediatric Gastroenterology Laboratory, and from the Gastroenterology Center at the University Hospital, namely, name, subject's age at the time of the examination, sex, indication for the ST (respiratory, digestive, or nutritional symptoms), *CFTR* mutation screening, immunoreactive trypsinogen (IRT) inclusion in our center, weight of the sweat sample collected, sweat chloride and sweat sodium concentrations, sweat chloride/sweat sodium ratio, and sweat sodium–sweat chloride in the ST samples. Samples were excluded according to the following criteria: sweat weight <75 mg and >400 mg, sweat chloride level ≤10 mEq/L or >160 mEq/L, sweat sodium level ≤10 mEq/L or >150 mEq/L, or absence of descriptive data (sweat weight and sweat sodium concentrations).

The age of patients was divided into three groups: (i) from birth to <6 months; (ii) ≥6 months to <18 years; (iii) ≥18 years (11, 15). The sweat chloride levels were used to group the samples according to the CF diagnosis: (i) chloride <30 mEq/L; (ii) chloride ≥30 to <60 mEq/L; (iii) chloride ≥60 mEq/L (group of patients with CF) (11).

All requested tests have been analyzed. For some subjects, the test involved more than one sweat sample.

Identification of Mutations in the *CFTR* Gene

CFTR mutations were analyzed by polymerase chain reaction techniques for F508del and enzymatic digestion for G542X, R1162X, R553X, G551D, and N1303K. Other mutations in *CFTR* were also identified by sequencing or using the SALSA MLPA technique (multiplex ligation-dependent probe amplification) Kit P091-C1 CFTR-MRC-Holland: S4X, 2183 A > G, 1717-G > A, I618T, with MegaBace1000® (GE Healthcare Biosciences, Pittsburgh, PA, USA) and ABI 3500 (Applied Biosystems-Thermo Fisher Scientific, São Paulo, Brazil) (16).

Considering the *CFTR* genotypes, patients were divided into three groups: (i) with two identified mutations belonging to class I, II, and/or III; (ii) with one identified mutation belonging to class I, II, and/or III; (iii) no identified mutation belonging to class I, II, and/or III. Other identified mutations in the *CFTR*

gene, belonging to class IV, V, and/or VI, were not included in the statistical analysis. The *CFTR* mutation classification is according to the literature (17).

Statistical Analysis

A descriptive analysis was used with a number of observations, mean value, standard deviation, median, and minimum and maximum values for continuous variables. Confidence interval (95%) was calculated for proportions. For categorical variables, the data are presented by frequencies and percentages.

Statistical analyses were performed using the Statistical Package for the Social Sciences software version 23.0 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) and OpenEpi version 3.03a. The comparison between the variables with categorical distribution was carried out by chi-square test and Fisher's exact test, depending on the data distribution. Mann–Whitney and Kruskal–Wallis non-parametric tests were used for the analysis of variables with numerical distribution. Spearman's rank correlation test and linear regression were used to compare the association between two variables with numerical distribution. Significance level was set at 0.05 for all analyses. The level of power in sample size calculations was >80%.

RESULTS

This study assessed 5,721 sweat samples of STs that had been requested due to clinical presentations compatible with CF and/or neonatal screening with abnormal IRT values. One excluded 444 (7.78%) of these sweat samples, as follows: (i) 23 showed sweat weight <75 mg; (ii) four lacked sweat weight described in the examination; (iii) one lacked laboratory data; (iv) one showed no sweat sodium value described in the examination; (v) 395 showed sweat chloride level <10 mEq/L; (vi) nine showed sweat chloride level >160 mEq/L; (vii) four showed sweat sodium level <10 mEq/L; (viii) one showed sweat sodium level >150 mEq/L; (ix) six showed sweat weight >400 mg. Thus, the final study included 5,277 sweat samples. Sex of 15 (0.28%) subjects was not obtained because the examination was carried out on the newborn's mother's name after neonatal screening. Thus, 2,786/5,262 (52.9%) samples of male subjects were included and analyzed.

The IRT inclusion in our center was dated since 2010. From our subjects, 4,020/5,265 (76.4%) were included before 2010 and 1,245/5,265 (23.6%) after this date. In this context, there were performed 174.78 and 177.86 ST/year on average before and after IRT inclusion, respectively.

The STs were carried out at an outpatient clinic which is primarily intended for individuals under 25 years of age. Therefore, only a few examinations were performed in adult individuals. This explains the low inclusion rate of adults compared with children and teenagers.

The mean age of subjects was 12.84 ± 18.28 years; median of five (ranging from 0 to 85.58) years. However, the age in 80/5,277 (1.5%) samples was not entered at the time of the examination. Thus, three age groups were built with the following frequencies: (i) birth to six months: 567/5,197 (10.9%) samples; (ii) ≥6 months to <18 years: 3,558/5,197 (68.5%) samples; (iii) ≥18 years: 1,072/5,197 (20.6%) samples. Moreover, in 61/5277 samples, we had the age groups without the exact age by years and/or months.

The mean level of sweat chloride was 34.01 ± 27.16 mEq/L, median of 23.74 (ranging from 10 to 159.2) mEq/L. The mean level of sweat sodium was 37.76 ± 21.31 mEq/L, median of 30.9 (ranging from 10 to 149.7) mEq/L. Subjects were referred to the ST due to the following symptoms (each symptom was analyzed separately): (i) respiratory symptoms: 2,623/3,400 (77.1%); (ii) digestive symptoms: 419/3,400 (12.3%); (iii) nutritional symptoms: 391/3,400 (11.5%). In addition, we analyzed the presence of simultaneous digestive and respiratory symptoms, and we observed (i) no symptom––577/3,400 (17%); (ii) one symptom––2,604/3,400 (76.6%); (iii) two symptoms––219/3,400 (6.4%).

Initial ST request information could not be obtained for 1,877/5,277 (35.57%) sweat samples. The chloride–sodium ratio showed mean level of 0.84 ± 0.23 mEq/L; median of 0.82 mEq/L (ranging from 0.29 to 2.31 mEq/L). The sodium–chloride difference showed mean level of 3.75 ± 10.38 mEq/L; median of 5.3 mEq/L (ranging from −82.7 to 42.4 mEq/L). The weight of the sweat samples showed mean level of 170 ± 40 mg and median of 170 mg (ranging from 80 to 370 mg).

The sweat samples were divided into three groups of sweat chloride levels: (i) <30 mEq/L: 3,249/5,277 (61.6%); (ii) ≥30 to <60 mEq/L: 1,326/5,277 (25.1%); (iii) ≥60 mEq/L: 702/5,277 (13.3%).

Table 1 shows the association between sweat chloride value, sex of the subject, and reason for the indication of the examination. The indication due to nutritional symptoms showed no

TABLE 1 | Association between sweat chloride levels, sex of the subject, and reason for indication of sweat test.

Chloride levels (mEq/L)	Sex			Odds ratio	95% CI
	Male	Female	Total		
<30	1,821	1,415	3,236	**1.415**	**1.266–1.582**
≥30 to <60	633	691	1,324	**0.76**	**0.67–0.86**
≥60	332	370	704	**0.77**	**0.657–0.903**
Chloride levels	**Indication—respiratory disease**			**Odds ratio**	**95% CI**
	Yes	**No**	**Total**		
<30	2,159	491	2,650	**2.71**	**2.271–3.235**
≥30 to <60	343	130	473	**0.749**	**0.601–0.933**
≥60	121	156	277	**0.193**	**0.15–0.248**
Chloride levels	**Indication—digestive disease**			**Odds ratio**	**95% CI**
	Yes	**No**	**Total**		
<30	278	2,372	2,650	**0.506**	**0.406–0.632**
≥30 to <60	63	410	473	1.11	0.832–1.479
≥60	78	199	277	**3.198**	**2.405–4.252**

Statistical analysis was made by chi-square test; odds ratio was calculated considering values set by Taylor series. For all analyses, P-value was <0.001. All positive values are represented in bold type. $\alpha = 0.05$.

95% CI, confidence interval of 95%; mEq/L, milliequivalents per liter.

positive association ($p = 0.678$). The indication due to respiratory reasons showed no correlation with the CF diagnosis, unlike the indication due to digestive reasons. In the ST, for sweat chloride, males had lower prevalence ratio for borderline values and values ≥60 mEq/L, as well as higher prevalence ratio for values <30 mEq/L.

Figure 1 shows the association of ST results with the weight of sweat, sweat sodium value and subject's age.

Table 2 presents the subjects' age distribution at the time of the ST, as well as the comparison between ST results and reason for the examination indication. There was a higher prevalence of sweat samples with sweat chloride levels <30 mEq/L in subjects aged ≥6 months to <18 years. The CF diagnosis (chloride ≥60 mEq/L) showed higher prevalence in the group aged over 18 years. Higher prevalence ratio for the group older than 18 years of age with borderline sweat chloride values was also observed. The indication for the ST due to respiratory manifestations was more often in the group aged ≥6 months to <18 years. For digestive manifestation, indication was more often in the group under six months of age.

There was no association between the reason for ST indication and the sex of the subjects [respiratory symptoms ($p = 0.362$), digestive symptoms ($p = 1$), or nutritional symptoms ($p = 0.384$)]. However, male patients were less prevalent in the group aged over 18 years and showed higher volume of sweat and lower levels of sweat sodium, sweat chloride, and chloride/sodium ratio (**Tables 2** and **3**).

Subjects referred to the ST because of respiratory symptoms were older and presented higher sweat weight, lower levels of sweat chloride, sweat sodium and chloride/sodium ratio, as well as higher sodium–chloride level. For the indication due to digestive reasons, there was no association with sweat weight ($p = 0.426$) (**Table 3**). However, there was association with digestive symptom and higher levels of sweat chloride, sodium and chloride/sodium ratio, as well as subject's younger age and lower sodium–chloride level (**Table 3**).

Figure 2 brings the correlation between sweat weight and levels of sweat chloride and sweat sodium, subject's age, difference between sodium and chloride concentrations and chloride/sodium ratio.

Figure 3 shows the sex of the subjects distribution by the result of CF diagnosis regarding the sweat weight and the sweat chloride.

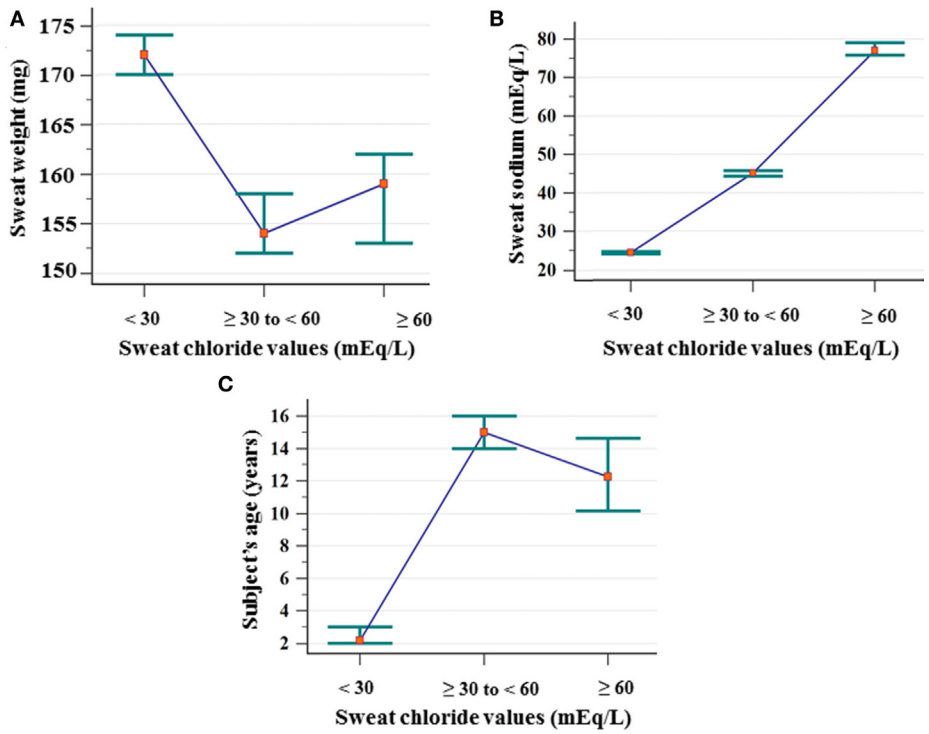

FIGURE 1 | Association between the ST results considering chloride cutoff values and sweat weight (mg), sodium value (mEq/L), and subject's age (years). **(A)** Sweat weight: (chloride <30 mEq/L) $N = 3,249$; mean of 175 ± 43; median of 172 (amplitude from 75 to 367); (chloride ≥30 to <60 mEq/L) $N = 1,326$; mean of 162 ± 41; median of 154 (amplitude from 78 to 299); (chloride ≥60 mEq/L) $N = 702$; mean of 164 ± 42; median of 159 (amplitude from 78 to 326). Chloride <30 mEq/L ≠ chloride ≥30 to <60 mEq/L, and chloride ≥60 mEq/L. **(B)** Sweat–sodium concentration: (chloride <30 mEq/L) $N = 3,249$; mean of 25.24 ± 7.03; median of 24.4 (amplitude from 10 to 68.5); (chloride ≥30 to <60 mEq/L) $N = 1,326$; mean of 45.82 ± 9.89; median of 45.11 (amplitude from 20.3 to 81.8); (chloride ≥60 mEq/L) $N = 702$; mean of 80.54 ± 17.98; median of 77 (amplitude from 42.9 to 149.7). All groups differ from each other. **(C)** Subject's age: (<30 mEq/L) $N = 3.233$; mean of 6.66 ± 11.91; median of 2.17 (amplitude from 0 to 76.33); (chloride ≥30 to <60 mEq/L) $N = 1,282$; mean of 24.34 ± 21.74; median of 15 (amplitude from 0 to 85.58); (chloride ≥60 mEq/L) $N = 621$; mean of 21.27 ± 22.52; median of 12.25 (amplitude from 0 to 81.17). All groups differ from each other. Statistical analysis was performed by Kruskal–Wallis test. *P*-value for all analyses was <0.001. $\alpha = 0.05$. The median is set in red marker and the 95% confidence interval is set in green line. mEq/L, milliequivalents per liter; ST, sweat test.

TABLE 2 | Association between sweat chloride levels, sex of the subjects, and reason for indication of sweat test with age and sweat test result.

Chloride levels (mEq/L)	**Age**				**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**
	0 to <6 m	**≥6 m to <18 years**	**≥18 years**	**Total**						
<30	426	2,579	230	3,235	**1.959**	**1.605–2.391**	**3.947**	**3.49–4.465**	**0.102**	**0.087–0.12**
≥30 to <60	46	657	587	1,290	**0.24**	**0.177–0.327**	**0.36**	**0.316–0.41**	**5.891**	**5.096–6.811**
≥60	95	322	255	674	**1.408**	**1.111–1.785**	**0.366**	**0.311–0.432**	**2.775**	**2.334–3.3**
Indication by two factors[b]	**0 to <6 m**	**≥6 m to <18 years**	**≥18 years**	**Total**	**Odds ratio**	**95% CI**	**Odds ratio**	**95 %CI**	**Odds ratio**	**95% CI**
No factor	167	405	5	577	**2.884**	**2.333–3.565**	**0.334**	**0.27–0.412**	**24.64**	**2.874–211.3[a]**
One factor	318	2,282	1	2,601	**0.421**	**0.345-0.513**	**2.449**	**2.009–2.984**	**0.061**	**0.007–0.522[a]**
Two factors	31	188	0	219	0.916	0.619–1.355	1.108	0.749–1.64	–	–
Sex	**0 to <6 m**	**≥6 m to <18 years**	**≥18 years**	**Total**	**Odds ratio**	**95% CI**	**Odds ratio**	**95 %CI**	**Odds ratio**	**95% CI**
Male	312	1,996	439	2,747	1.157	0.969–1.381	**1.497**	**1.331–1.684**	**0.539**	**0.471–0.618**
Female	243	1,559	634	2,436	1	–	1	–	1	–
Respiratory	**0 to <6 m**	**≥6 m to <18 years**	**≥18 years**	**Total**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**
Yes	282	2,337	1	2,620	**0.28**	**0.23–0.341**	**3.668**	**3.015–4.463**	**0.059**	**0.007–0.505**
No	234	538	5	777	1	–	1	–	1	–
Digestive	**0 to <6 m**	**≥6 m to <18 years**	**≥18 years**	**Total**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**
Yes	98	321	0	419	**1.87**	**1.458–2.398**	**0.544**	**0.424–0.697**	–	–
No	418	2,554	6	2,978	1	–	1	–	–	–
Indication by two factors[b]	**Sweat test-chloride values (mEq/L)**				**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**
	<30	**≥30 to <60**	**≥60**	**Total**						
No one	368	99	110	577	**0.417**	**0.344–0.507**	**1.356**	**1.064–1.728**	**3.746**	**2.889–4.858**
One factor	2,127	342	135	2,604	**2.328**	**1.95–2.778**	**0.768**	**0.617–0.956**	**0.252**	**0.196–0.324**
Two factors	155	32	32	219	**0.666**	**0.492–0.902**	1.063	0.721–1.567	**2.051**	**1.379–3.05**

[b]No factor = absence of respiratory and digestive symptoms at the moment that the ST was performed; one factor = presence of respiratory or digestive symptoms at the moment that the ST was performed; two factors = presence of respiratory and digestive symptoms at the moment that the ST was performed. Statistical analysis was made by chi-square test; odds ratio was calculated considering values set by Taylor series, except for the data marked with [a] (odds ratio was calculated considering values set by Fisher's exact test). For all analyses, P-value was <0.001. All positive values are represented in bold type. α = 0.05. 95% CI, confidence interval of 95%; m, months; mEq/L, milliequivalents per liter; ST, sweat test.

Figure 4 shows the correlation between the levels of sweat sodium and sweat chloride by the result of CF diagnosis.

In addition, the association between the IRT inclusion in our sample and sex of the subjects, CF diagnosis, and age are presented in **Table 4**. The association with indication to the ST performance was not made regarding the bias by the IRT inclusion.

In our sample, 408 subjects were screened for at least F508del mutation in the *CFTR* gene. From these subjects, we found (i) 169/408 (41.4%) patients with unknown mutation in the *CFTR* gene, or with one or two mutations in the *CFTR* gene belonging to classes IV, V, or VI; (ii) 87/408 (21.3%) patients with one mutation in the *CFTR* gene belonging to class I, II, or III, and unknown mutation or one mutation in the *CFTR* gene belonging to class IV, V, or VI; (iii) 152/408 (37.3%) patients with two identified mutations in the *CFTR* gene belonging to class I, II, and/or III (see **Table 5).**

The *CFTR* genotype was associated with sex, reason for indication of ST, result achieved from ST, age of subjects, and ST markers regarding *CFTR* mutation screening genotype and F508del genotype (**Tables 6** and **7**).

The normal variability enrolled with the ST could direct the diagnosis to CF, mainly in the limits associated with the ST cutoff points. In addition, the sex, *CFTR* mutation, sweat weight, digestive symptoms, pulmonary symptoms, and neonatal screening influenced the values achieved in the ST, and this influence is present in all possible ST classes: (i) <30 mEq/L; (ii) ≥30 to <60 mEq/L; and (iii) ≥60 mEq/L (**Figure 5**).

DISCUSSION

High levels of sweat chloride with the presence of CF indicative signs and symptoms have been the gold standard for the CF diagnosis for more than 70 years (3). The amounts of ions in sweat have high specificity and sensitivity to demonstrate dysfunction or absence of chloride channels (CFTR protein) (18). However, despite the efficacy and importance of the ST for CF diagnosis, many methodological aspects of the stages related to its realization and interpretation continue and should be discussed as follows: (i) How do sweat chloride and sweat sodium concentrations vary

TABLE 3 | Association between sweat weight, sex of the subjects, sweat chloride, sweat sodium, and reason for indication of sweat test.

Variable	Category	N	Mean	Standard variation	Median	Minimum	Maximum
Sweat weight (mg)							
Sex	Male	2,786	174	43	172	76	367
	Female	2,476	165	41	161	75	337
Respiratory	Yes	2,623	170	41	168	76	367
	No	777	159	38	157	75	295
Indication by two factors[a,b]	No factor	577	157	37	155	75	276
	One factor	2,604	170	41	167	76	367
	Two factors	219	171	47	164	76	296
Age (years)							
Sex	Male	2,712	10.41	15.75	4	0	80.83
	Female	2,409	15.65	20.44	6	0	85.58
Respiratory	Yes	2,620	4.28	4.03	3	0	44
	No	777	4.2	4.94	2	0	19
Digestive	Yes	419	3.42	4.27	2	0	17
	No	2,978	4.38	4.24	3	0	44
Sweat sodium (mEq/L)							
Sex	Male	2,786	36.16	20.77	29.3	10	149.7
	Female	2,476	39.66	21.8	32.75	10.1	145.08
Respiratory	Yes	2,623	30.52	15.14	26.4	11.1	119.3
	No	777	41.79	25.01	32.2	10	149.7
Digestive	Yes	419	39.26	25.23	29.6	10	124.6
	No	2,981	32.23	17.17	27.2	11.1	149.7
Indication by two factors[c]	No one	577	41.38	23.83	32.2	12.9	149.7
	One factor	2,604	31.03	16.17	26.5	10	124.6
	Two factors	219	35.86	21.7	28	11.6	117
Sweat chloride (mEq/L)							
Sex	Male	2,786	32.27	26.44	22.25	10	159.2
	Female	2,476	36.07	27.86	25.8	10	158.6
Respiratory	Yes	2,623	23.91	18.6	18.3	10	136.2
	No	777	38.19	33.29	22.6	10	152.3
Digestive	Yes	419	36.94	33.93	21	10	141.8
	No	2,981	25.8	21.38	18.8	10	153.3
Indication by two factors[c]	No factor	577	37.25	32	22.6	10	152.3
	One factor	2,604	24.42	19.66	18.4	10	141.8
	Two factors	219	33.33	30.82	19.7	10.4	130.4
Chloride/sodium							
Sex	Male	2,786	0.835	0.229	0.806	0.31	2.1
	Female	2,476	0.854	0.239	0.834	0.29	2.31
Respiratory	Yes	2,623	0.747	0.182	0.731	0.31	1.96
	No	777	0.824	0.26	0.776	0.32	2.16
Digestive	Yes	419	0.843	0.26	0.791	0.42	2.16
	No	2,981	0.754	0.193	0.733	0.31	1.96
Indication by two factors[d]	No factor	577	0.81	0.26	0.77	0.32	1.81
	One factor	2,604	0.75	0.18	0.73	0.31	2.16
	Two factors	219	0.83	0.26	0.78	0.42	1.81
Sodium–chloride							
Respiratory	Yes	2,623	6.608	7.752	6.9	−66.6	33.7
	No	777	3.602	12.807	6.2	−60.7	42.4
Digestive	Yes	419	2.32	12.382	5.4	−51.4	26.6
	No	2,981	6.427	8.592	7.04	−66.6	42.4
Indication by two factors[e]	No factor	577	4.13	12.96	6.8	−60.7	42.4
	One factor	2,604	6.6	7.69	6.9	−66.6	33.7
	Two factors	219	2.53	12.51	5.6	−49.6	22

[a]No factor = absence of respiratory and digestive symptoms at the moment that the ST was performed; one factor = presence of respiratory or digestive symptoms at the moment that the ST was performed; two factors = presence of respiratory and digestive symptoms at the moment that the ST was performed.

[b]The no factor group showed difference regarding the others groups.

[c]All the groups are different among them.

[d]One factor group showed difference regarding the others groups.

[e]Two factor group showed difference regarding the others groups. Statistical analysis was made by Mann–Whitney test and Kruskal-Wallis test.

For all analyses, P-value was <0.001, except $p = 0.001$. $\alpha = 0.05$.

N, number of subjects; mg, milligram; mEq/L, milliequivalents per liter; ST, sweat test.

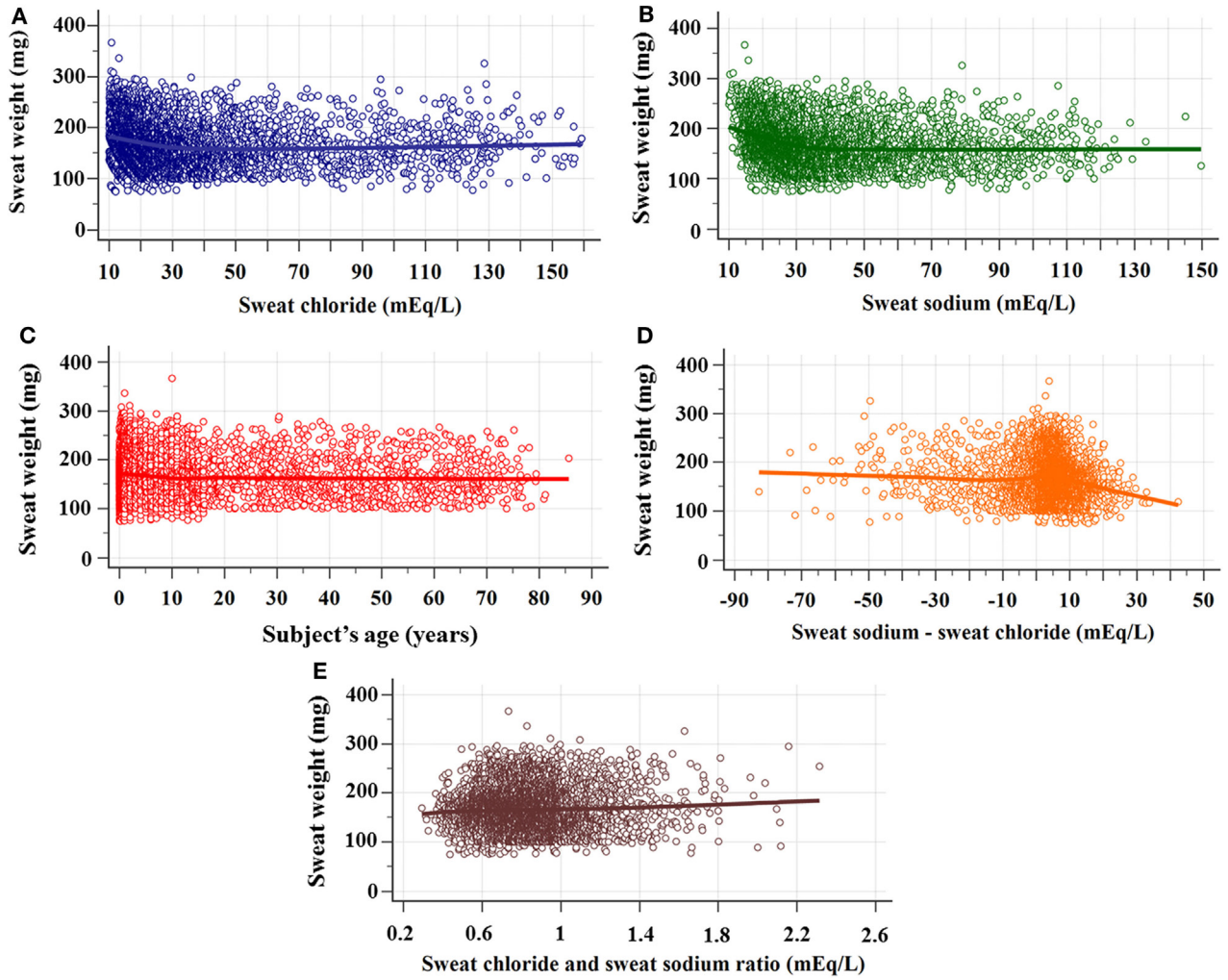

FIGURE 2 | Correlation between sweat weight and sweat chloride and sodium concentrations, subject's age, difference between sweat chloride and sodium concentrations, and sodium/chloride ratio. **(A)** Correlation between sweat weight and chloride concentrations. $N = 5,277$; rho = –0.201 (95% CI = –0.227 to –0.175), $p < 0.0001$. **(B)** Correlation between sweat weight and sweat sodium concentrations. $N = 5,277$; rho = –0.257 (95% CI = –0.282 to –0.232), $p < 0.001$. **(C)** Correlation between sweat weight and subject's age. $N = 5,136$; rho = –0.075 (95% CI = –0.102 to –0.048), $p < 0.0001$. **(D)** Correlation between sweat weight and the difference between sweat sodium and chloride concentrations. $N = 5,277$; rho = –0.094 (95% CI = –0.121 to 0.067), $p < 0.0001$. **(E)** Correlation between sweat weight and chloride/sodium ratio. $N = 5,277$; rho = 0.007 (95% CI = –0.02 to 0.034), $p = 0.621$. The statistical analysis was performed by Spearman's rank correlation test. $\alpha = 0.05$. 95% CI, confidence interval of 95%.

with age? (15, 19); (ii) Is it necessary to measure sweat chloride alone or together with the analysis of sweat sodium? (15, 20); (iii) Which are the most appropriate types of waves and current intensity to stimulate sweating in the ST? (4); (iv) How are the ST stages performed at different reference centers? (21); How has the evolution of the ST been since its implementation for nearly 70 years? (3); Are the values of electrolytes obtained in the ST dependent on the variants of the *CFTR* gene? (16).

The importance of the ST is increasing day by day, and recently the sweat chloride level has been described as a predictive marker of CF severity lung disease (22).

In this context, in the present study we intended to demonstrate the characterization of the data presented in the ST regarding the influence of subject's sex and age, *CFTR* mutation screening, and, mainly, ST clinical indication (nutritional, respiratory, and digestive symptoms). All aspects analyzed were discussed in part as follows.

ST Clinical Indication

Our laboratory, at a university referral center, receives requests to measure sweat sodium and sweat chloride ions in sweat from several specialties, mainly pediatric care. STs were more frequently requested for patients with chronic respiratory and digestive diseases.

Most patients with non-classic phenotype show symptoms later in life, often during adolescence or later, namely, less severe form of the disease, pancreatic sufficiency, mild chronic respiratory symptoms, and obstruction of the vas deferens with azoospermia. Levels of sweat chloride may be normal, abnormal, or

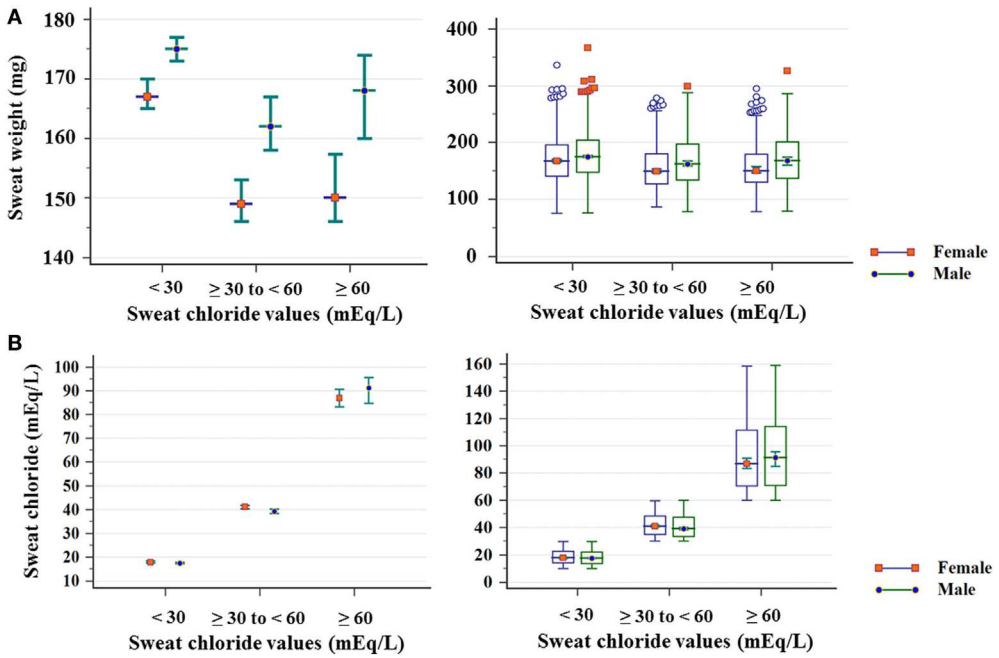

FIGURE 3 | Distribution of sex of subject, considering sweat chloride (mEq/L) and sweat weight. **(A)** Sweat weight (mg). (chloride <30 mEq/L) Female: $N = 1{,}415$; mean of 171 ± 43; median of 167 (amplitude from 75 to 337); male: $N = 1{,}821$; mean of 178 ± 43; median of 175 (amplitude from 76 to 367) ($p < 0.001$); (chloride ≥30 to <60 mEq/L) female: $N = 691$; mean of 156 ± 38; median of 149 (amplitude from 87 to 279); male: $N = 633$; mean of 168 ± 44; median of 162 (amplitude from 78 a 299) ($p < 0.001$); (chloride ≥60 mEq/L) Female: $N = 370$; mean of 157 ± 39; median of 150 (amplitude from 78 to 295); male: $N = 332$; mean of 170 ± 44; median of 168 (amplitude from 79 to 326) ($p < 0.001$). **(B)** Sweat chloride concentrations obtained in the sweat test. (<30 mEq/L) Female: $N = 1{,}821$; mean of 18.15 ± 5.26; median of 17.4 (amplitude from 10 to 29.9); male: $N = 1{,}415$; mean of 18.54 ± 5.29; median of 17.7 (amplitude from 10 to 29.9) ($p = 0.038$); (chloride ≥30 to < 60 mEq/L) female: $N = 691$; mean of 42.08 ± 8.29; median of 41.1 (amplitude from 30 to 59.8); male: $N = 633$; mean of 40.89 ± 8.28; median of 39.3 (amplitude from 30 to 59.9) ($p = 0.006$); (chloride ≥60 mEq/L) female: $N = 370$; mean of 91.87 ± 24.53; median of 86.85 (amplitude from 60 to 158.6); male: $N = 332$; mean of 93.28 ± 25.06; median of 91.2 (amplitude from 60 to 159.2) ($p = 0.618$). The statistical analysis was performed by Mann–Whitney test. $\alpha = 0.05$. The median is set in red marker and the 95% confidence interval is set in green line. The second graphic is a box plot. mEq/L, milliequivalents per liter.

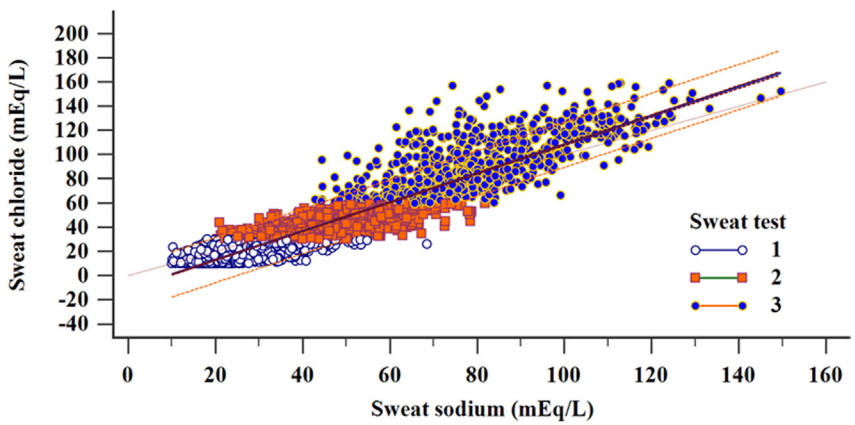

FIGURE 4 | Regression and correlation of sweat sodium and chloride concentrations distributed according to the diagnostic results considering the sweat test values (total sample). Linear regression: $N = 5{,}277$; $R^2 = 0.877$; $y = -11.05 + 1.1932x$ ($p < 0.001$). Correlation: $N = 5{,}277$; correlation coefficient $r = 0.9365$; 95% CI = 0.9331–0.9397 ($p < 0.001$); (1) $N = 3{,}249$; $R^2 = 0.4347$; $y = 5.8091 + 0.4955x$ ($p < 0.001$); (2) $N = 1{,}324$; $R^2 = 0.4913$; $y = 14.5228 + 0.5888x$ ($p < 0.001$); (3) $N = 702$; $R^2 = 0.5336$; $y = 11.4797 + 1.0064x$ ($p < 0.001$). (i) chloride <30 mEq/L; (ii) chloride ≥30 to <60 mEq/L; and (iii) chloride ≥60 mEq/L. $\alpha = 0.05$. mEq/L, milliequivalents per liter; 95% CI, confidence interval of 95%.

borderline in patients with non-classical phenotypes. However, these levels are lower in patients with classic phenotypes (16, 23). In this context, the clinical manifestation could drive to the early or late CF diagnosis by the ST. In addition, early CF diagnosis provides numerous benefits, such as prevention of early malnutrition, reduced pulmonary complications, and long-term

TABLE 4 | Association between immunoreactive trypsinogen (IRT) inclusion in our center and subject's sex and age and cystic fibrosis diagnosis.

Chloride levels (mEq/L)	Inclusion of IRT			Odds ratio	95% CI
	After	Before	Total		
<30	503	2,749	3,252	**0.313**	**0.275–0.357**
≥30 to <60	500	824	1,324	**2.603**	**2.27–2.985**
≥60	242	447	689	**1.929**	**1.625–2.289**
Sex	**Yes**	**No**	**Total**	**Odds ratio**	**95% CI**
Male	574	2,202	2,776	**0.702**	**0.618–0.797**
Female	670	1,804	2,474	1	–
Subject's age	**Yes**	**No**	**Total**	**Odds ratio**	**95% CI**
0 to <6 months	52	515	567	**0.3**	**0.224–0.403**
≥6 months to <18 years	633	2,916	3,549	**0.395**	**0.345–0.451**
≥18 years	529	541	1,070	**4.898**	**4.236–5.663**

Statistical analysis was made by chi-square test; odds ratio was calculated considering values set by Taylor series. For all analyses, P-value was <0.001. All positive values are represented in bold type. $\alpha = 0.05$.
95% CI, confidence interval of 95%; IRT, immunoreactive trypsinogen; mEq/L, milliequivalents per liter.

TABLE 5 | Distribution of patients for the *CFTR* gene genotype and classes of identified mutations*.

Genotype	N	Group of patients with cystic fibrosis
Unknown/unknown	137	169/408 (41.4%) patients with unknown mutation in the *CFTR* gene, or with one or two mutations in the *CFTR* gene belonging to classes IV, V, or VI
V562I/unknown	1	
G576A/R668C	3	
p.Glu528G > A/TG11-5T	3	
R334W/R334W	1	
D110H/V232H	1	
I507V/unknown	2	
D614G/unknown	4	
F508del/unknown	61	87/408 (21.3%) patients with mutation in the *CFTR* gene belonging to class I, II, or III, and unknown mutation or one mutation in the *CFTR* gene belonging to class IV, V, or VI
G542X/unknown	6	
G542X/P205S	2	
G542X/R334W	2	
622-2 A > G/711 + 1G > T	1	
G542X/I618T	3	
3120+ 1G > A/L206W	3	
F508del/D1152H	2	
F508del/R334W	2	
R1066C/R334W	1	
F508del/P205S	3	
R1162X/unknown	1	
F508del/F508del	88	152/408 (37.3%) patients with two mutations identified in the *CFTR* gene belonging to class I, II, and/or III
F508del/G542X	22	
F508del/N1303K	8	
F508del/R1162X	8	
F508del/R553X	5	
F508del/1584-18672pbA > G	4	
F508del/c.1717 – 1G > A	3	
3120 + 1G > A/R1066C	3	
F508del/2183AA > G	1	
F508del/2184insA	1	
F508del/6B to 16 exon duplication	2	
F508del/G85E	2	
F508del/S549R (T > G)	2	
G542X/2183AA > G	1	
G542X/R1162X	2	
F508del/S4X	3	
F508del/R1066C	4	
F508del/1812 – 1G > A	4	
R1162X/R1162X	4	
2183AA > G/2183AA > G	2	
3120 + 1G > A/3120 + 1G > A	1	

F508del/other mutation = 136/408 (33.3%); F508del/F508del = 88/408 (21.6%).
CFTR, cystic fibrosis transmembrane regulator; N, sample size.

minor deterioration in lung function, allowing higher life quality and expectancy.

Our study found that the main reason for the ST requests was related to respiratory signs and symptoms (77.1%), followed by digestive symptoms (12.3%) and nutritional symptoms (11.5%). These data are in line with a recent study performed in France with 523 STs (24). Moreover, the ST request due to digestive manifestations prevailed in the group aged under six months, showing higher correlation with chloride values ≥60 mEq/L.

According to Ribeiro et al. (25), exocrine pancreatic insufficiency is often the earliest and most prevalent symptom in about 75% of patients with CF at birth, in 80–85% at the end of the first year and in 90% at adult age (25). As digestive symptoms are early identified, it allows differential diagnosis (26, 27). This fact was observed in our data, where the indication of ST due to digestive symptoms was also associated with higher values of sweat chloride, sweat sodium, and chloride/sodium ratio, as well as lower sodium–chloride level.

The low concordance between the ST indication by respiratory symptoms and the CF diagnosis might be related with the wide variability in the pulmonary symptoms, in addition to the higher number of diseases associated with pulmonary tract as compared with digestive tract. In this context, many solicitations of ST were performed to exclude the CF diagnosis hypothesis among many other pulmonary tract diseases.

Finally, the mutual association between respiratory and digestive symptoms enabled a better odds ratio for CF diagnosis and a lower odds ratio to achieve normal values in the ST. In literature, the clinical decision rule might be cited as a reliable index of clinical suspicion and timely referral for sweat testing in settings without newborn screening (NBS) programs, and may also be applied to false-negative individuals where such programs already exist (28).

Demographic Data Related with the Variability Presented in the ST

In this study, the analysis of a larger ST sample size indicated that the levels of sweat chloride and sweat sodium vary with age and sex.

Sex

In this study, males showed higher sweat weight. In addition, males showed higher sweat chloride levels only among those with sweat chloride values <30 mEq/mL cutoff in the ST as compared with females. This is likely to be explained by the fact that the sweat volume was higher in men due to the production of their sweat glands. Males have fewer active glands with higher sweat rate per gland and increased response to cholinergic and

TABLE 6 | Association between sex of the subject, reason for indication of sweat test, result achieved from sweat test, and subjects' ages with *CFTR* mutation screening.

Chloride levels (mEq/L)	*CFTR* genotype				Odds ratio	95% CI	Odds ratio	95% CI	Odds ratio	95% CI
	IM/IM	IM/NIM	NIM/NIM	Total						
<30	6[a]	0	6	12	1.429	0.453–4.51	–	–	1.712	0.542–5.407
≥30 to <60	4[b]	3	48	55	**0.089**	**0.023–0.252**[a]	**0.185**	**0.056–0.607**[a]	**16.42**	**7.193–37.47**
≥60	159	84	98	341	**4.98**	**2.461–10.08**	**6.973**	**2.135–22.77**	**0.097**	**0.05–0.186**
Subject's age										
0 to <6 m	50	17	1	68	**5.242**	**2.922–9.401**	1.294	0.703–2.383	**0.018**	**0.001–0.095**[a]
≥6 m to <18 years	106	47	75	228	**1.664**	**1.106–2.504**	0.916	0.565–1.483	**0.634**	**0.421–0.954**
≥18 years	9	21	74	104	0.085	0.041–0.175	0.732	0.425–1.263	**7.14**	**4.34–11.75**
Respiratory										
Yes	36	17	18	71	**0.356**	**0.188–0.674**	1.425	0.682–2.979	**4.118**	**1.678–10.1**
No	78	19	8	105	1	–	1	–	1	–
Chloride levels (mEq/L)	**Presence of F508del mutation**				**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**
	+/+	+/–	–/–	Total						
<30	2	4	6	12	0.722	0.075–3.479[a]	1	0.216–3.815[a]	1.225	0.388–3.863
≥30 to <60	2	4	49	55	**0.117**	**0.014–0.463**[a]	**0.131**	**0.046–0.372**[a]	**13.19**	**5.501–31.62**
≥60	84	128	129	341	**5.148**	**1.819–14.57**	**4.432**	**2.052–9.574**	**0.133**	**0.069–0.257**
Subject's age										
0 to <6 m	31	29	8	68	**4.042**	**2.318–7.048**	1.701	0.997–2.902	**0.121**	**0.056–0.261**
≥6 m to <18 years	52	83	93	228	1.116	0.691–1.804	1.522	0.99–2.341	**0.642**	**0.431–0.957**
≥18 years	5	18	81	104	**0.13**	**0.051–0.33**	0.344	0.197–0.602	**6.799**	**4.036–11.46**
Sex										
Male	53	60	69	182	**2.242**	**1.385–3.63**	0.971	0.641–1.469	**0.589**	**0.396–0.877**
Female	35	76	115	226	1	–	1	–	1	–
Respiratory										
Yes	12	32	27	71	**0.282**	**0.136–0.586**	1.094	0.597–2.007	**3.413**	**1.668–6.985**
No	44	45	16	105	1	–	1	–	1	–

NIM—unknown mutation in the CFTR gene, or with one or two mutations in the CFTR gene belonging to classes IV, V, or VI; IM—known mutations in the CFTR gene belonging to class I, II, and/or III; (+) shows that the F508del allele is present; (–) shows that the F508del allele is not present.

[a]*The cystic fibrosis patients showed the following genotypes: four patients F508del/N1303K and two patients F508del/F508del.*

[b]*One patient F508del/S4X, one patient F508del/N1303K, and two patient F508del/F508del. Statistical analysis was made by chi-square test; odds ratio was calculated considering values set by Taylor series, except for the data marked with [a] (odds ratio was calculated considering values set by Fisher's exact test).*

For all analyses, P-value was <0.002. All positive values are represented in bold type. $\alpha = 0.05$.

CFTR, cystic fibrosis transmembrane regulator; 95% CI, confidence interval of 95%; IM, identified mutation; m, months; mEq/L, milliequivalents per liter; NIM, non-identified mutation.

beta-adrenergic stimulation, as compared with females (5). In this context, these observations indicate prospects for the diagnosis of functional changes of the CFTR protein that include the difference between sexes (29).

Subject's Age

Notably, the onset age of CF symptoms is widely variable: they may occur in the first years of life, childhood, adolescence, or even adulthood. This complex onset of symptoms can be explained by environmental factor, modifier genes, and different mutations in the *CFTR* (1). Therefore, the CF diagnosis should be made as soon as possible, with a high degree of certainty due to medical, financial, and psychosocial implications. Moreover, in our study group, we showed the correlation between subject's age and sweat chloride levels regarding the ST results (19).

Subjects under six months and over 18 years of age showed greater probability of positive ST (≥60 mEq/L) and higher prevalence of sweat samples with chloride levels <30 mEq/L. This can be explained by the fact that STs had been indicated for children younger than six months due to clear clinical signs and probability of a CF diagnosis.

The highest prevalence ratio for borderline sweat chloride levels could be observed in the group over 18 years of age. The CFTR dysfunction causes a series of disorders in the organism, and the ST does not always distinguish CF from other pathological conditions also mediated by CFTR (30). However, we observed high probability to perform the ST with values ≥60 mEq/L in patients over 18 years of age, and this fact can be associated with the exclusion of other diseases, mainly for lung pathologies, during the first years of life. After the exclusion of common diseases, as asthma, the indication for the ST with a positive result could show higher probability.

The indication of the ST due to respiratory symptoms prevailed among subjects aged six months to 18 years. Lung diseases are more frequent after six months of age and their presence and deterioration have been assessed by lung function tests, bacteriological

TABLE 7 | Association between sweat test markers and *CFTR* mutation screening.

Variable	Category	N	Mean	Standard Variation	Median	Minimum	Maximum
***CFTR* mutation**							
Sweat weight (mg)[a]	IM/IM	169	163	4.4	161	79	326
	IM/NIM	87	174	4.6	174	78	276
	NIM/NIM	152	160	3.9	152	96	268
Sweat sodium (mEq/L)[a]	IM/IM	169	84.16	21.86	83.7	10.1	145.08
	IM/NIM	87	85.51	18.16	85.2	44.4	128.76
	NIM/NIM	152	68.62	20.76	67.2	14.23	113.8
Sweat chloride (mEq/L)[b]	IM/IM	169	107.29	27.08	109.42	13.1	159.2
	IM/NIM	87	107.03	23.41	113.6	46.8	152.5
	NIM/NIM	152	71.03	25.68	66	12.4	140.16
Sodium–chloride[b]	IM/IM	169	−23.13	18.21	−21.1	−82.7	22
	IM/NIM	87	−21.52	17.5	−19.2	−71.97	13.27
	NIM/NIM	152	−2.41	11.71	−0.46	−46.01	32.7
Chloride/sodium[b]	IM/IM	169	1.292	0.261	1.25	0.57	2.11
	IM/NIM	87	1.269	0.255	1.226	0.8	2.12
	NIM/NIM	152	1.029	0.174	1.007	0.49	1.73
F508del mutation screening							
Sweat sodium (mEq/L)[c]	+/+	88	84.4	20.8	84.95	10.1	124.6
	+/−	136	84.02	21.71	83.05	30.2	145.08
	−/−	184	71.95	21.15	70.35	14.23	118.81
Sweat chloride (mEq/L)[c]	+/+	88	107.69	25.86	106.8	13.1	159.2
	+/−	136	105.52	26.89	111.36	21.6	158.6
	−/−	184	78.33	29.51	72.61	12.4	152.5
Sodium–chloride[c]	+/+	88	−23.27	16.78	−23.2	−64.3	11.5
	+/−	136	−21.5	18.39	−18.65	−82.7	22
	−/−	184	−6.39	15.88	−1.85	−73.42	32.7
Chloride/sodium[c]	+/+	88	1.294	0.23	1.263	0.87	1.98
	+/−	136	1.273	0.275	1.21	0.57	2.12
	−/−	184	1.077	0.22	1.03	0.49	2.04

NIM—unknown mutation in the CFTR gene, or with one or two mutations in the CFTR gene belonging to classes IV, V, or VI; IM—known mutations in the CFTR gene belonging to class I, II, and/or III; (+) shows that the F508del allele is present; (–) shows that the F508del allele is not present.

[a]IM/NIM genotype showed difference regarding the other groups.

[b] NIM/NIM genotype showed difference regarding the other groups.

[c]–/– genotype showed difference regarding the other groups.

Statistical analysis was made by Kruskal–Wallis test. For all analyses, P-value was <0.001. $\alpha = 0.05$.

CFTR, cystic fibrosis transmembrane regulator; IM, identified mutation; mEq/L, milliequivalents per liter; mg, milligram; N, number of subjects; NIM, non-identified mutation.

markers, and high-resolution computed tomography (31). The early diagnosis should be done once the lungs of patients with CF are often colonized and infected with microorganisms that cause damage to the epithelial surface at early childhood. Chronic infections are the main reason for reduced lung function and are associated with increased morbidity and mortality in CF (32).

Finally, clinical indication should consider the subject's age in order to induce the ST performance. Even if the NBS was performed, the presence of false-negative tests may occur. In the presence of IRT false-negative test and symptoms related with CF, the ST performance should be recommended.

Correlation between Sweat Chloride and Sweat Sodium

The amounts of sweat sodium did not add discriminatory value to CF diagnosis. Current guidelines do not recommend using amounts of sweat sodium to CF diagnosis. However, some laboratories use this value for quality control purposes. The use of sweat sodium as quality control is given by the positive correlation between both ions (3), and this fact was showed in our study.

The association between sweat chloride and sweat sodium parameter to the ST quality control was analyzed by us previously, and we considered that the sweat sodium may be an indicative for quality discrimination, mainly in cases of doubts regarding the sweat chloride values (20). Even so, other studies should be done to clarify the biological variability, considering both ions dosage, observed in the ST among subjects that performed the test. Moreover, the studies should consider an ST performance overview, as a wide number of limitations and problems related in this examination at public and private centers is known (21). In this case, sweat sodium dosage should be stimulated in daily routine.

Finally, in all ages, levels of sweat sodium and sweat chloride were positively correlated, which had been documented in previous studies (3, 15).

CFTR Mutation Influencing the Data Presented in the ST

In 1989, mutations in the *CFTR* gene were described as a causative factor of CF. The identification of the *CFTR* mutations directed the diagnosis toward molecular biology, allowing greater understanding of CF, revision of the diagnostic criteria, and new therapeutic possibilities (1, 33–35). Moreover, the *CFTR* mutations classification showed changes along the years (17). In this context, with the advances in the molecular field, more

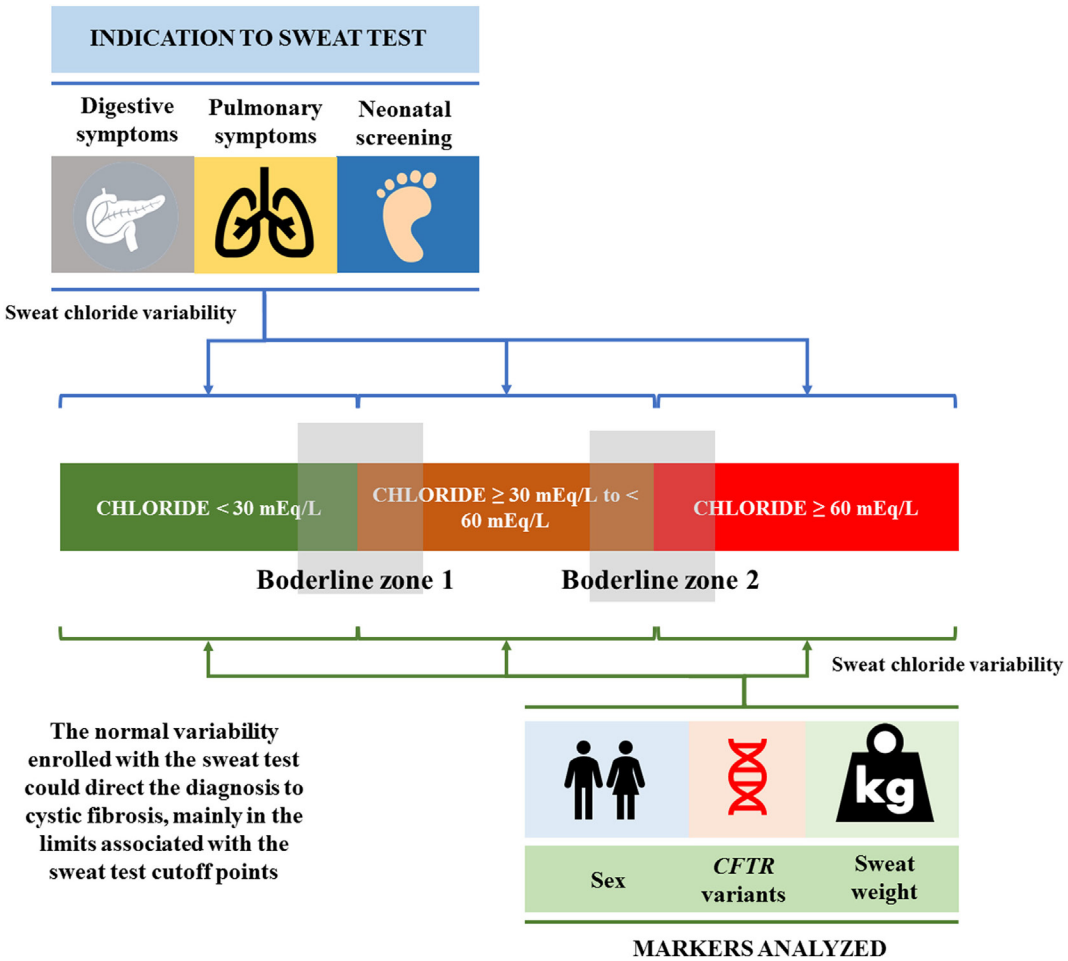

FIGURE 5 | The normal variability enrolled with the sweat test, which could direct the diagnosis to cystic fibrosis, mainly in the limits (borderline zones 1 and 2) associated with the sweat test cutoff points. In addition, sex, *CFTR* mutation, sweat weight, digestive symptoms, pulmonary symptoms, and neonatal screening influenced the values achieved in the sweat test, and this influence is present in all possible sweat test classes: (i) <30 mEq/L; (ii) ≥30 to <60 mEq/L; and (iii) ≥60 mEq/L. *CFTR*, cystic fibrosis transmembrane regulator.

than 2,000 mutations have been identified in the *CFTR* gene (33); however, its association with the results presented in the ST is not well known. Advances in this field are important to define the reference and levels of sweat chloride in the ST for each class of mutations in the *CFTR* gene (16, 36).

In addition, variants in the *CFTR* gene may cause several clinical phenotypes, such as chronic sinusitis, gastrointestinal disorders, and pulmonary diseases (1, 37). Thus, the ST should be an important tool for the CF diagnosis, particularly in the absence of the identification of mutations in the *CFTR* gene. It has also contributed to deeper understanding of the physiopathology aspects of CF and of the effects of some drugs to restore the function of the CFTR protein. The ST may show controversial results (although rare); therefore, further diagnostic methods are needed in some cases, as we showed in our data, when we identified 10 patients with CF and two *CFTR* mutations belonging to classes I, II, or III, and absence of positive ST.

Newborn Screening by Immunoreactive Trypsinogen

The implementation of NBS tends to increase the number of CF diagnosis in newborn infants. In CF, the release of trypsinogen in blood is increased as a result of the obstruction caused by secretion accumulation in the pancreatic ducts. High IRT levels are a marker of neonatal screening test for CF (38) and the diagnosis is confirmed by duplicate STs and/or the detection of two mutations in the *CFTR* gene (1).

In our data, the annual number of the ST performed was the same as before and after the implementation of IRT dosage. In addition, we showed a higher number of ST performed in patients aged over 18 years after the implementation of IRT dosage. This fact can be associated with a higher number of solicitations by the adult pneumology, correlated with the improvement of knowledge of (i) CF and residual CFTR function; (ii) presence of *CFTR* mutations included in the classes IV, V, and VI; and (iii) better registry from the adult clinic in the last years.

Limitations of the Study

Our study shows the following limitations: (i) it did not include a control group, i.e., there may be patients with negative ST with CF; (ii) it was not possible to confirm a CF diagnosis for all patients through genetic studies; (iii) the reason for the ST referral for 1,877/5,277 (35.57%) sweat samples could not be obtained; (vi) there was inclusion of a higher number of subjects under 18 years of age than of older subjects.

Highlight

(i) High levels of sweat chloride with the presence of CF indicative signs and symptoms are the gold standard for the CF diagnosis.
(ii) Amount of chloride is greater in men than women in all ST reference ranges.
(iii) Indication to perform the ST regarding respiratory symptoms was associated with minor probability of presenting CF disease and age between six months and 18 years.
(iv) Indication to perform the ST regarding digestive symptoms was associated with higher probability of presenting CF disease and age under six months.
(v) CF diagnosis regarding the age cutoffs showed association between them.
(vi) Subjects with digestive symptoms showed higher chloride levels than subjects without this type of symptom. In addition, subjects with respiratory symptoms showed lower chloride levels in their tests than subjects without this type of symptom.
(vii) Indexes [(sweat chloride/sweat sodium) and (sweat sodium–sweat chloride)] used in the article showed association with sex, reason for ST indication, and *CFTR* mutations.
(viii) After the NBS implementation by the IRT, there was a higher chance of borderlines values in the ST and CF diagnosis.
(ix) A wide spectrum of *CFTR* mutation was found with the highest prevalence of F508del identification.
(x) *CFTR* mutation identification or presence of F508del/F508del genotype was associated with higher probability of ST chloride levels ≥60 mEq. Moreover, the absence of *CFTR* mutations identified was associated with borderlines values in the ST, as well as with presence of respiratory symptoms.

Perspectives

Some issues are not well known in CF disease. For example, a large number of subjects with intermediate amounts of ions in sweat will develop classic forms of CF in the future (39). This fact was observed by Groves et al. (39), who carried out a 15-year monitoring on patients with sweat chloride concentrations between 30 and 59 mEq/L. From all patients with positive NBS by IRT, a positive allele for F508del, and intermediate ST, 14/29 (48%) developed classic CF. These data should be analyzed prospectively in many centers worldwide regarding the values of chloride longitudinally and the inclusion of other ST markers, as we analyzed in the present study (sweat chloride and sweat sodium ratio; sweat sodium–sweat sodium; correlation with sweat chloride and sweat sodium). In addition, the intervals of chloride concentration to perform the ST should be analyzed and revised. The variability in the ST might be associated with the populational structure.

The outlier data from the ST should be considered, taking into account that 2% of US Americans with CF have normal sweat chloride levels in the ST (3, 5). Patients with CF and normal ST should be recognized among the health subjects, or regarding other disease. This fact is more important in the genetic era, when the personalized/precision medicine is available for some *CFTR* mutations and probability will be available to all classes of *CFTR* mutations. The innate variability of ST is associated with variants of the *CFTR* gene, presence of modifier genes, and individual biological variation in the production of chlorides, possibly through other ion channels (3, 30), as long as laboratory errors have not occurred. Furthermore, sweat chloride as a biomarker of CFTR activity in the personalized/precision medicine was already used and should be better understood (40).

The function of CFTR should be better studied. The sweat glands in patients with CF, unlike the glands of healthy individuals, do not excrete sweat in response to beta-adrenergic stimulation, but to cholinergic stimulation. Healthy individuals with one allele with mutation in the *CFTR* gene (e.g., parents of patients with CF) excrete 50% of sweat, due to beta-adrenergic stimulation. Function evaluation tests of the CFTR protein may provide, indirectly, identification of carriers of *CFTR* gene mutations, which enables proper genetic counseling (5, 9). However, this fact is not well studied to be used as daily practice. Other tools could be studied, including the sweat induction by evaporimeter, chloride dosage from saliva, nasal potential difference, rectal biopsies, and organoids so that to evaluate the CFTR presence and function (2–9).

Finally, it is necessary but not sufficient to organize and publish CF diagnosis consensus processes. In addition, monitoring implementation efforts and practices seem essential as recommended in the literature (41–43).

Conclusion

The ST data showed wide variability, which was dependent on age, sex, reason for examination indication (respiratory and digestive symptoms), *CFTR* mutations, and weight of the sweat sample collected. Sweat sodium concentration is directly correlated with sweat chloride levels and it should be used as a quality parameter.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethics Committee of the University of Campinas (Protocol no. 474326), with written informed consent from the institution in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AGF, FALM, and JDR made substantial contributions to conception and design, acquisition, analysis, and interpretation of data; they were involved in drafting the manuscript and revising it critically for important intellectual content; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work by ensuring that questions related to the accuracy or integrity of any part of the work have been

appropriately investigated and resolved. CCSG, MFS, and AFR made substantial contributions to conception and design of the study, acquisition, analysis, and interpretation of data.

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6. Discussão

As manifestações clínicas na FC são complexas e, mesmo décadas após a descrição da doença e identificação de *CFTR*, a totalidade do espectro clínico e as repercussões laboratoriais da doença (incluindo o TS) não são completamente conhecidas ou compreendidas (Marson *et al.*, 2017).

A quantificação dos eletrólitos no suor tem sido realizada há mais de 60 anos e, até os dias atuais, ainda é utilizada e considerada o "padrão ouro" para o diagnóstico de FC.

Nos últimos anos surgiram desafios no diagnóstico e manejo clínico de indivíduos com FC e de acordo com o consenso desenvolvido pela CFF (2017), indivíduos identificados pela triagem neonatal, com sinais e sintomas clínicos e/ou histórico familiar para FC podem ser diagnosticados pelos valores do TS ≥ 60 mEq/L ou com o íon cloreto entre 30 a 59 mEq/L, se houver a presença de duas mutações de *CFTR* (Farrell *et al*., 2008; Levy e Farrell, 2015; Farrel *et al.*, 2017).

As medidas das concentrações do íon cloreto no TS fornecem um indicador sensível da atividade da proteína CFTR e, apresenta associação com os fenótipos da FC (Rowe *et al.,* 2007; Marson *et al.,* 2013; Tiddens *et al.*, 2015).

Recentemente, um novo papel foi acrescentado para o TS e, mais precisamente, a quantificação do íon cloreto no TS, que se tornou uma medida robusta para avaliar a eficácia de agentes corretores e potencializados da CFTR.

Como os níveis do íon cloreto no suor mudam de forma significativa com pequenas mudanças na atividade da CFTR e, como a função da CFTR varia muito entre os pacientes, com e sem FC, é importante conhecer, cada vez mais, a fisiopatologia envolvida com os mecanismos da função e/ou presença da CFTR.

As variações que ocorrem nos níveis dos íons cloreto no suor podem estar ligadas a influência de variações biológicas intraindividuais. Tem sido mostrado que indivíduos saudáveis apresentam variação intraindividual entre ~14,2 a 32,8% nos valores de íons cloretos no suor (Koerbin *et al.*, 2008; Collie *et al.* 2014). Nesse contexto, um paciente com resultado de TS de 50 mEq/L, e com variação biológica de 20%, como desvio padrão, terá uma faixa de resultado, que oscila entre 30 a 70 mEq/L, desconsiderando a imprecisão analítica (Collie *et al.*, 2014). Variações fisiológicas no estado de hidratação podem estar relacionadas com a variação biológica intraindividual, mas as explicações para essas alterações ainda não são compreendidas.

Outro fator que dificulta o diagnóstico de FC, pela avaliação do TS, foi descrita pela CFF, em 2010, que relata a prevalência de 5% de TS com valores do íon cloreto < 60 mEq/L na FC. Os fatores que sustentam a especificidade do TS podem estar associados com a influência de diferentes mutações do *CFTR*, variações biológicas individuais e devido a limitações do próprio teste.

Duas investigações sobre a variação dos níveis do íon cloreto no TS estudaram as diferenças nos valores do TS entre coletas simultâneas nos braços direito e esquerdo. Mackay e colaboradores utilizaram o método de Gibson e Cooke para comparar 295 TS em pares. Os resultados mostraram um coeficiente de variação de 20,2% para o íon cloreto entre os dois braços. Mesmo com a elevada variabilidade, entre os dois braços, o TS, definiu claramente a maioria dos resultados de pacientes saudáveis e com FC. Em contrapartida, demonstra a incerteza considerável para aqueles sujeitos que produzem resultados intermediários (Mackay *et al.*, 2008; Collie *et al.* 2014). Dessa forma, destacam-se a variabilidade e a importância da interpretação do resultado do TS.

Em contrapartida, a presença de dados clínicos sugestivos de FC associada a quantidade de íons no suor maiores que 60 mEq/L, possui elevada especificidade e sensibilidade para demonstrar disfunção ou ausência da CFTR (Farrell *et al.*, 2017).

Pacientes com FC tem proporcionalmente elevados valores para ambos os eletrólitos, sódio e cloreto, com diferença entre eles que não excede 15 mEq/L. O nível do íon sódio no TS, na FC, é usualmente menor que o do íon cloreto, e a relação contrária ocorre nos indivíduos sem FC (Beauchamp e Lands, 2005).

Um aspecto prático, que tem provocado controvérsias entre os investigadores, é se as quantidades do íon sódio adicionam valor discriminativo no diagnóstico de FC. Os *guidelines* se dividem quanto a utilização do íon sódio no diagnóstico de FC e muitos não recomendam a utilização desse eletrólito (Collie *et al*., 2014). No entanto, muitos laboratórios utilizam a dosagem do íon sódio com o propósito de controle de qualidade do TS (Collie *et al.*, 2014). A utilização do íon sódio como controle de qualidade é dada pela correlação positiva de ambos os íons e nós mostramos a importância de sua dosagem em nossos estudos, principalmente, em casos duvidosos dos valores do íon cloreto obtido no TS.

A idade é uma variável significativa para a interpretação do TS. A variação pode ser explicada por fatores ambientais, genes modificadores e mutações do *CFTR*. Os pacientes com fenótipo não clássico da FC mostram sintomas mais tardiamente na vida (adolescência ou vida adulta), demonstrando formas menos graves da doença, com suficiência pancreática e sintomas respiratórios moderados. Nesses casos, os níveis do íon cloreto podem ser normais, anormais ou limítrofes e as manifestações clínicas podem direcionar para um diagnóstico precoce ou tardio.

Nós observamos que o íon cloreto no TS varia ao longo da vida; com comportamentos diferentes entre os pacientes com e sem FC. No entanto, os mecanismos para estas alterações

com a idade não puderam ser esclarecidos no trabalho de Traeger e colaboradores e nem no nosso. Quando analisamos a população total dos dois trabalhos podemos constatar que os níveis dos íons cloreto diminuem no primeiro de vida e aumentam com a idade. Este fato poderia ser explicado por instabilidade da proteína CFTR em saudáveis, naqueles com FC e naqueles com o TS limítrofe. No entanto, se estudarmos separadamente por *follow-up* das populações, com e sem FC, seria mais provável discriminar com mais acurácia a evolução dos níveis de eletrólitos no suor e quem sabe permitir o entendimento do funcionamento da CFTR via o TS.

Em contrapartida, fica difícil entender para todos os indivíduos, nos dois trabalhos, a redução nos níveis do íon cloreto, a partir dos 18 anos. Acreditamos que, com o avançar da idade e o "envelhecimento" da CFTR, haveria aumento nas concentrações de eletrólitos no suor. Portanto, como ocorreu o contrário do esperado, nos dois estudos, pode-se hipotetizar que o processo de envelhecimento causa mudanças em outros canais reguladores do íon cloreto.

Além da idade os nossos estudos demonstraram que há diferença no peso do suor quando comparamos o sexo feminino ao masculino com maiores valores no sexo masculino, de peso do suor e níveis do íon cloreto, quando comparado com ao feminino. Nesse contexto, as observações indicam perspectivas para o diagnóstico de alterações funcionais da proteína CFTR que incluem a diferença entre os sexos.

Segundo Collaco e colaboradores, mutações do *CFTR* é causa predominante de variações dos níveis do íon cloreto no TS. Este fato foi documentado no trabalho recente do nosso grupo por Bonadia colaboradores. Ainda, a maior parte da variação não-CFTR é causada por variabilidade individual, idade, sexo, fatores ambientais e genéticos que

interferem diretamente e indiretamente nos níveis dos íons no TS (Bonadia *et al.*, 2014; Cutting, 2015; Collaco *et al.*, 2016).

Porém, mesmo com os avanços no campo da biologia molecular e mais de 2.000 mutações identificadas do *CFTR*, a associação das mutações com os resultados do TS ainda não está bem esclarecida. Avanços nessa área são importantes para definir os valores de referência e os níveis de cloreto no TS para cada classe de mutação de *CFTR* (Bonadia *et al.*, 2014; Cutting, 2015).

Apesar da busca constante de um biomarcador único, o TS, pela alta especificidade e sensibilidade, vem sendo realizado há mais de 60 anos e ainda é o padrão ouro para o diagnóstico da FC.

7. Perspectiva

O conhecimento da fisiologia dos órgãos envolvidos na FC, como a glândula sudorípara, tem contribuído, enormemente para o entendimento da doença. A avaliação da função da proteína CFTR foi e será um campo promissor.

Pacientes com FC, diferentemente de indivíduos saudáveis, não excretam suor em resposta a estimulação beta adrenérgica, somente colinérgica. Indivíduos saudáveis com somente um alelo com mutação de *CFTR* (como pais de pacientes com FC) excretam 50% do suor, devido a estimulação beta adrenérgica. Portanto, testes de avaliação da função da proteína CFTR podem fornecer, indiretamente, informações das mutações do *CFTR* que permitem um adequado aconselhamento genético.

Dentre as ferramentas que constituem boas perspectivas, para avaliar a presença e a função da CFTR, podemos citar:

- Indução do suor pela evaporimetria (Quinton *et al.*, 2012);
- Dosagem do íon cloreto na saliva (Gonçalves *et al.,* 2013);
- Diferença de potencial nasal (Ng *et al.,* 2015)
- Biópsia retal e organoides (Servidoni *et al.,* 2013);
- Espectometria de massa (Estevez *et al.,* 2018).

8. Conclusões

Durante todos esses anos o TS provou ser um teste robusto no diagnóstico de FC e, tem contribuído para o entendimento da fisiopatologia da doença. Apesar disso, o TS mostra ampla variabilidade dependente da idade, sexo, razão para indicação do exame, mutações de *CFTR* e peso da amostra de suor.

Padronização rigorosa para realização do TS, novas técnicas de análise do íon cloreto e maior aderência às diretrizes podem ser parâmetros para atingir maior sensibilidade e especificidade do TS para o diagnóstico de FC.

Conclusão do artigo 1

A qualidade do TS pode ser avaliada pelas concentrações dos íons sódio e cloreto. A qualidade do TS pode estar associada com importantes fatores como: sexo, resultado do diagnóstico de FC e idade. Embora seja considerado padrão ouro no diagnóstico de FC, o TS, tem limitações e pode produzir resultados falso positivos e falso negativos. Esforços constantes, para o entendimento dos resultados e a busca de marcadores de qualidade para a realização do TS devem ser continuos.

Desse modo, considerando a proporção adotada nesse estudo a quantificação do uso do íon sódio ainda é necessária no TS e atenção especial deve ser dirigida aos pacientes com resultado limítrofes, onde pode ser observado grande quantidade de resultados com proporções inadequadas dos eletrólitos.

Conclusão do artigo 2

Em indivíduos com FC o íon cloreto aumenta no primeiro ano de vida e, após o primeiro ano de vida diminui com a idade. Na população total podemos constatar que os níveis do íon cloreto diminuem no primeiro de vida e aumentam com a idade. Este fato poderia ser explicado por instabilidade da proteína CFTR em saudáveis, naqueles com FC e naqueles com TS limítrofe. Outros estudos devem ser realizados para avaliar a interferência da idade nos níveis do íon cloreto em saudáveis e na FC.

Conclusão artigo 3

Os dados do TS mostraram variabilidade dependente da idade, sexo, razão para indicação do exame, mutações de *CFTR* e peso da amostra de suor. A concentração do íon sódio está correlacionada com os níveis do íon cloreto. O sódio pode ser usado como parâmetro de qualidade do TS.

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10. ANEXOS

Anexo 1 – Autorização do artigo 1

Roberto Asturias Jr. <robert... 03/01/2018

Para: Alethéa Guimarães Faria Guimarãe... Detalhes

RJ

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Anexo 2 – Autorização artigo 2

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Please do let me know if you have any further questions.

Best regards,
Victor
--

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Anexo 4 - Questionário para coleta dos dados dos pacientes

Nome: _____ Data da coleta: _____

Data de nascimento: ____/____/____

Idade na data do exame (anos e meses): _____

Sexo: ( ) feminino ( ) masculino

Cor:

( ) Branca

( ) Pardo

( ) Negro

( ) Oriental

Indicação do teste:

( ) Sintoma trato respiratório

( ) Sintoma trato digestório

( ) Sintoma nutricional ( ) IMC: _____

( ) Outro: _____

Triagem neonatal: ( ) sim ( ) não

Dosagem 1: IRT _____ ng/mL

Dosagem 1: IRT _____ ng/mL

Análise genética:

Mutações identificadas: _____

Dosagem - Teste de suor (TS)

TS	Data	Peso amostra (mg)	Sódio (mEq/L)	Cloreto (mEq/L)	Membro testado	Tempo de coleta do suor (minutos)	Tempo de dosagem dos eletrólitos (minutos)
1							
2							
3							
4							
5							
6							
7							
8							
9							

Após três dosagens:

Repetir teste: ( ) sim não ( )

Motivo: _____

Data: ___/___/___