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CIBELE ZANARDI ESTEVES

APLICAÇÃO DA METABOLÔMICA EM COLESTASE NEONATAL E FIBROSE CÍSTICA: A BUSCA DE MARCADORES PARA DIAGNÓSTICO UTILIZANDO ESPECTROMETRIA DE MASSAS

APPLICATION OF METABOLOMICS IN NEONATAL CHOLESTASIS AND CYSTIC FIBROSIS: THE SEARCH FOR MARKERS FOR DIAGNOSIS USING MASS SPECTROMETRY

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Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos necessários para obtenção do título de Doutora em Ciências.

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RESUMO

Seguindo os avanços da genômica e da proteômica, novos campos das “ômicas” estão emergindo. A metabolômica complementa naturalmente os estudos na área de genômica e proteômica, no entanto a grande diversidade físico-química do metaboloma conduz a uma subdivisão dos metabólitos. Assim, a lipidômica está atualmente emergindo como uma disciplina na interface da bioquímica de lipídeos, tecnologia e medicina. Esta expansão do campo da lipidômica pode ser atribuída a avanços na tecnologia analítica, em particular, no desenvolvimento de novas técnicas de espectrometria de massas para a caracterização e quantificação das diversas espécies de lipídeos presentes em um lipidoma. O objetivo desta tese foi a busca por biomarcadores que representem o estado de doença e que possam auxiliar no desenvolvimento de novos métodos diagnósticos baseados em espectrometria de massas. Desta forma, foram estudadas duas doenças: colestase neonatal e fibrose cística analisando-se amostras de papel filtro com sangue seco e pele e saliva, respectivamente. As amostras foram coletadas de forma simples e não invasiva e foram preparadas de maneira rápida para serem injetadas diretamente em espectrômetro de massas, sem etapas de separação. A espectrometria de massas de alta resolução associada a validação estatísticas dos marcadores encontrados através de análise discriminante por mínimos quadrados parciais ofereceu aos métodos desenvolvidos alta sensibilidade e especificidade possibilitando uma potencial implementação na rotina de diagnóstico de colestase neonatal e fibrose cística.

Palavras chave: colestase neonatal, fibrose cística, espectrometria de massas de alta resolução, biomarcadores, metabolômica.

ABSTRACT

Following the advances of genomics and protection, new fields of "omics" are emerging. The metabolomics complement naturally the studies in the area of genomics and proteomics, however a great physicochemical diversity of the metabolism leads to a subdivision of the metabolites. Thus, lipidomics is present emerging as a discipline at the interface of biochemistry of lipids, technology and medicine. This expansion of the lipid field can be attributed to advances in analytical technology, in particular, there is no development of new mass spectrometry techniques for a characterization and quantification of the various lipid species present in a lipidoma. The objective of this research was a search for biomarkers that represent the disease state and that does not help any development of new diagnostic methods based on mass spectrometry. In this way, two diseases were studied: neonatal cholestasis and cystic fibrosis, analyzing dryed blood spots and skin imprinting and saliva, respectively. Samples collection was simple and non-invasive and were prepared quickly to be directly infused in mass spectrometer, without separation steps. High resolution mass spectrometry associated with statistical validation of the markers on the partial least squares discriminant analysis offered to the developed methods high sensitivity and specificity allowing a potential implementation in the routine of diagnosis of neonatal cholesterol and cystic fibrosis.

Keywords: neonatal cholestasis, cystic fibrosis, high resolution mass spectrometry, biomarkers, metabolomics.

LISTA DE ABREVIATURAS

BA	Atresia bililar (*Biliary Atresia*)
BAc	Ácidos biliares (*Bile Acids*)
CF	Fibrose cística (*Cystic fibrosis*)
CFTR	Regulador da condutância transmembrana da fobrose cística (*cystic fibrosis transmembrane conductance regulator*)
CFSPID	Fibrose cística com diagnóstico inconclusivo (Cystic fibrosis screen positive inconclusive diagnosis)
Chol	Colestase (*Cholestasis*)
DAG	Diacilglicerol (*Diacylglycerols*)
ESI	Ionização por spay de elétrons (*Electronspray ionization)*
FA	Ácidos Graxos (Fatty Acids)
GCL	Glutamilcisteína ligase (*Glutamate-Cysteine Ligase*)
GGT	Gama-glutamil transferase (*γ-Glutamyl Transferase*)
GSH	Glutationa (*Gluthatione*)
GSSG	Glutationa dissulfito (*Gluthatione dissulfide*)
HRMS	Espectrometria de massas de alta resolução (*High-resolution mass spectrometry*)
MPO	Mieloperoxidase (*Myeloperoxidase*)
MS	Espectrometria de Massas (*Mass spectrometry*)
NRS	Espécies reativas de nitrogênio (*Reactive nitrogen species*)
OPLS-DA	Análise discriminante dos mínimos quadrados parciais ortogonais (*Ohthogonal partial least squares discriminant analysis*)
PA	Ácido Fosfatídico (*Phosphatidic acid*)
PC	Fosfatidilcolina (*Phosphatidylicholine)*
PE	Fosfatidiletanolamina (*Phosphatidylethanolamine)*
PEMT	Fosfatidiletanolamina metil transferase (*Phosphatidylethanolamine-N-methyl transferase*)
PG	Fosfatidilglicerol (*Phosphatidylglycerol)*
PGF2α	Prostaglandina F2 alfa (*Prostaglandin F2 alpha*)
PI	Fosfatidilinositol (*Phosphatidylinositol*)
PKC	Proteína quinase C (*Protein kinase C*)
PLS-DA	Análise discriminante dos mínimos quadrados parciais (*Partial least squares discriminant analysis*)
PS	Fosfatidilserina (*Phosphatidylserine)*
ROC	Característica de reação do receptor (*Receiver operating curve*)

ROS	Espécies reativas de oxigênio (*Reactive oxigen species*)
SAH	Adenosil homocisteína (*S-Adenosylhomocysteine*)
SAM	Adenosil metionina (*S-Adenosylmethionine*)
SM	Esfingomielina (*Sphingomyelin*)
SMase	Eesfingomielinase (*Sphingomyelinase*)
TAG	Triacilglicerol (*Triacylglycerols*)
TNFα	Fator de necrose tumoral alfa (*Tumor necrosis factor alpha*)
VIP	Variação da importância na progressão (*Variation importance in projection*)

LISTA DE FIGURAS

GSH levels due impairment in CFTR secretion and enzymatic modifications. In **B)** the consequent diminish in GSH:GSSG and overall antioxidant capacity levels, lead to the formation of Nitrotyrosine and Dityrosine, biomarkers of the enhanced MPO and NOS activity in oxidative stress.

GCL - Glutamate-Cysteine Ligase; GGT - γ-Glutamyl Transferase; GSH - Glutathione; GSSG - Glutathione dissulfide; MPO - Myeloperoxidase; NOS - Nitric Oxide Synthase; PEMT - Phosphatidylethanolamine-N-Methyl Transferase; SAH - S-Adenosylhomocysteine; SAM - S-Adenosylmethionine.

Figura 9 Representative fingerprinting spectra on negative ion mode (150-700 *m/z*) of the groups: Control and Cystic Fibrosis. The mass of each group's biomarkers are highlighted, and full information on these molecules can be seen in Tabela 4

Figura 10 Partial Least Square Discrimination Analysis (PLS-DA), with the comparison between the Control (green) and Cystic Fibrosis (red) groups. The 95% confidence interval of the model is displayed as the shaded colors among samples within each group

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INTRODUÇÃO GERAL

Metabolômica

A grande quantidade de genomas disponíveis em domínio público tem ocupado os pesquisadores com o desafio de conectar os genes com suas funções, ou seja, o genótipo com o fenótipo. O impulso para entender a função dos genes descobertos recentemente impulsionou a análise sistemática dos níveis de expressão de componentes de um sistema biológico, tais como mRNA, proteínas e metabólitos, e o catalogamento global destes componentes tem dado origem a vários “OMAs” (o genoma, o proteoma, o metaboloma). Entender a rede de componentes e com eles interagem é a base do acesso aos sistemas biológicos (1, 2).

Os metabólitos são reguladores chave da homeostase do sistema e fornecem uma leitura funcional da bioquímica cellular (3). Metabolômica é o estudo sistemático completo da série de intermediários de baixo peso molecular, não protéicos, sintetizados endogenamente (o metaboloma) e contidos em um sistema biológico, e que representam o produto final da expressão gênica. Dentre eles estão os aminoácidos, ácidos nucléicos, açúcares e lipídeos. Assim, a partir da metabolômica surgiram subáreas dentre elas a glicômica e a lipidômica (1,2,3).

A Figura 1 mostra como a metabolômica representa o ponto final da cascata “ÔMICA” e o ponto mais próximo ao fenótipo. A metabolômica se desenvolve como uma ferramenta funcional da genômica e quando combinada com genômica, proteômica, transcriptômica pode fornecer uma poderosa série de ferramentas para examinar mudanças fenotípicas (2).

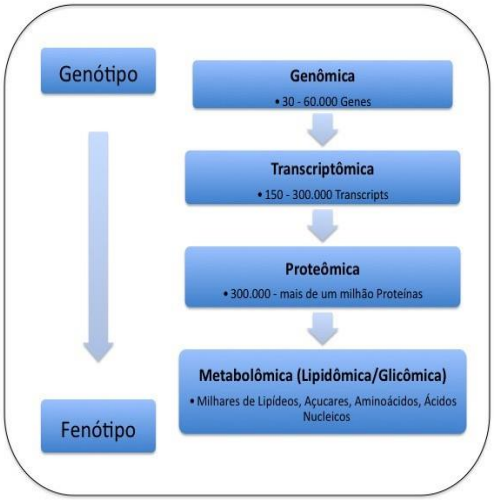

Figura 1: Diferentes níveis das "ÔMICAS" demonstrados como uma cascata que relaciona o genótipo com o fenótipo (2).

O interesse em metabolômica está se expandindo rapidamente em vários campos de pesquisa, tais como pesquisa de câncer, descoberta e desenvolvimento de fármacos, investigação toxicológica, biomédica e clínica e pesquisa nutricional (3). A maioria dos testes de química clínica que dependem de tecnologias antigas não são nem sensíveis nem específicos para doenças. Uma tarefa fundamental na medicina diagnóstica é detectar a doença o mais cedo possível. Para alcançar este objetivo, o uso da metabolômica aliada a espectrometria de massas (MS) oferece novas oportunidades para a descoberta de biomarcadores em doenças complexas e pode proporcionar uma compreensão fisiológica de doenças para além das tecnologias tradicionais (4, 5).

Análise de metabólitos

Existem duas abordagens analíticas utilizadas para a análise dos metabólitos: com e sem alvo previamente estipulado. A abordagem mais tradicional da metabolômica com alvo (*Target Metabolomics*) os ensaios são projetos para a quantificação rotineira de menos de 200 metabólitos predefinidos. Por outro lado, na

metabolômica sem alvo (*Untarget Metabolomics)* se faz-se um levantamento exploratório e qualitativo do conjunto de metabólitos presentes na amostra dependendo da instrumentação e do método de extração escolhidos. Essa abordagem sem alvo exploratória, também chamadas de fingerprints pois é considerada a impressão digital da amostra, pode complementar os métodos tradicionais através da busca de novos alvos que podem ser úteis para o desenvolvimento de métodos quantitativos mais focalizados em biomarcadores específicos para a doença estudada (3, 4, 6, 7).

De acordo com o tipo de abordagem analítica escolhida deve-se proceder com a escolha do pré-tratamento da amostra que é um passo-chave nos estudos em metabolômica, uma vez que tem uma grande influência na cobertura global e na qualidade dos perfis metabólicos obtidos e na interpretação biológica dos dados. Além disso é também necessário para libertar os metabolitos da matriz da amostra e transportar os analitos para um meio compatível com o instrumento analítico. O principal desafio do pré-tratamento de amostras é a grande diversidade de estruturas químicas e propriedades físico-químicas e, ao mesmo tempo, a falta de técnicas analíticas abrangentes universais com uma gama dinâmica suficiente e cobertura físico-química. Portanto, o preparo da amostra pode ser considerado o passo mais demorado e é talvez a principal fonte de inconsistências entre laboratórios (3).

A técnica analítica tradicionalmente aplicada aos estudos em metabolômica é a MS utilizada em combinação com cromatografia líquida de alta eficiência (LC-MS) ou cromatografia gasosa (CG-MS) para a separação dos compostos ates da detecção principalmente devido à baixa resolução de alguns analisadores de massas e ao efeito causada pela matrix biológicada amostra (8).

Porém esta estratégia de análise exige um controle cuidadoso sobre o processo cromatográfico para assegurar a reprodutibilidade e requerem tempo, esforço e conhecimentos significativos para o pré-processamento dos dados, a fim de descontaminar, alinhar e anotar os picos correctamente. Infelizmente, qualquer matriz

de coluna de cromatografia sofrerá deteriação gradual com a utilização repetitiva, resultando em alterações significativas nas características dos dados após um período de operação constante em experiências de perfilamento maiores, ou seja, em torno de 200 amostras por dia (8, 9).

Uma abordagem alternativa para capturar informações relacionadas com o conteúdo total de metabólitos é omitir completamente etapa de separação cromatográfica, introduzindo a amostra diretamente em espectrômetro de massas por infusão direta (8, 9).

A ausência de um passo de cromatografia antes da ionização da amostra tem uma grande influência tanto na concepção experimental como nos resultados. A separação prévia realizada pela cromatografia simplifica grandemente as abordagens de pré-processamento de dados quando comparada com a complexidade da conversão de dados brutos de análises realizadas a partir de infusão direta por MS (9).

Espectrometria de Massas

O princípio fundamental da MS é a representação de cada metabólito em qualquer matriz biológica por medição do espectro de sinais que refletem a relação massa/carga (*m/z*) de seus produtos de ionização (5,4,9).

Dentre várias fontes de ionização utilizadas em MS a ionização por spay de elétrons (*Electronspray ionization - ESI*) é a mais utilizada para a realização de *fingerprints*, ou seja, análise sem alvo. Durante o processo de pulverização, a ionização ocorre pela perda ou ganho de um prótons, ou outros adutos, e as moléculas de analito carregadas podem transportar cargas únicas ou múltiplas. Embora idealmente adequado para produtos químicos mais polares, mesmo as moléculas que não têm grupos ácidos ou básicos podem ser carregadas através da formação de adutos com vários íos tais como Cl- em modo negativo ou normalmente K+ ou Na+ no modo positivo. A formação de tais adutos é altamente dependente do

teor de sal da matriz de amostra bruta. A metodologia de *fingerprinting* realizada ESI-MS pode ser adaptada para analisar tanto extratos polares quanto lipofílicos nos modos de ionização positivo e negativo para proporcionar uma cobertura abrangente de uma ampla variedade de íons presentes na amostra (3,4,9).

A configuração dos analisadores utilizados para as análises de f*ingerprinting* realizada ESI-MS pode contra com quadrupolos simples ou híbrido, tempo de vôo (TOF), Ion trap e Orbitrap, que oferecem diferentes capacidades em termos de resolução, precisão de massa e robustez. E por isso a escolha do instrumento tem grande impacto no desenho experimental (9, 10).

Os analisadores de alta resolução, dentre eles o Orbitrap, possuem erro de massa de sub-ppm permitindo a predição eficiente das estruturas dos íons. A coleta de dados com alta resolução de massa efetivamente melhora o número de metabólitos a serem detectados quando comparados com instrumentos de baixa resolução e a precisão da massa é tal que os íons pseudo-parentais, isótopos e adutos de sal podem ser reconhecidos por diferenças de massa com muito mais confiança, embora seja necessário ter cuidado com a interpretação de sinais de artefatos (9, 10).

Análise Estatística

Devido à complexidade e ao volume dos dados gerados pelas análises por MS, os métodos de reconhecimento de padrões tornaram-se predominantes nas ciências médicas e podem ser adequados para certas aplicações médicas em diagnóstico. Ao aplicar avançadas ferramentas analíticas e estatísticas, os biomarcadores que estão associados com o estado de doenças são selecionados do metaboloma. Métodos estatísticos multivariados e programas de reconhecimento de padrões foram desenvolvidos para lidar com os dados adquiridos e para procurar as características discriminantes entre os dados adquiridos a partir de dois conjuntos de amostras, saudáveis e doentes (5,11).

A análise discriminante por mínimos quadrados parciais (PLS-DA) é um dos procedimentos de classificação mais conhecidos em quimiometria e vem sendo amplamente utilizado em metabolômica. A finalidade da análise PLS-DA é calcular modelos que diferenciam entre grupos e entre sujeitos modelo e controle. Na modelagem PLS-DA, ocorre a ligação entre duas matrizes de dados: X, referente aos dados brutos extraídos da análise por MS e Y que são os grupos de amostras visando a maximizar a covariância entre as variáveis independentes X e a correspondente variável dependente Y de dados altamente multidimendionais, encontrando um subespaço linear das variáveis explicativas. Este novo subespaço permite a predição da variável Y com base em um número reduzido de fatores (componentes PLS, ou o que também são conhecidos como variáveis latentes). Esses fatores descrevem o comportamento das variáveis dependentes Y e abrangem o subespaço no qual as variáveis independentes X são projetadas (11,12).

Uma forma especializada de PLS-DA, chamada de análise discriminante dos mínimos quadrados parciais ortogonais (OPLS-DA) correspondem a projeções ortogonais para estruturas latentes, nas quais a variância sistemática não-correlacionada é removida do modelo (5,11).

A principal vantagem desta abordagem PLS-DA é a disponibilidade e manipulação de dados altamente colineares e ruidosos, que são resultados comuns aos experimentos por MS. Além disso, isso fornece várias estatísticas que podem ser utilizadas para identificar as variáveis mais importantes, dentre elas a importância variável na projeção (VIP). Esta técnica fornece uma interpretação visual de conjuntos de dados complexos através de um gráfico de contagens de baixa dimensão, facilmente interpretáveis, que ilustra a separação entre grupos diferentes. A comparação das massa/carga e da pontuação da plotagem apóia as investigações em termos da relação entre as variáveis importantes que podem ser específicas para o grupo de interesse (11,12).

Colestase Neonatal

Colestase (Chol) neonatal é a interrupção ou redução do fluxo biliar, que ocorre dentro dos primeiros 90 dias de vida (13,14,15). A incidência sugerida em diferentes populações é entre 1 e 1.500 a cada 9000 bebês nascidos, além disso, sua manifestação está associada a diversas patologias intra ou extra hepáticas (16,17,18).

Os casos intra-hepáticos correspondem a aproximadamente 70% de todas as causas de Chol neonatal, e as principais condições envolvidas são doenças infecciosas, metabólicas e endócrinas congênitas, hipoplasia intra-hepática dos dutos de bile, medicamentos, infecções e doenças genéticas e origem idiopática. Os casos extra-hepáticos representam os outros 30% de todas as causas de Chol neonatal, e a principal doença envolvida é atresia biliar (BA), uma doença grave que conduz a cirrose e a morte prematura, se a interferência cirúrgica não for executada tão rapidamente quanto possível (17,18,19,20,21).

Os principais sinais clínicos são icterícia e fezes claras além de duas semanas de idade. No entanto, outros sintomas como urina escura, prurido, esteatorréia e hemorragias profusas inexplicáveis também podem ser manifestações colestáticas. Não obstante estes sinais serem inespecíficos, a Sociedade Norte- Americana de Gastroenterologia Pediátrica, Hepatologia e Nutrição recomenda a medição dos níveis séricos de bilirrubina total e direta para confirmar a Chol. Uma vez confirmada, outros exames podem auxiliar no diagnóstico etiológico, dentre eles a ultrassonografia de fígado e vias biliares, biópsia hepática percutânea, cintilografia hepatobiliar, e aspirado duodenal (13,15,18,22).

Neste contexto, os métodos para o reconhecimento precoce da doença têm sido desenvolvidos, tais como o cartão de fezes. Este método é um ensaio colorimétrico diretamente relacionado com a quantidade de bilirrubina eliminada nas fezes O mesmo foi introduzido no rastreio de recém-nascidos em Taiwan e é referido

como sendo favorável em 5 anos de resultados no diagnóstico da Chol causada pela BA, com 73,6% de sensibilidade, dentro dos primeiros 60 dias de vida. No entanto, apesar de o cartão de fezes apresentarem níveis aceitáveis de sensibilidade e especificidade, é usado apenas após o início dos sintomas de Chol. Além disso, os resultados de sensibilidade, especificidade e mortalidade são relacionados apenas com Chol causada pela BA, e não para Chol relacionada a outras causas etiológicas (23,24,25).

Métodos baseados em cromatografia e espectrometria de massas (MS) foram desenvolvidos para ajudar no diagnóstico da Chol, e perfilamento metabólico foi avaliado para eleger biomarcadores potenciais da doença. As amostras de manchas de sangue seco em papel filtro recolhidos no primeiro ano de vida, têm sido o foco de estudos nos últimos 25 anos (26,27,28).

Mills, et al., em 1998, publicou o primeiro estudo para medir os ácidos biliares (BAc) em amostras de sangue seco coletadas de crianças com menos de um ano de idade utilizando ESI- MS e os padrões de BAc marcados com deutério. Dados preliminares mostraramgrande potencial do método para medir BAc em casos de Chol causados pela BA, galactosemia, hepatite citomegalovírus, síndromes hipoplasia do ducto biliar, colangite esclerosante neonatal, tyrosemia, hemangiomata hepática, hepatite de células gigantes idiopática e distúrbios peroxissomais. No entanto, o estudo não apresentava valores de especificidade e sensibilidade, e não é possível afirmar se o método é eficaz para detectar esta doença obtida de crianças de 7-10 dias após o nascimento (29).

Mushtaq, et al., em 1999, com o mesmo objetivo, usaram um método semelhante com base em MS e padrões de deutério em manchas de sangue seco de amostras de triagem neonatal. Depois de avaliar a concentração de BAc em hepatite neonatal idiopática, síndrome Allagile, BA, distúrbios metabólicos, hepatite por citomegalovírus, alfa 1 - antitripsina e outras causas de Chol, o estudo obteve, respectivamente, 96% e 62% de especificidade e sensibilidade para todas as doenças

colestáticas hepatobiliares (30).

Recentemente, Janzen et al., em 2010, desenvolveu um método baseado em cromatografia líquida acoplada a MS (LC-MS) para detectar e quantificar BAc e seus precursores em manchas de sangue seco de triagem neonatal em casos de BA, galactosemia e defeitos peroxisomais. Para tal, apresentaram um método útil para o diagnóstico e monitoramento destas doenças, no entanto, o estudo não mostra dados de sensibilidade e especificidade (31).

Zhou, et al., em 2012, desenvolveu um método semelhante com base na LC-MS para quantificar BAc em manchas de sangue seco de triagem neonatal para BA, outras causas de icterícia neonatal e indivíduos saudáveis. A sensibilidade e especificidade alcançadas apenas para diagnóstico BA foram, respectivamente, 79,1% e 62,5%, o que não é tão bom como o teste que avalia bilirrubina conjugada e os cartões colorimétricos para fezes, embora possam permitir o diagnóstico precoce apenas para BA antes do aparecimento dos sintomas (32).

Portanto, o método de triagem neonatal ideal para Chol deve ser realizado o mais cedo possível, incluindo todas as causas etiológicas, usando um fluxo de trabalho simples, direto e com sensibilidade e especificidade de quase 100%.

Fibrose Cística

A fibrose cística (FC) é uma doença autossômica recessiva causada por mutações no gene CFTR (regulador da condutância transmembrana da fibrose cística), que codifica uma proteína expressa na membrana das células epiteliais e atua como um canal de cloreto/bicarbonato. O comprometimento da função do canal CFTR leva a anormalidades no transporte iônico, sinalização inflamatória, proliferação

celular, metabolismo de macromoléculas e eliminação bacteriana pelo sistema imunológico (33,34,39). A doença afeta todos os grupos raciais e étnicos, mas os caucasianos são os mais afetados, uma vez que 1 em 2000-3000 recém- nascidos europeus apresenta FC (35).

Logo que a doença foi caracterizada, em 1953 (36), a FC foi considerada quase inevitavelmente letal, principalmente na primeira década de vida. No entanto, as melhorias nos métodos de diagnóstico e gestão clínica levaram a um aumento da expectativa de vida para pacientes com FC ao longo dos anos. Dados estatísticos recentes indicam que a coorte de nascimento do ano 2000 pode apresentar uma mediana de sobrevivência de 50 anos (37). Hoje em dia, a maioria dos pacientes com FC lidam bem com a doença, embora sua qualidade de vida ainda seja afetada por limitações no funcionamento fisiológico, principalmente infecção pulmonar e insuficiência pancreática (38).

Mais de 2000 mutações foram descritas e divididas em seis categorias, dependendo de seus efeitos na produção e atividade da proteína. Essas categorias também podem estar correlacionadas com o prognóstico do paciente. Embora haja múltiplas mutações no gene CFTR associado a FC, a mutação mais comum corresponde a uma deleção de uma fenilalanina na posição 508, denominada mutação F508del e é o mais frequente clássico alelo mutante FC, representando cerca de 70% dos cromossomos em todo o mundo (34,39,40).

A variabilidade fenotípica da FC pode ser atribuída não apenas ao tipo de mutação CFTR, mas também a outros genes modificadores e fatores ambientais, como estilo de vida e acesso aos tratamentos (34,40).

As manifestações clínicas mais comuns são desidratação e acidificação das secreções glandulares e outras, insuficiência pancreática, má absorção de macro e micronutrientes, motilidade intestinal anormal e viscosidade e reologia anormal do muco do trato respiratório, levando a uma depuração mucociliar anormal. Este último leva o paciente a ser mais propenso a infecção bacteriana, o que provoca inflamação

aumentada e função pulmonar prejudicada. A detecção precoce e o início de terapias, portanto, resultam em melhor prognóstico e melhora da qualidade de vida do paciente (33,34,41). Estas diferenças no locus da mutação do gene CFTR geram grande heterogeneidade das manifestações clínicas, tornando difícil o diagnóstico clínico e até o diagnóstico laboratorial (42).

Em muitos países, a triagem neonatal que mede os níveis de tripsinogênio imuno-reativos de amostras de manchas de sangue seco é usada para prever a necessidade de realizar outros testes diagnósticos para confirmação de FC, devido à sua baixa especificidade para a doença. Assim, um segundo teste é obrigatório para melhorar a precisão diagnóstica (33,41,43).

Atualmente, a metodologia padrão-ouro para o diagnóstico de FC é o teste de suor, onde a concentração de íons cloreto é determinada pelo teste de iontoforese quantitativa de pilocarpina (QIPT) (44).

Para a coleta de suor, a transpiração é induzida através da administração local de nitrato de pilocarpina, seguida de uma aplicação de corrente elétrica operada por bateria, que estimula a taxa de sudorese rápida e elevada. Em seguida, o fluido de suor é coletado da pele do antebraço e os níveis de cloreto são avaliados. Neste teste, as concentrações de cloreto <30 mmol/L são consideradas normais, enquanto a gama entre 30 e 59 mmol/L são consideradas limítrofes e as concentrações ≥60 mmol/L são positivas para FC. Para um resultado conclusivo, o QPIT requer pelo menos dois testes e é considerado positivo quando as concentrações de cloreto estão acima de 60 mmol/L (43,44,45,46).

Avaliando o QPIT como o método de escolha para o diagnóstico de FC, enfrenta-se uma janela de diagnóstico impreciso para concentrações de cloreto entre 40 e 60 mmol/L, classificados como casos indeterminados, que requerem mais avaliação (46). Considerando a quantificação de cloreto < 40 mmol/L como normal e ≥ 40 mmol/L como risco para FC, a sensibilidade e a especificidade de QPIT são 100% e 92,8%, respectivamente. Entretanto, considerando a janela limítrofe, o método de

diagnóstico padrão atual não diferencia pacientes positivos e negativos para FC, apesar da alta sensibilidade e sensibilidade expostas acima. Levando-se em conta as muitas questões críticas associadas a este método, o resultado do teste de suor pode ser um desafio e erros técnicos e interpretações erradas são comuns e podem levar a resultados falsos (47,48).

Uma abordagem mais avançada, testes genéticos, por outro lado, emergiu recentemente como uma ferramenta poderosa para o diagnóstico de FC (40,45). Porém estes testes são aplicados para reconhecer painéis de mutação de CFTR limitados por um número de alterações de genes predefinidos para testes laboratoriais comerciais (por exemplo, 97 mutações) (49). Embora seja possível analisar a sequência completa do gene CFTR, esta análise genética não detecta mutações que ocorrem fora das regiões codificadoras, é um processo caro e demorado permanece, contudo, uma abordagem que se restringe apenas aos laboratórios clínicos especializados em genética e molecular. Além disso, a análise de genes do CFTR mostra limitações inerentes à tecnologia de genotipagem, variância de acordo com genes monitorados em diferentes etnias e interpretações errôneas de acordo com o contexto clínico em que o teste é desenvolvido (50).

Atualmente, a abordagem metabolômica tem sido uma ferramenta promissora para o monitoramento de vias bioquímicas através da análise de metabólitos em sistemas biológicos (51).

Comparando com genes ou proteínas, a alteração da concentração de metabólitos é consideravelmente mais perceptível e representa os fenótipos biológicos numa perspectiva integrada (52). A avaliação metabolômica de amostras biológicas permite que os pesquisadores identifiquem biomarcadores químicos de diferentes condições, como as de saúde e de doença, por exemplo. Esta abordagem facilita a caracterização de biomarcadores específicos ou conjuntos de biomarcadores que poderiam ser úteis para diferenciar perfis de saúde e de doença e que poderiam levar a uma ferramenta diagnóstica mais precisa (53).

A EM vendo sendo amplamente utilizadas para o estudo e avaliação do metaboloma e m relação a FC, os estudos com pacientes baseados em EM utilizaram uma grande variedade de fluidos biológicos, tais como urina, sangue, ar de condensação do ar expirado, escarro e lavado broncoalvear (51) Neste tese escolhemos como material de amostra a saliva e o imptinting de pele que são de coleta fácil e não invasiva e requer apenas treinamento profissional básico em serviço de saúde. Essa abordagem de coleta de amostra juntamente com a análise por EM podem auxiliar na determinação de biomarcadores de forma robusta que podem ser empregados para o desenvolvimento de novos métodos diagnóstico para FC

CAPÍTULO 1 - COLESTASE NEONATAL

The discovery of new biomarkers for the diagnosis of cholestasis in newborn screening using mass spectrometry

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ABSTRACT

Clinical identification of cholestasis in newborns is a multifactor condition that may be indicative of several underlying diseases. Manifestations may be from intra- or extra-hepatic disorders, many of which lead to severe conditions and even death. The early diagnosis of cholestasis remains an unmet medical need; moreover, differentiating the etiology of cholestasis would improve patients' quality of life by allowing earlier and hence more effective interventions for treatment. This contribution has employed a simple mass spectrometry-based metabolomics strategy to elect potential biomarkers that may provide molecular evidence of cholestasis, compared to a control group, using dried blood spot samples from patients enrolled in the newborn screening program. We obtained 7 statistically-validated biomarkers for cholestasis, which presented 95% of specificity and 85% of sensitivity, and are now being presented as proposals for inclusion in routine analyses for newborn screening tests.

Keywords: Neonatal Cholestasis, Biomarkers, Mass Spectrometry

INTRODUCTION

Neonatal cholestasis (Chol) is the reduction or interruption of bile flow, which occurs within the first 90 days of life [1-3]. The suggested incidence in different populations is between 1 in 1,500 – 9,000 term infants and its manifestation is associated with several intra or extra hepatic pathologies [4-6]. Intra-hepatic cases correspond to approximately 70% of all causes of neonatal Chol, and the main involved conditions are congenital infection, metabolic and endocrinological diseases, intrahepatic bile ducts hypoplasia, drugs, infections, and genetic-chromosomal and idiopathic origin. The extra-hepatic cases represent the other 30% of all causes of neonatal Chol, and the main disease involved is Biliary Atresia (BA), a serious condition that leads to biliary cirrhosis and early death if the surgical interference is not performed as soon as possible [5-9].

Etiological diagnosis is a major challenge for the pediatrician who needs to determine quickly the differentiation of hepatocellular from obstructive Chol, in order to define certain situations where early treatment or surgical management reduces patient morbidity and mortality of patients [3,5,6,10]. The main clinical signs are jaundice and pale stools beyond 2 weeks of age, however other symptoms such as dark urine, pruritus, steatorrhea and unexplained profuse bleedings may be cholestatic manifestations as well [1,3-6]. Nonetheless these signals are unspecific and the recommendation of North American Society for Pediatric Gastroenterology, Hepatology and Nutrition is the measurement of serum levels of total and direct bilirubin to confirm Chol [3]. Once confirmed, other tests may help in the etiological diagnosis among them ultrasonography of liver and bile ducts, percutaneous liver biopsy, hepatobiliar radionuclide scan, and duodenal aspirate [3,5].

Therefore early recognition of the etiology of neonatal Chol is essential to establish the diagnosis and treatment effectively [1,3-6]. In this context, methods for early and fast recognition of the disease have been developed, such as the stool card, a

colorimetric assay directly related to the amount of bilirubin eliminated in the feces, which was introduced in newborn screening in Taiwan and is reported to have favorable 5-year results in the diagnosis of Chol caused by BA with 73.6% of sensitivity at the first 60 days of life and to decrease the mortality associated with biliary atresia [11,12]. The same positive 10-year experience was observed in Japan in which the sensitivity of the method reached 76.5% in the check-up with 1 month of life and a specificity of 99.9% [13]. However, although the stool card has acceptable levels of sensitivity and specificity, it is only used after the onset of symptoms of Chol [11,13].

Methods based on chromatography and mass spectrometry (MS) have been developed to assist the etiological diagnosis of Chol and metabolomic profiling has been assessed to elect potential biomarkers of the disease [14,15]. The dried blood spots samples collected at newborn screening program within the first 7 days of life are the focus of studies for the past 25 years [16-18]. Mills, et al. [17], in 1998 published the first study to measure bile acids (BAc) in dried blood spot samples collected 7-10 days after birth using ESI-MS and their preliminary data showed good potential of the method to measure BAc however not presented specificity and sensitivity values . Mushtaq, et al. [18] in 1999 with the same aim and using a similar method obtained respectively 96% e 62% of specificity and sensitivity for cholestatic hepatobiliar disease and 96% and 79% of corresponding values for BA. Recently, Janzen, et al. [19] in 2010 developed a method based on liquid chromatography coupled with MS to detect and quantify bile acids in dried blood spots with good precision and sufficient sensitivity for a clinical exam that is 80%. Zhou, et al. [20] in 2012 developed a similar method and the sensitivity and specificity achieved was respectively 79.1% and 62.5% which is not as good as serum-conjugated bilirubin and the stool color card.

The ideal newborn screening method for Chol should be carried out as early as possible, and with sensitivity and specificity close to 100%, which has not yet been achieved by any of the other previously studies. The aim of this study,

therefore, was to propose a new strategy based on mass spectrometry with minimal sample preparation to identify biomarkers in neonatal screening that may assist in the fast and accurate diagnosis of Chol.

METHODS

Newborn screening samples selection

Sample collection was approved by the Research Ethics Committee of the School of Medical Sciences – University of Campinas/Brazil (Protocol number: 988.199). Chol group included 8 patients who developed intra or extra hepatic Chol (IHC and EHC respectively) within the first weeks of life, while the control group included 14 patients at the same age, which did not develop Chol. Blood samples collected as dried blood spots on filter paper by the Newborn Screening Program within the first hours of life were used for this retrospective study. All experiments were performed in accordance with relevant guidelines and regulations regarding samples from human origin.

Sample preparation

The filter paper containing the dried blood samples of the newborn screening was cut in 6-mm diameter circles, which were extracted with 1 mL of Methanol: Water: Tetrahydrofuran (45:45:10). 10 µL of the resulting solution were diluted in 990 µL Methanol: Water (1:1). All the samples were filtered through 0.22-µm polyvinylidiene difluoride membranes and 1 µL of formic acid was added to each sample.

Mass spectrometry analysis

Samples were directly injected in an ESI-LTQ-XL Orbitrap Discovery instrument (Thermo Scientific, Bremen, Germany) with nominal resolution of 30,000 (FWHM) under the following conditions: flow rate of 10 µL.min^{-1}, spray voltage of 5 kV, sheath gas at 10 arbitrary units, capillary temperature of 280°C. The analyses

were performed in triplicates and all data were acquired in the positive ion mode using the mass range at 400-1200 *m/z*.

Statistical analysis and biomarker election

An Orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA) using the online software MetaboAnalyst 3.0 [21] was performed to select characteristic ions from each group; this choice was carried out considering the VIP score list (Variable Importance in Projection).

To verify the accuracy of the biomarkers candidates chosen by VIP score list, the Receiver Operating Characteristic (ROC) Curve was performed.

Lipid MAPS online database (University of California, San Diego, CA – www.lipidmaps.org), HMDB version 3.6 (Human Metabolome database - www.hmdb.ca), METLIN (Scripps Center for Metabolomics, La Jolla, CA) and KEGG Pathway Database (Kyoto Encyclopedia of Genes and Genomes – www.genome.jp/keeg/pathway.html) were consulted to elect the potential biomarkers with mass accuracy no greater than 2 ppm.

RESULTS

The age of admission of patients from the Chol group ranged from 11 days to 90 days, with a median of 51 days. The age of onset of jaundice ranged from birth to 60 days, with a median of 15 days. The final diagnoses in this group were: BA (4), cytomegalovirus infection (1), galactosemia (1), idiopathic (1) and multifactor (1). Figura 2 shows representative mass spectra from each group.

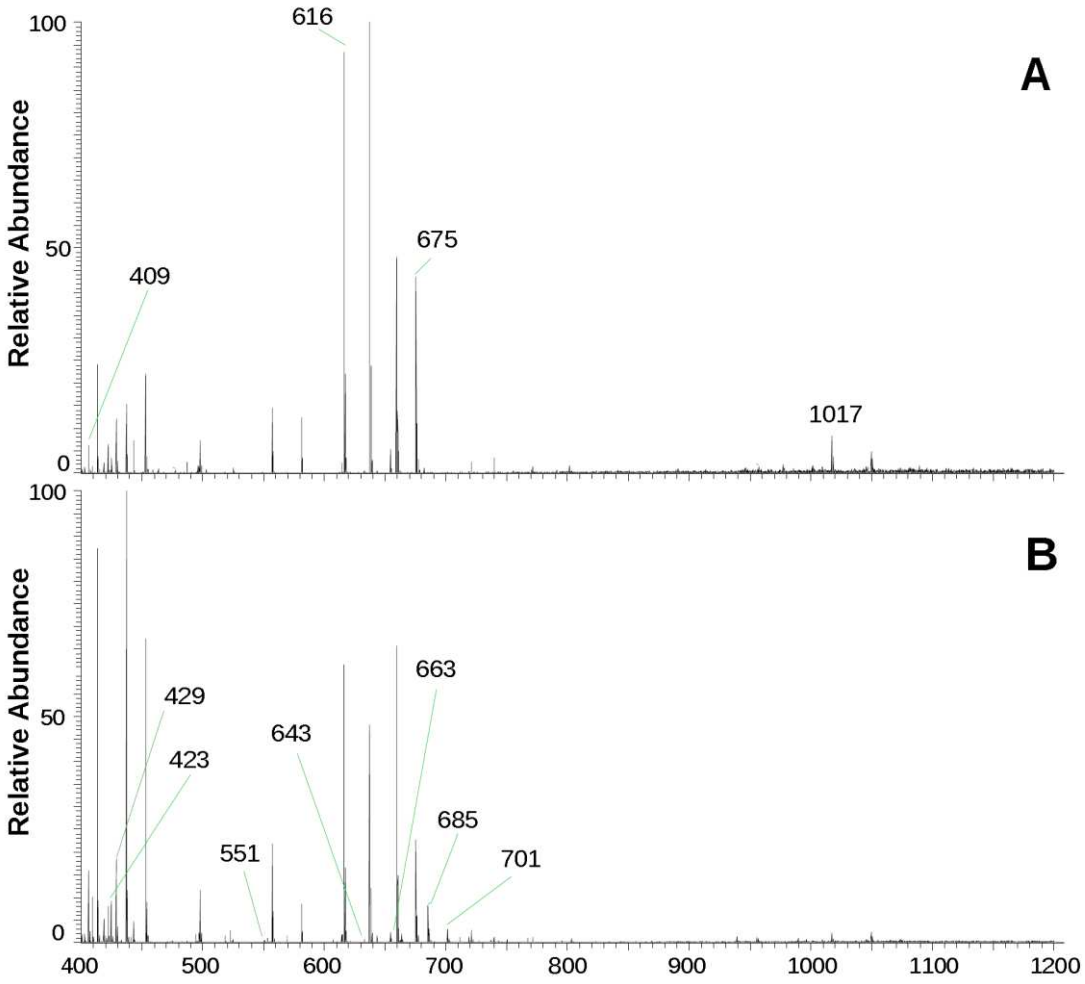

Figura 2: Representative fingerprinting spectra on positive ion mode (400-1200 *m/z*) of the groups: (A) Control and (B) Cholestasis. The mass of each group's biomarkers are highlighted, and full information on these molecules can be seen in Table 1.

Statistical analyses were performed using OPLS-DA comparing the control and Chol groups to elect specific biomarkers for Chol. OPLS-DA loadings (Figura 3) show complete separation of control and Chol groups; from these analyses, 7 biomarkers were selected for Chol and 4 biomarkers for the control group, all with VIP scores above 4.

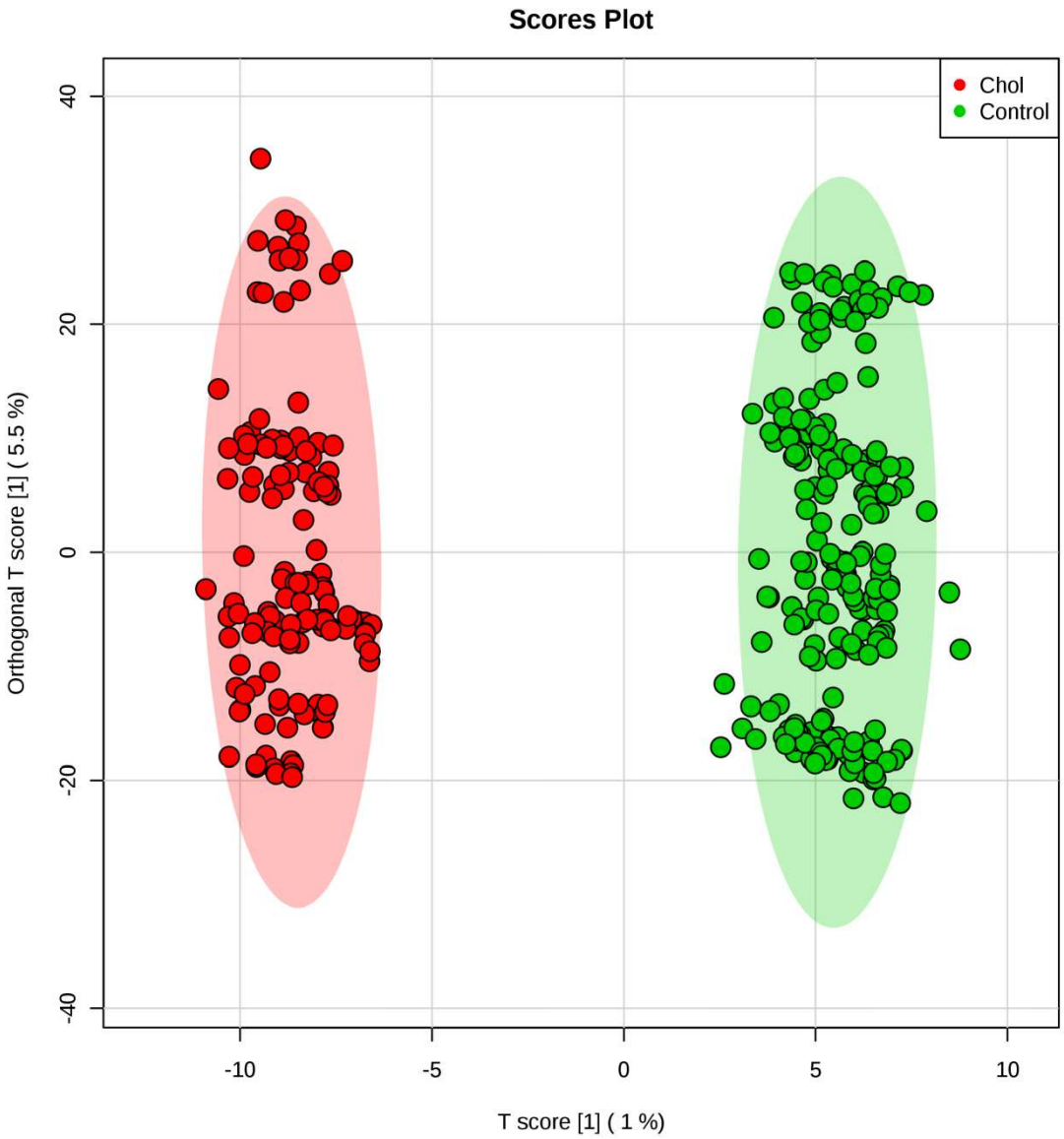

Figura 3: Orthogonal Partial Least Square Discrimination Analysis (OPLS-DA), with the comparison between Control and Cholestasis groups.

To evaluate the relevance of these biomarkers, a ROC curve was built, presenting 95% of specificity (95%CI: 85.8 – 98.7) and 85% of sensitivity (95%CI: 70.1 – 93.9) (Figure 3).

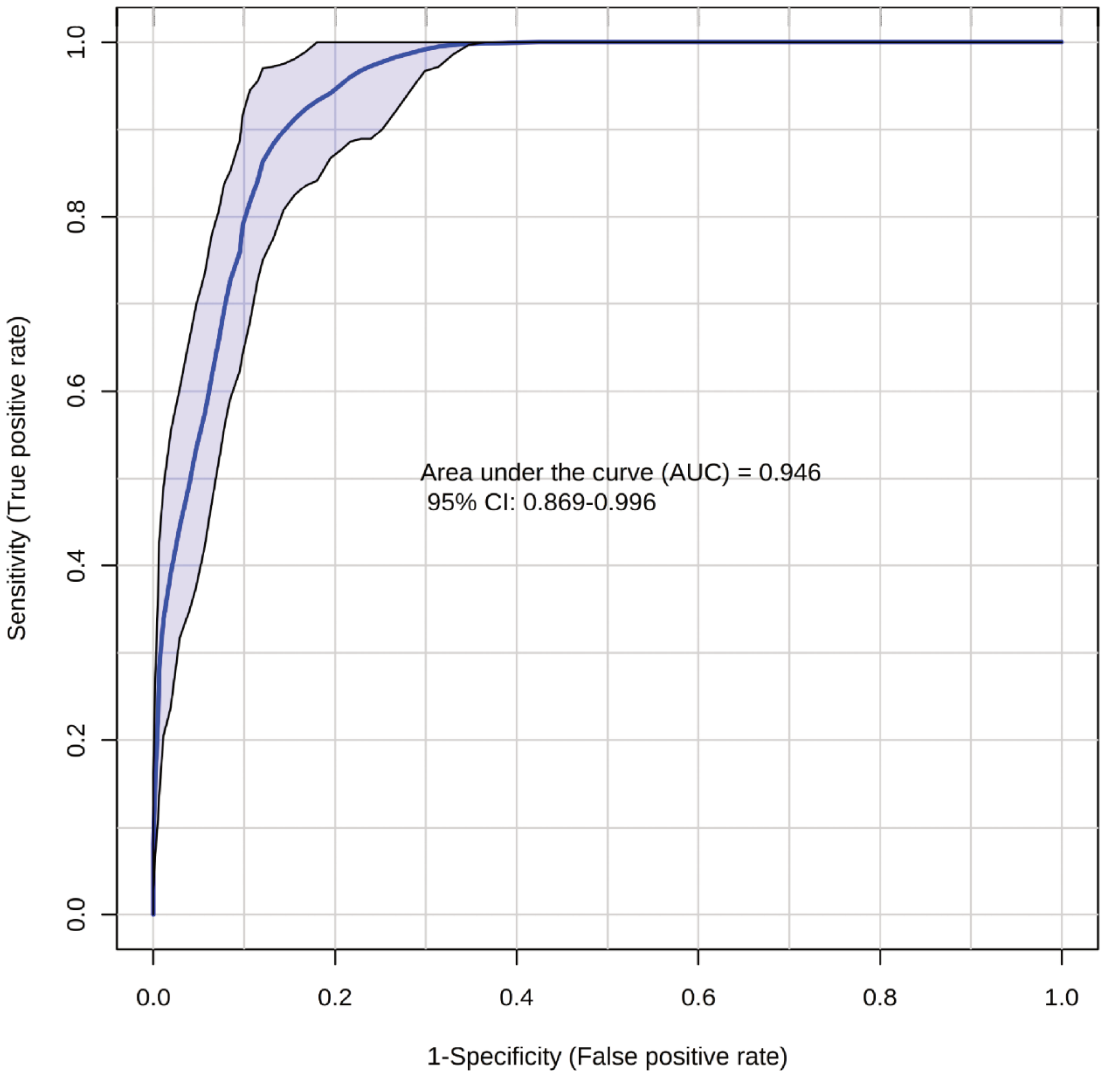

Figura 4. Receiving Operating Characteristic (ROC) between Control and Cholestasis. Calculated results led to 95% of specificity and 85% of sensitivity.

Detailed information on the biomarkers elected for control and Chol

groups can be seen in Tabela 1.

Tabela 1: Biomarkers identified via ESI-MS (positive ion mode) for Control, Cholestasis and Biliary Atresia groups. Identification is based on the comparison between the exact and theoretical masses of each compound in Lipid Maps, METLIN and HMDB.

Groups	Molecule	Adduct	ID	Experimental Mass	Theoretical Mass	Error (PPM)
Control	20-hydroxy-PGF2a	$[M+K]^+$	MID3821/HMDB04049/ LMFA03010029	409.1991	409.1987	-0.9775
	3'-Sialyllactose	$[M-H_2O+H]^+$	MID3628/HMDB00825	616.2086	616.2089	0.4868
	6-Sialyl-N-acetyllactosamine and/or 3'-N-Acetylneuraminyl-N-acetyllactosamine and/or a-N-Acetylneuraminyl-2,6-b-D-galactosyl-1,4-N-acetyl-b-D- glucosamine	$[M-H]^-$	MID58472/HMDB06584 MID58470/HMDB06581 MID5990/HMDB01081	675.2467	675.2455	-1.7771
	PI(22:0/22:0)	$[M+K]^+$	MID80937/LMGP06010915	1017.6749	1017.6768	1.8670
Cholestasis	Palmitoylcarnitine	$[M+Na]^+$	MID36667/LMFA07070004	422.3234	422.3241	1.6575
	7-Ketodeoxycholic acid and/or 3-Oxocholic acid	$[M+Na]^+$	MID84498/HMDB00391/ LMST04010184 MID57908/HMDB00502/ LMST04010443	429.2603	429.2611	1.8637
	(3a,5b,7a)-23-carboxy-7-hydroxy-24-norcholan-3-yl, b-D-Glucopyranosiduronic acid	$[M-H_2O+H]^+$	MID6678/HMDB02430	551.3230	551.3220	-1.8138

	Leukotriene C4	$M+NH_4]^+$	MID3681/HMDB01198/ LMFA03020003	643.3360	643.3371	1.7098
	PA(P-18:0/18:4(6Z,9Z,12Z,15Z)) and/or PA(P-16:0/20:4(5Z,8Z,11Z,14Z)) and/or PA(O-16:0/20:5(5Z,8Z,11Z,14Z,17Z))	$[M-H_2O+H]^+$	MID82283/LMGP10030043 MID82327/LMGP10030087 MID82222/LMGP10020076	663.4744	663.4754	1.5072
	SM(d18:1/12:0)	$[M+K]^+$	MID41585/LMSP03010002	685.4668	685.4681	1.8965
	DG(22:5(7Z,10Z,13Z,16Z,19Z)/18:4(6Z,9Z,12Z,15Z)/0:0) and/or DG(22:5(4Z,7Z,10Z,13Z,16Z)/18:4(6Z,9Z,12Z,15Z)/0:0) and/or DG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:3(9Z,12Z,15Z)/0:0) and/or DG(20:5(5Z,8Z,11Z,14Z,17Z)/20:4(8Z,11Z,14Z,17Z)/0:0) and/or DG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:3(6Z,9Z,12Z)/0:0) and/or DG(18:4(6Z,9Z,12Z,15Z)/22:5(7Z,10Z,13Z,16Z,19Z)/0:0) and/or DG(20:4(8Z,11Z,14Z,17Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0) and/or DG(18:4(6Z,9Z,12Z,15Z)/22:5(4Z,7Z,10Z,13Z,16Z)/0:0) and/or DG(18:3(6Z,9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) and/or DG(20:5(5Z,8Z,11Z,14Z,17Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	$[M+K]^+$	MID59207/HMDB07744 MID59178/HMDB07715 MID59233/HMDB07772 MID59065/HMDB07577 MID59232/HMDB07771 MID58891/HMDB07352 MID59037/HMDB07549 MID58890/HMDB07351 MID58847/HMDB07295 MID59064/HMDB07576	701.4529	701.4542	1.8533

1

Abbreviations: DAG: Diacylglycerol; PA: Phosphatidic Acid; PI: Phosphatidylinositol; SM: Sphingomyelin.

DISCUSSION

ESI-MS Method

Dried blood spot samples of neonatal screening program have been used for a long time to detect many inborn metabolic diseases. Regarding Chol, current practices are focused on the detection and quantification of BAc; our method, however, proposes a simple and fast-paced sample preparation without chromatography steps and direct injection using ESI-MS. Furthermore, the analyses were performed on positive ion mode at the mass range 400-1200 *m/z* to not only focus in BAc but for potential biomarkers for Chol that had not been explored before.

Results from the Chol group showed that regardless of the major cause that leads to Chol development, the method was efficient enough in providing accurate separation from the control group within the first few days after birth, before the onset of the symptoms. Therefore, this method may be understood as a potential early diagnostic approach for Chol, to be incorporated in neonatal screening tests, since there are no specific markers for this disease, with diagnosis being relied on symptoms and clinical observations.

Control and Cholestasis Biomarkers

Breast milk is the only source of feeding for newborns and provides nutrition, growth and development [22-25]. Human milk is mainly composed of water, macro and micronutrients. Among macronutrients, proteins are the most abundant, followed by lipids [22,23], which are secreted in globules, with a central core composed mainly of triacylglycerols (TAG) surrounded by a phospholipid bilayer with a glycosylated surface. The composition of these globules varies with stage of lactation and maternal nutrition [23,24,26]. Among phospholipids, phosphatidylinositol (PI) (1017 *m/z*) was elected as biomarker and is more abundant in the control group compared to the Chol group. This happens possibly because this particular lipid class is used as a

messenger in response to the inflammation initiated by Chol, and therefore it is being more consumed by the inflammatory process present in the Chol group. Immunosuppressant lipids are also present in human milk such as prostaglandins, which regulate the intestinal mucosa immune system response in the first days of life, avoiding intestinal mucosa damaging [25]. Prostaglandin F2 alpha (PGF2α) is reported to be produced in elevated concentrations in human milk at the first days after birth [23,25,27] which corroborates the election of the human metabolite 20-hydroxy- PGF2α (209 *m/z*) as a control biomarker.

Oligosaccharides are the third most abundant macronutrients present in human milk, and their biological role is closely related with their structure [22,25]. The biomarkers found in the control group, namely 3'-Sialyllactose (616 *m/z*) and 6-Sialyl-N-acetyllactosamine (675 *m/z*) are oligosaccharides with terminal sialic acid, and are reported to be absorbed by the human gut and to remain in blood at high concentrations. The structure of these carbohydrates present anti-inflammatory properties, since the terminal sialic acid reduces the glycan-mediated adhesion of leukocytes to endothelial cells [22]. Therefore, when the ability of the liver to transport solutes into the canaliculus is compromised for various mechanisms and there is decrease in bile formation, the development of Chol occurs, as well as a decrease in milk nutrients absorption, with accumulation of many molecules in the liver and in the systemic circulation [10,28].

Hepatocelullar bile formation is regulated for multiple pathways and mediators, such as protein kinase C (PKC). The activation of different isoforms of PKCs is involved in bile acid-induced Chol and apoptosis and requires binding of diacylglycerols (DAG) and/or Ca^{2+} and interaction with acidic membrane lipids [28,29] which explain the election of DAG for Chol group. This biomarker is also supported by the alterations in TAG liver metabolism, since the elevation of BAc also activates many lipases, responsible for the decrease of TAG levels and production of DAG

(701 *m/z*) and phosphatidic acid (PA) (663 *m/z*), also elected as Chol biomarkers [30,31].

The decrease of BAc in intestine prevents the absorption of cholesterol, TAG and DAG, whereas the absorption of fatty acids (FA) is maintained at a certain extent, providing a source of energy to the body, although limited [23,30]. Carnitine helps long-chain FA enter into the mitochondria for β-Oxidation, and Palmitoylcarnitine (422 *m/z*), elected as biomarker, is reported to be elevated in children with Chol [14,32].

Due to the impairment of bile secretion and flow for biliary ducts and BAc, phospholipids, cholesterol and bilirubin are now accumulated in liver and blood [5,14,19,31,32], explaining the election of the BAc 3-Oxocholic acid and/or 7-Ketodeoxycholic acid (429 *m/z*) as Chol biomarkers. However, at the initial stages of the disease, the accumulation of BAc is slower than bilirubin, which is reported to decrease lipid peroxidation in liver, the production of Tumor Necrosis Factor - Alpha (TNFα) and sphingomyelinase (SMase) activity. SMase is responsible for the hydrolysis of sphingomyelin to ceramide and sphingosine, mediators of apoptosis. In animal models, within the firsts 9 days of Chol, the activity of SMase is reduced by the accumulation of bilirubin; however, after this period, bilirubin is constantly produced by the organism and the increase in BAc concentration intensifies lipid peroxidation and the production of TNFα [33]. These findings corroborate the election of Sphingomyelin (685 *m/z*) as Chol biomarkers, suggesting the development of Chol in the first hours after birth, and indicate that the early diagnose of the disease, even before the arising of the symptoms, is possible.

The accumulation of BAc in high concentrations causes cytotoxicity, as their detergent properties and accumulation in liver cells lead to oxidative stress, apoptosis and subsequent damage to the liver parenchyma [10,34]. Glucoronidation of BAc is the major detoxification pathway of the organism and, after conjugation with

a highly hydrophilic glucoronide group, the molecules are generally more easily excreted in urine [31,34]. This mechanism justifies the election of (3a,5b,7a)-23-carboxy- 7-hydroxy-24-norcholan-3-yl, b-D-Glucopyranosiduronic acid (551 *m/z*), product of glucoronidation of chenodeoxycholic acid, as a Chol biomarker.

Cysteinyl-leukotrienes, such as the biomarker Leukotriene C4 (643 *m/z*), are mediators of liver injury in many diseases, including Chol. Under normal conditions, this class of leukotrienes is predominantly eliminated via bile; however, the accumulation in liver leads to an increase in vascular permeability that prompts hepatic edema resulting in an increasing of bile flow and thus contributing to the onset of Chol [35].

Therefore, our findings indicate that the method developed presents interesting results of specificity and sensibility for early cholestasis diagnosis, in addition to presenting a potential diagnostic method to be incorporated in newborn screening program. The next steps include improving this method to encompass an etiological diagnosis for biliary atresia and other causes, thus enhancing and increasing its specificity even further.

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CAPÍTULO 2 - FIBROSE CÍSTICA

Biomarkers in saliva for cystic fibrosis screening

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ABSTRACT

Genetic disorders such as cystic fibrosis are conditions that lay a heavy burden over the individuals affected by it. Detection of the disease may not always be readily performed, as many variables are involved in its accurate diagnosis, directly affecting the quality of life of affected individuals. We used high-resolution mass spectrometry as the main analytical tool, associated with partial least squares discriminant analysis to establish molecular targets that will be used for potential biomarkers for the early detection of cystic fibrosis' most common genetic variant, F508del, within a biochemical context that makes sense on the pathways that are most affected by this condition. Statistical analysis using PLS-DA showed an efficient separation between CF and control groups and the election of biomarkers that represents the systemic manifestation of CF disease (section below). The ROC curve using these 8 biomarkers promoted the achievement of 68% of sensibility and 90% of specificity, better specificity values than compared to other methods using the same matrix. The developed method is the first described for the evaluation of metabolic profiling in saliva using HRMS approach. Easier sample collection associated with little sample preparation, absence

of separation steps, and direct infusion into ESI-MS, in addition to statistical robustness that validates the obtained data, leads to highly sensitive and specific method that allowed us proposing a suitable method for targeted CF screening with high potential for implementation in routine analyses.

Keywords: Cystic Fibrosis, Saliva, High-resolution mass spectrometry, Metabolomics, Biomarkers.

1. NTRODUCTION

Cystic fibrosis (CF) (OMIM: 219700) is an autosomal recessive disease caused by mutations in the CFTR [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)] gene (OMIM: 602421), which codes a protein expressed at the membrane of epithelial cells and acts as a chloride/bicarbonate channel. Impairment of CFTR channel function leads to abnormalities in ion transport, inflammatory signaling, cell proliferation, metabolism of macromolecules and bacterial elimination by the immune system[1-3].

Over 2000 mutations have been described and divided into six categories, depending on their effects in protein production and activity; these categories may also be correlated with patient prognosis. Although more common in caucasian, CF also affects other ethnic groups, and F508del is the most frequent classic CF mutant allele, accounting for approximately 70% of worldwide chromosomes[2-4].

The phenotypic variability of CF may be attributed not only to the CFTR mutation type, but also to other modifier genes and environmental factors such as lifestyle and access to treatments[2,4]. Common clinical manifestations are dehydratation and acidification of glandular and other secretions, pancreatic insufficiency, malabsorption of macro and micronutrients, abnormal intestinal motility,

and abnormal respiratory tract mucus viscosity and rheology, leading to abnormal mucociliary clearance. The latter leads the patient to be more prone to bacterial infection, which causes increased inflammation and impaired lung function [1,2]. Early detection and initiation of therapies therefore, result in better prognosis and improved quality of life for the patient [1,5].

In many countries, neonatal screening measuring immuno-reactive trypsinogen levels from dried blood spots samples is used to predict the need for performing other diagnostics tests for CF confirmation, due to its low specificity for CF; therefore, a second test is mandatory to improve diagnostic accuracy [1,5,6]. Currently, the gold standard methodology for CF diagnosis is the sweat test where the concentration of chlorine ions is determined by the quantitative pilocarpine iontophoresis test. In this test, chloride concentration ranges <30 mmol/L are considered normal, while the range between 30 and 59 mmol/L are considered borderline and concentrations ≥60 mmol/L are positive for CF. Sample collection for this method is often uncomfortable for the patient, and the procedures and analyses require strict protocols, since methodological errors are not uncommon [6,7]. A more advanced approach, genetic testing, on the other hand, recently emerged as a powerful tool for CF diagnosis; it remains, however, an approach that is restricted to specialized clinical molecular, and genetics laboratories only. In addition, CFTR gene analysis shows limitations inherent to genotyping technology, variance according monitored genes in different ethnic and misinterpretation according to the clinical context in which the test is developed [4,7].

More recently metabolomic profiling has been used fo theassessment of how metabolic pathways behave in the context of disease, and mass spectrometry (MS) is widely used for this purpose. Regarding CF, MS- based studies with patients have utilized a wide variety of biological fluids such as urine, blood, exhaled breath condensate air, sputum, and bronchoalveoar lavage [1]. Additionally, saliva has been

used as an alternative biological sample, since collection is easy - it is a noninvasive approach -, and requires only basic professional training in healthcare services. Recent studies also showed the presence of biomarkers that can be applied for the diagnosis of several diseases[8]; in the past decade, a few contributions used saliva and its applications to CF studies, determining ion concentration[9] and oxidative stress[10]. Our proposal herein is to develop a practical metabolomics strategy using saliva as the biological sample and high-resolution mass spectrometry (HRMS) as the tool to determine biomarkers that may be used to assist in a potential and accurate CF diagnosis that will provide basis for early medical monitoring in affected individuals.

2. METHODS

2.1. Patients' selection

Patients (n = 21) were selected retrospectively (i.e. patients were already diagnosed upon inclusion) during a four-month period at the CF Clinic at the Clinics Hospital of the University of Campinas. The inclusion criteria were (i) to have at least one sweat test with chloride concentration greater than or equal to 60 mEq/L, and (ii) presence of homozygosis for the mutation F508del, characterized by genetic studies. Exclusion criteria were either the presence of either oropharynx infection or fever. The control group was composed by healthy individuals at the same age and gender as the CF group, recruited from schools and universities. Sample collection was approved by the Research Ethics Committee of the School of Medical Sciences – University of Campinas/Brazil (Protocol number: 1.100.978). All experiments were performed in accordance with relevant guidelines and regulations regarding samples of human origin.

2.2. Sample collection and preparation

After rinsing the mouth with water for one minute to stimulate the salivary gland, saliva samples were collected using a sterile cotton roll, Salivet® (Sarstedt AG & Co., Germany), by chewing it for one minute in each side of mouth. The Salivet® was centrifuged at 500 *g* for 10 minutes, and 30 µL of the resulting saliva were extracted with 970 µL of Methanol: Water (1:1). All samples were filtered through 0.22-µm polyvinylidiene difluoride membranes and 1 µL ammonium hydroxide was added to each sample to facilitate ionization. A blank Salivet® sample set was also prepared by the same protocol for comparison purposes.

2.3. Mass spectrometry analysis

Samples were directly injected in an ESI-LTQ-XL Orbitrap Discovery instrument (Thermo Scientific, Bremen, Germany) with nominal resolution of 30,000 (FWHM) under the following conditions: flow rate of 10 µL.min^{-1}, sheath gas at 10 arbitrary units, spray voltage of 5 kV and capillary temperature of 280°C. Analyses were performed in triplicates and all data were acquired in the negative ion mode at the mass range of 150-700 *m/z*. All selected biomarkers for CF were elucidated via MS/MS reactions, which were performed in the instrument's linear trap, using helium as the buffer gas, with energies ranging from 30 to 50 units. The fragmentation profile was then evaluated with the assistance of the High Chem Mass Frontier software (v. 6.0, Thermo Scientific, San Jose, CA), which calculated theoretical fragmentation pathways.

2.4. Statistical analysis and biomarker election

A Partial Least Squares Discriminant Analysis (PLS-DA) using the online software MetaboAnalyst 3.0 (www.metaboanalyst.ca)[11] was performed to select

characteristic ions from all groups (control, CF and blank); the choice considered the VIP score list (Variable Importance in Projection) generated by the software. To verify the accuracy of the biomarkers candidates chosen by the VIP score list, a Receiver Operating Characteristic (ROC) Curve was performed. METLIN (Scripps Center for Metabolomics, La Jolla, CA), HMDB version 3.6 (Human Metabolome database - www.hmdb.ca), Lipid MAPS online database (University of California, San Diego, CA – www.lipidmaps.org) and KEGG Pathway Database (Kyoto Encyclopedia of Genes and Genomes – www.genome.jp/keeg/pathway.html) were consulted to elect potential biomarkers with mass accuracy no greater than 2 ppm.

3. RESULTS

In 4 months, 21 CF patients were selected for this study, with ages ranging from 5 to 19 years old (mean: 12 years; median: 13 years), with 52% males and 48% females. The routine of the CF Clinic at the Clinics Hospital of the University of Campinas works by requesting follow-up visits of each registered patient at least every three-month cycle. Hence, in the 4-month period of patient inclusion, all CF patients who receive care from the CF Clinic were screened; those who met the inclusion criteria were, therefore, included.

Sweat test presented an average concentration of chloride of 106 mEq/L (minimum: 76 mEq/L; maximum: 166 mEq/L). All patients presented deficits in lung function, 14% with hemoptysis, 5% with pulmonary hypertension and 5% with atelectasis. In 78% of the patients, two or more different microorganisms were isolated from sputum within the past 12 months: *Staphylococcus aureus* (86%), *Pseudomonas aeruginosa* (67%), *Aspergillus sp.* (24%), *Burkholderia cepacia* complex (19%), *Stenotrophomonas maltophilia* (10%) and *Haemophilus influenzae* (5%). All patients also presented pancreatic insufficiency, 67% have evidence of liver disease, 19% developed diabetes and 10% developed distal intestinal obstruction syndrome. The

control group was composed of 21 healthy patients within the same sex and age range as the CF group and collected at the same period.

HRMS analysis on negative ion mode was performed, and Figura 5 shows representative mass spectra from each group. Information extracted from spectral data served as basis for the statistical analyses using PLS-DA, which compared the control and CF groups to elect biomarkers for CF.

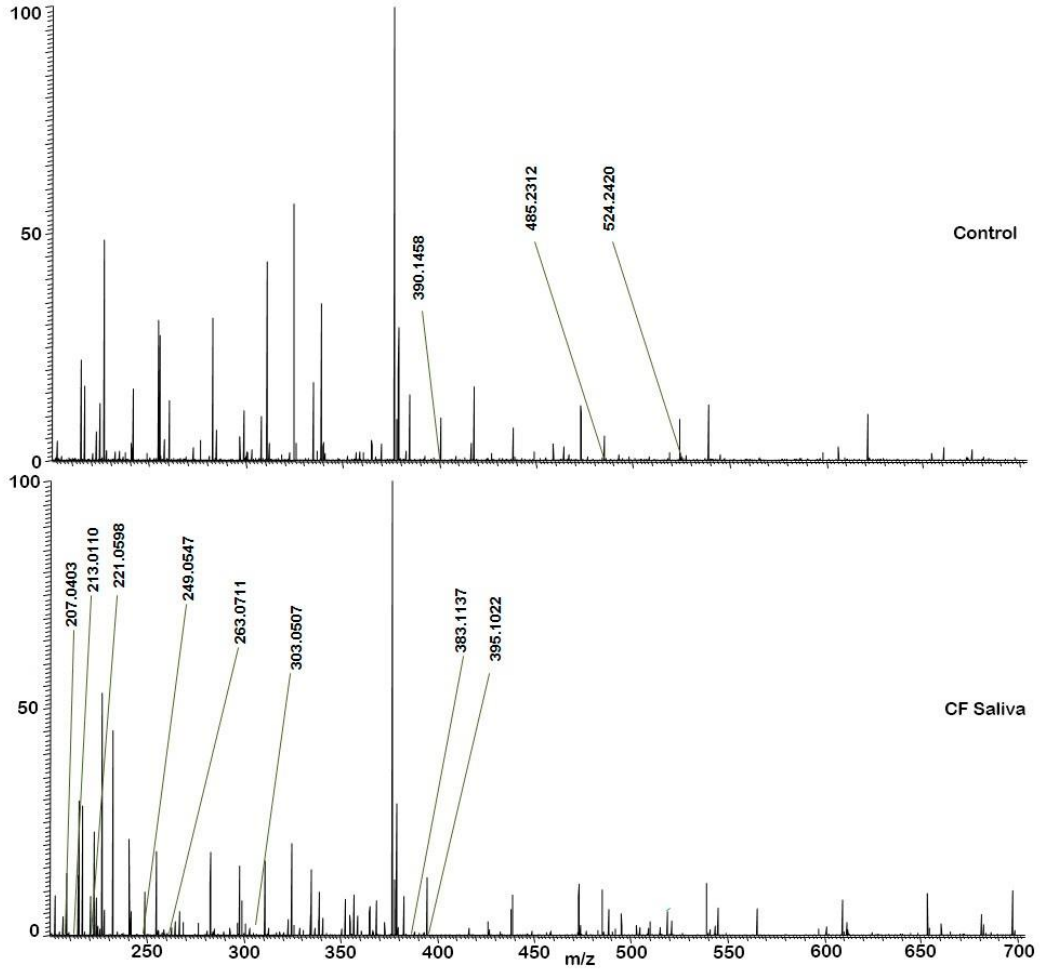

Figura 5: Representative fingerprinting spectra on negative ion mode (150-700 *m/z*) of the groups: Control and Cystic Fibrosis. The mass of each group's biomarkers are highlighted, and full information on these molecules can be seen in Tabela 2.

PLS-DA plot (Figura 6) shows complete separation of control and CF groups; from these analyses, 3 biomarkers were selected for control group and 8 biomarkers for the CF group, all with VIP scores above 4.

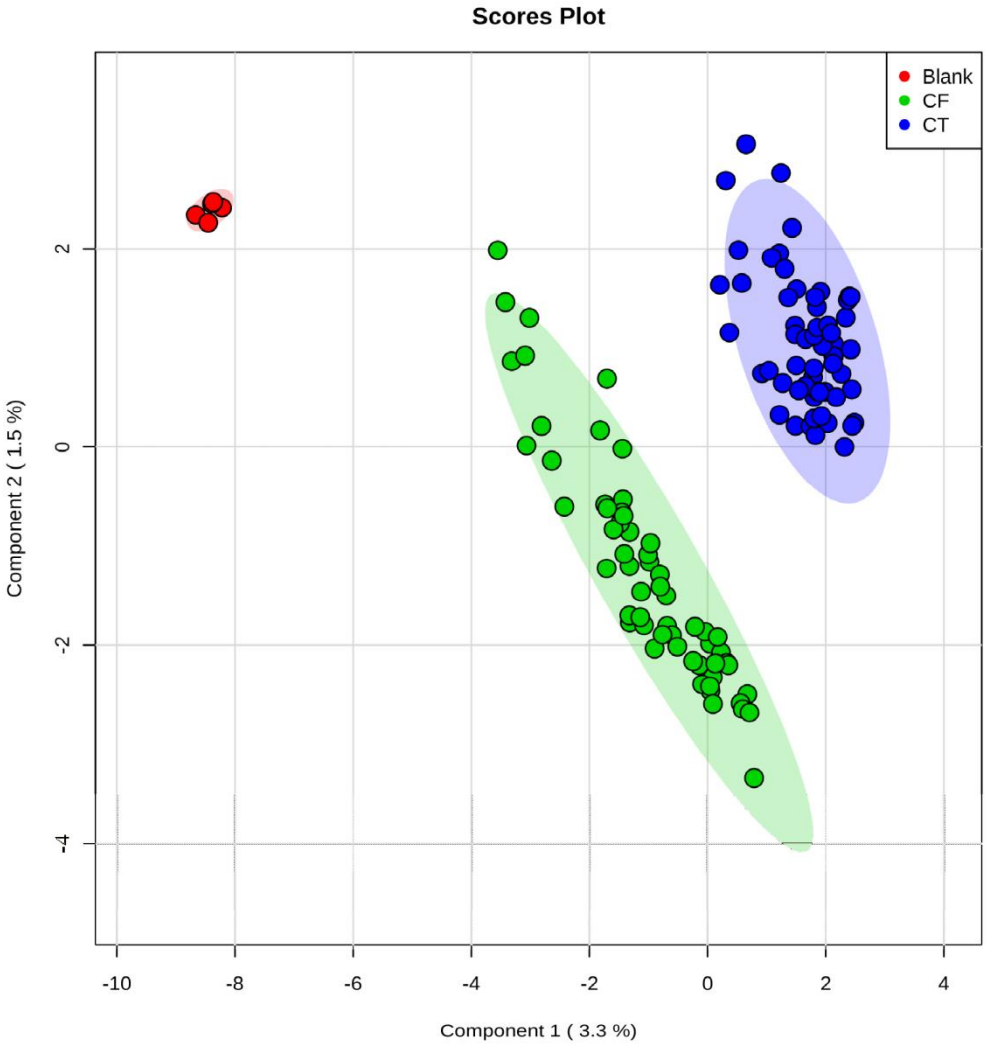

Figura 6: Partial Least Square Discrimination Analysis (PLS-DA), with the comparison between the Blank (red), Control (blue) and Cystic Fibrosis (green) groups. The 95% confidence interval of the model is displayed as the shaded colors among samples within each group.

Detailed information on the biomarkers elected for control and CF groups can be seen in Table 1, and the MS/MS fragments that helped elucidating the molecules are described in Table 2

Tabela 2: Biomarkers identified via ESI-MS (negative ion mode) for Control, Cystic Fibrosis groups. Identification is based on the comparison between the exact and theoretical masses of each compound in Lipid Maps, METLIN and HMDB.

Groups	Molecule	Conj	ID	Experimental Mass	Theoretical Mass	Error (PPM)
Control	PC(0:0/6:0) and/or PC(6:0/0:0)	$[M+Cl]^-$	MID40359/ LMGP01050093 MID40328/ LMGP01050062/ HMDB29207	390.1458	390.1454	1.0253
	PG(18:4/0:0) and/or PG(0:0/18:4)	$[M-H_2O-H]^-$	MID80009/ LMGP04050021	485.2312	485.2304	1.6487
	PS(20:5/0:0) and/or PS(0:0/20:5)	$[M-H_2O-H]^-$	MID78854/ LMGP03050027	524.2420	524.2413	1.3353
Cystic Fibrosis	Nitrotyrosine	$[M-H_2O-H]^-$	MID6383/ HMDB01904	207.0403	207.0406	1.4490
	Cysteinylglycine	$[M+Cl]^-$	MID85689/ HMDB00078	213.0110	213.0106	1.8778
	L-Cystathionine	$[M-H]^-$	MID39/ HMDB00099	221.0598	221.0602	1.8095
	Gamma-Glutamyl-Cysteine	$[M-H]^-$	MID3379/ HMDB01049	249.0547	249.0551	1.6061
	Gamma-Glutamyl-S-Methylcysteine	$[M-H]^-$	MID88096/ HMDB31985	263.0711	263.0707	1.5205
	Inosine	$[M+Cl]^-$	MID84/ HMDB00195	303.0507	303.0502	1.6499
	S-Adenosylhomocysteine	$[M-H]^-$	MID296/ HMDB00939	383.1137	383.1143	1.5661
	Dityrosine	$[M+Cl]^-$	MID58352/ HMDB06045	395.1022	395.1015	1.7717

Abbreviations: PC: Phosphatidylcholine; PG: Phosphatidylglycerol; PS: Phosphatidylserine.

Tabela 3. MS/MS fragments of the CF biomarkers elected by PLS-DA.

Mass	Molecules	MS/MS Fragments
207	Nitrotyrosine	192, 179, 163, 149
213	Cysteinylglycine	195, 169, 151
221	L-Cystathionine	206, 205, 193, 177
249	Gamma-Glutamyl-Cysteine	221, 206, 205, 192, 137
263	Gamma-Glutamyl-S-Methylcysteine	245, 243, 235, 221, 219
303	Inosine	287, 285, 259, 243, 217, 215
383	S-Adenosylhomocysteine	363, 337, 335, 321
395	Dityrosine	377, 375, 353, 351, 315

. To evaluate the relevance of these biomarkers, a ROC curve was built, presenting 90% of specificity (95%CI: 78.8 – 95.9) and 68% of sensitivity (95%CI: 54.9 – 79.4) (Figura 8).

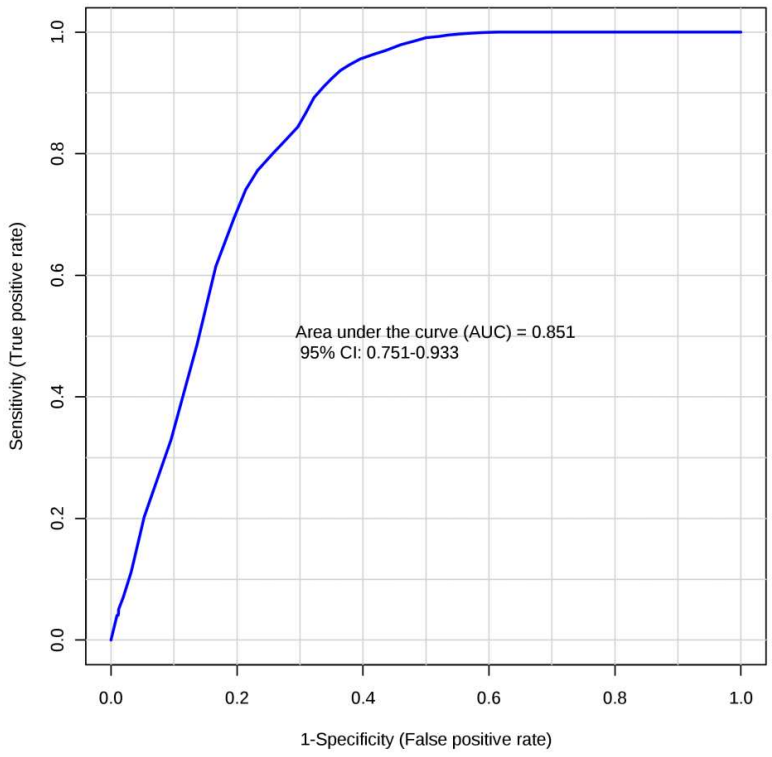

Figura 7: Receiving Operating Characteristic (ROC) between Control and Cystic Fibrosis groups. Calculated results led to 90% of specificity and 68% of sensitivity.

4. DISCUSSION

4.1. A potential ESI-MS method for Cystic Fibrosis diagnosis

Cystic fibrosis diagnosis is not a simple task; it involves a multistep process that begins with newborn screening for CFTR mutation. Although the genetic identification of default CFTR gene itself does not confirm diagnosis, it leads to a Cystic Fibrosis Screen Positive Inconclusive Diagnosis (CFSPID) in many cases[5]. CFSPID patients will require close clinical monitoring for symptoms, and additional tests such as the repeated evaluation of chloride sweat testing and extensive genotyping[2,4,5]. Our developed method has presented evidence to work regardless of age and gender of the patients, and was efficient when applied to two F508del mutations. Due to the generic nature of the elected biomarkers, i.e. they are not linked to any mutation - governed biochemical pathway, the same test may be applied to other mutation types. Hence, the proposed method shows great potential to be applied in CF screening, in addition to assess existing CFSPID cases.

Mass spectrometry-based methods have been reported as a tool for diagnosis through measurement of chloride and sodium in sweat[12] and through biomarkers in bronchoalveolar lavage fluid[13]. The identification of biomarkers in saliva with mass spectrometry opened a favorable history to the proposal of a reliable MS approach for CF investigation[14,15]. In addition to creating a simpler, faster, noninvasive method, our proposition brings a major advantage: the availability of saliva and its easier accessibility is crucial when dealing with children and young adults[16,17].

4.2. Control and Cystic Fibrosis biomarkers

Saliva presents over 99% of water in its composition, followed by other minor components such as low molecular weight molecules, lipids, peptides, proteins,

enzymes, mucus, electrolytes and metabolites derived from bacteria activity[18]. Neutral lipids, such as cholesterol, cholesterol esters, glycerides and free fatty acids re present over 95% of total lipid composition of saliva from submandibular and labial gland, while glycerophospholipids compose only a small fraction[19]. Regarding glycerophospholipids, phophatidylcholine (PC) and phosphatidylserine (PS) are reported to be the major contents in saliva[20] and also in lung spuntum fluid, a mixture of both saliva and mucus[21]. Analyzing the control group data PS (*m/z* 524), PC (*m/z* 390) and phosphatidylglycerol (PG) (*m/z* 485) were elected as control biomarkers, which, in addition to demonstrating a normal lipid composition of saliva, may also indicate a decrease in lipid metabolism in the CF group when compared to the control group.

The mutation in the encoding gene of CFTR transporter is especially important in the epithelial cells of exocrine glands, such as those involved in saliva and sweat secretion and respiratory and digestive tract[22]. The amylase, mucin, and electrolyte-rich saliva composition is altered throughout the gland ducts by reabsorption mechanisms. Molecules from blood can also reach saliva through passive diffusion or active transport, thus corroborating a chemical composition directly related to the whole organism system, with similarities in the metabolomic profile[14,15].

Therefore, the biomonitoring of biochemical alterations from cystic fibrosis disorder in saliva is a real possibility. The selected biomarkers, even if not directly related or produced by salivary glands, may be detected in the salivary fluid, expressing the systemic manifestation of cystic fibrosis disease. The pronounced oxidative stress observed in CF patients is caused both by the disease itself, and by the inflammatory status brought by recurrent pulmonary infections, common to most patients, leading to changes in metabolic homeostasis, cellular damage through lipid peroxidation,

oxidation of proteins and DNA fragmentation[10,16].

Consequently, the effects of oxidative stress exacerbation are observed in the deficits of lung function of the patients studied and in the form of biomarkers in salivary fluid. Due to oxidative stress and inflammation, myeloperoxidases (MPO) present in saliva (lactoperoxidases) and in the lining fluid of epithelial airway cells are increased under CF conditions[23]. MPO usually catalyze the reaction between the thiocyanate anion and hydrogen peroxide to form oxidation products with antimicrobial properties (O_2SCN^-, O_3SCN^-, $(SCN)_2$, HOSCN and $OSCN^-$)[24]. While hydrogen peroxide is an important known airway inflammation biomarker, the presence of thiocyanate in the airways and saliva in CF patients is impaired. With the abnormal function of the CFTR transporter, the accumulation of thiocyanate in apical epithelial cells is restrained, affording major availability of MPO to act over tyrosine residues and leaving the oral cavity vulnerable to bacteria colonization[10,25].

The formation of Dityrosine (*m/z* 395) and Nitrotyrosine (*m/z* 207), elected as biomarkers in the CF group, are due to the increase in MPO activity and protein nitration. The upregulation in nitric oxide synthase (NOS2), and the abnormal NO metabolism in CF patients due the airway inflammatory process also contribute to the presence of Nitrotyrosine. Both biomarkers have already been reported to be present in the lung of CF patients and polymorphisms of MPO gene promoter influence on the severity of pulmonary disease[23,25-27].

The NO pathway significantly contributes to the increase of oxidative damage through the production of nitrogen reactive species (NRS). In addition to NRS, reactive oxygen species (ROS) and free radicals arising from bacteria activity and inflammation needs the accentuated antioxidant profile of saliva to be controlled. Under normal conditions, saliva presents several antioxidant molecules such as glutathione, vitamins,

uric acid, peroxide and superoxide dismutase[14,24,27]. Exacerbated inflammation present in CF may appear due to the elevated level of ROS and oxidative stress. Increased ROS in turn, could be a consequence of the absence of normal levels of antioxidant substances. Glutathione (GSH) is part of the antioxidant capacity in salivary fluid[16,24]; children with CF present significant systemic decrease in extracellular GSH due to the diminished capacity of the abnormal CFTR chloride channel in GSH secretion[10,28]. Besides CFTR abnormality, the impairment of glutathione levels seems to be associated to an alteration of GSH metabolism and/or further modification in genotype[29,30].

GSH synthesis and *de novo* synthesis depend on the availability of its precursors, glutamate and cysteine, and require as catalyst Glutamylcysteine Ligase (GCL). Gamma-glutamyl-S- cysteine (*m/z* 249) and its methylated form, Gamma-glutamyl-S-methylcysteine (*m/z* 263) were both elected as CF group biomarkers, since the alterations in glutathione metabolism, secretion, enzymes and related pathways (Figure 4) might alter precursors levels. Additionally, the transcription of GCL is affected by the exacerbation of oxidative stress and inflammation. The pre-existent abnormal efflux of GSH in CF condition is aggravated when a low level in GSH:GSSG ratio is established, favoring the activation of the transcription factors NF-KB and AP-1. These transcription factors are involved in the GCL transcription and inflammation process[31]. Possible modifications in GSH degradation could also determine low levels of GSH. Gamma-glutamyl transferase (GGT) is the enzyme that converts GSH in Cysteinyl-Glycine and L-Glutamate[29].

The rise of GGT activity in homozygous F508del due to the induced genetic expression by oxidative stress and pro inflammatory cytokines might favor the turnover of GSH. Therefore, low levels of extracellular GSH might also be associated with excess of GSH turnover leading to an increase in GSH precursor levels, such as

gamma-glutamyl-S-cysteine, and GSH metabolite Cysteinyl-glycine (*m/z* 213), elected as a CF group biomarker[29,31,32].

In addition to enzymatic modifications in the biosynthesis of glutathione by transcription factors from inflammation and oxidative stress, genetic modifiers contribute to CF phenotypes, such as the determination of lung disease severity and pulmonary infections susceptibility. Several studiesassociate gene candidates with CF phenotype, most of them from the immune and inflammatory classes[33].

Recently, polymorphisms in the gene that encodes GCL, a rate limiting enzyme in the formation of Gamma-glutamyl- cysteine, were also discovered in patients with moderate genotype of CFTR mutation (one allele)[30]. The correlation between gene modifiers and CFTR/ non-CFTR genetic variation influences the variability of phenotypes, and can be explored by the test developed for diagnosis, even when the patients have on F508del and other mutations. The low GSSG:GSH ratio might also be a consequence of choline deficiency expected in CF patients.

Due to impaired secretion of bicarbonate and pancreatic enzymes in CF patients suffering from pancreatic insufficiency, the absorption of nutrients, including vitamins and lipids from dietary intake is compromised. Even with the administration of enzymes and food supplements, the correction of patients' nutrition is not completely established[34].

Malabsorption of choline-contained lipids due the 9- fold increase in PC fecal losses, affects in the *de novo* biosynthesis of PC. PC plays an important role in the synthesis of acetylcholine and in the secretion of triacylglycerol from liver through very low-density lipoprotein. Thus, the impairment of the enterohepatic bile PC cycle affects not only liver function but also the biochemical homeostasis of PC synthesis[32,34]. An alternative pathway for PC formation involves the transference of methyl groups from phosphatidylethanolamine (PE) to S-Adenosylmethionine (SAM) leading to the

formation of Adenosilhomocysteine (SAH) and PC, in a reaction catalyzed by Phosphatidylethanolamine-N-methyl transferase (PEMT).

Therefore, in addition to the lower GSH:GSSG ratio in plasma of CF children compared to control, plasmatic increase of homocysteine, SAH, adenosine levels and decrease in the SAM:SAH ratio have also been reported[32,34]. SAH (*m/z* 383) was elected as biomarker of CF group due the alterations observed in CF patients associated to choline deficiency.

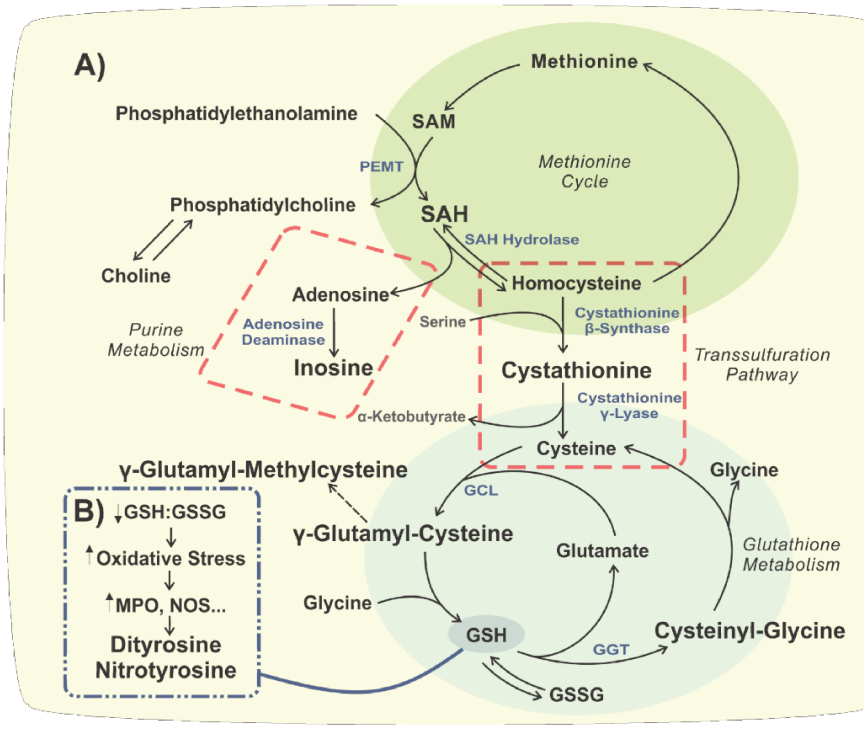

Figura 8: Schematic metabolic pathways interconnection of the 8 CF group biomarkers. In **A)** due to the malabsorption of choline-rich molecules, the formation of phosphatidylcholine occurs preferably by the PEMT pathway, leading to an increase in SAH and adenosine levels. The possible down-regulation of adenosine deaminase in chronic pulmonary disease affects the formation of Inosine, elected biomarker, in purine metabolism. The metabolic interconnection with glutathione metabolism happens through the biomarker Cystathionine in the transsulfuration pathway and consequent convertion to cysteine, which enters in glutathione cycle. The alterations in precursors and glutathione degradation products, γ-Glutamyl-Cysteine, γ-Glutamyl-Methylcysteine and Cysteinyl-Glycine might be associated with the proven decrease in GSH levels due impairment in CFTR secretion and enzymatic modifications. In **B)** the consequent diminish in GSH:GSSG and overall antioxidant capacity levels, lead to the formation of Nitrotyrosine and Dityrosine, biomarkers of the enhanced MPO and NOS activity in oxidative stress.

GCL - Glutamate-Cysteine Ligase; GGT - γ-Glutamyl Transferase; GSH - Glutathione; GSSG - Glutathione dissulfide; MPO - Myeloperoxidase; NOS - Nitric Oxide Synthase; PEMT - Phosphatidylethanolamine-N-Methyl Transferase; SAH - S- Adenosylhomocysteine; SAM - S-Adenosylmethionine.

Following the metabolic route (Figura 8), SAH originates homocysteine in a reaction catalyzed by SAH hydrolase. Homocysteine present in elevated levels, in turn, enters a transsulfuration pathway, being converted to Cystathionine in the first step of this reaction by cystathionine β-synthase[34].As previously described by Endres *et al*.[35], Cystathionine *(m/z* 221), was elected CF biomarker. This molecule is converted to cysteine, a precursor of GSH, in the second step of transsulfuration reactions using cystathionine γ-lyase. Cystathionine is present in basal concentrations under normal conditions; the full activation of cystathionine β-synthase is achieved with the allosteric modulator SAM. As increased SAH levels over SAM are established in CF patients due to an increased turnover in the parallel pathway of choline production (Figura 8), lower levels of SAM impacts the conversion of homocysteine into cystathionine, which would result in further less cysteine and GSH[36]. CF patients, however, require a higher activity of the choline production[32,34] pathway than a normal, healthy individual would; this causes, therefore, SAM production to be much increased and, even though the SAH:SAM ratio is high in these patients, when compared to an individual of the control group the production of Cystathionine stands out, corroborating the election of this molecule as a biomarker for CF.

When SAH is converted to homocysteine, it also originates adenosine. Adenosine is a nucleoside that plays an important role in cell-signaling pathways and in the composition of energy molecules, such as adenosine- triphosphate. Adenosine participates in purine metabolism originating Inosine by catalysis of adenosine deaminase. However, in patients with cystic fibrosis, purine metabolism is also impaired[37]. Some references state that purine metabolite inosine is significantly

decreased in CF cells; nevertheless, this molecule was elected as a CF group biomarker (*m/z* 303), possibly due to the upregulated production of choline; as inosine participates as a side reaction in the SAH:SAM metabolism (Figure 4), the increased levels of this molecule in CF patients corroborate even further our hypothesis.

5. CONCLUSIONS

Our findings show a strong link between the 8 biomarkers, highlighting the interconnection of metabolic pathways, which reflects in the high sensitivity and specificity achieved. Since some biomarkers are associated with genetic modification in the CFTR function or induction of enzyme genes, this methodology may be applied to other mutations in addition to F508del. Others may be consequence of the pronounced oxidative stress, inflammation and lung disease, which are corroborated by the clinical conditions of the patients select for this study. Finally, the choice of saliva as sample matrix, the simple preparation approach and the reliability of mass spectrometry and statistical validation combined make the proposed method a valuable potential and more assertive alternative method for CF screening.

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Skin imprinting: a potential method for cystic fibrosis screening

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ABSTRACT

This contribution describes an accurate strategy to provide biomarkers that are related to cystic fibrosis by using skin imprints on silica plate followed by direct analysis in high-resolution mass spectrometry. The presented approach relies on changes in skin composition caused by the mutation in the cystic fibrosis transmembrane conductance regulator (F508del) observed in affected patients. Due to these metabolic changes, specific biomarkers were selected using multivariate data analysis, providing a specificity of 90% and a sensitivity of 73%, a remarkable measure of relevance for this condition, considering existing diagnostic methods.

Keywords: Cystic Fibrosis, Skin, High-resolution mass spectrometry, Metabolomics, Biomarkers.

INTRODUCTION

Cystic Fibrosis (CF) is a recessive genetic exocrine disorder that affects the transport of fluids and electrolytes through cell membrane, resulting in abnormal viscous secretion in multiple organs. Although this disease affects all racial and ethnic groups, Caucasians are the most affected; 1 in every 2000-3000 European newborns presents CF (FÍRMIDA and Lopes, 2011). Soon after this disease was characterized, in 1953 (di Sant'Agnese et al., 1953), cystic fibrosis was considered almost inevitably lethal, mainly at the first decade of life. However, improvements at diagnostic methods and clinical management have led to increased life expectancy for CF patients over the years. Recent statistical data indicate that the birth cohort of the year 2000 may present a survival median of 50 years (Dodge et al., 2007). Currently, most CF patients are able to manage well disease, albeit their quality of life is still affected by limitations such as physiological functioning, endobronchial infection and pancreatic insufficiency (De Jong et al., 1997).

All cystic fibrosis manifestations are caused by mutations affecting the cystic fibrosis transmembrane conductance regulator (CFTR). Although there are multiple mutations at CFTR gene associated with CF disorders, the most common corresponds to a deletion of a phenylalanine at position 508, called F508del mutation (Riordan, 1989). More than 1000 CFTR mutations are known to cause CF; some may be responsible for milder CF phenotypes, while others present more severe manifestations. These differences at CFTR gene mutation locus generates great heterogeneity, making clinical and even laboratory diagnosis difficult tasks (De Boeck et al., 2006).

The two most used tests for CF laboratory diagnosis are DNA analysis and sweat test; the first is applied to recognize mutations on CFTR gene, but the mutation panels are limited by a number of gene alterations predefined for commercial laboratory tests (e. g. 97 mutations) (Hamosh et al., 1998). Although it is possible to

analyze the complete sequence of the CFTR gene, this genetic analysis does not detect mutations that occur outside the coding regions, and it is an expensive and time consuming process that is performed in specialized clinical molecular and genetics laboratories, making it a test with limited access for patients (Wine et al., 2001).

Also a laboratory diagnosis test used for CF diagnosis is the quantitative pilocarpine iontophoresis testing (QPIT), also known as sweat test (Gibson and Cooke, 1959). Although an uncomfortable method, QPIT is considered the Gold standard test, which consists in determination of chloride concentration in sweat; in this test, the normal upper limit can be <30 mmol/L for infants or <40 mmol/L for older adults (LeGrys et al., 2007). For sweat collection, perspiration is induced through the local administration of pilocarpine nitrate, followed by a battery-operated electric current application that stimulates rapid and increased sweating rate. After sweat collection from the forearm skin, chloride levels are evaluated. For a conclusive result, QPIT requires at least two tests, and it is considered positive when chloride concentrations are above 60 mmol/L (Gibson and Cooke, 1959, LeGrys et al., 2007, LeGrys VA, 2000, Grosse et al., 2004).

By having QPIT as the method of choice for CF diagnosis, there is an obvious window of inaccurate diagnosis for chloride concentrations between 40 and 60 mmol/L, which classifies patients within this range as indeterminate cases, requiring further evaluation (LeGrys et al., 2007). Considering chloride quantification < 40 mmol/L as normal and ≥ 40 mmol/L as risk for CF, QPIT sensitivity and specificity are 100% and 92.8%, respectively (Warwick et al., 1986). However, considering the borderline window, the current standard diagnostic method does not differentiate positive and negative patients for CF, despite the high sensitivity and sensibility exposed above. Taking into account the many critical issues associated with this method, accurate sweat test results may be challenging, and technical

errors and misinterpretation are common and can lead to false results (LeGrys, 1996, Mattar et al., 2014; Shaw and Littlewood, 1987). Therefore, new techniques that enhance or solve CF diagnosis issues for all cases are mandatory.

The metabolomics approach has been growing as a promising tool for monitoring biochemical pathways and their relationship with biological phenotypes in an integrated perspective (Medina et al., 2014). Metabolomics evaluation of biological samples allows researchers to identify specific biomarkers or sets of biomarkers that could be useful to differentiate health and disease profiles, which may lead to more accurate diagnostic tools (El-Aneed et al., 2009; Gowda et al., 2008). In this context, this contribution intended to provide a set of potentially diagnostic biomarkers for cystic fibrosis, based on an innovative and noninvasive sweat collection method, associated with high-resolution mass spectrometry. The identified molecules present great potential to be part of a new diagnostic tool for cystic fibrosis, thereby reducing questionable diagnostic results.

METHODS

Patients' selection

Sixteen patients were selected in a retrospective fashion at the CF Clinic of the Clinics Hospital at the University of Campinas, during four months, which is a period that covered scheduled visits for all treated patients in this facility. Inclusion criteria were to have at least one sweat test with chloride concentration greater than or equal to 60 mEq/L, and presence of homozygosis for the mutation F508del, characterized by genetic studies. Exclusion criteria were the presence of skin lesions on the back, local of sample collection and/or another skin disease. The control group was composed by healthy individuals at the same age and gender as the CF group, recruited from schools and universities. Sample collection was approved by the Research Ethics Committee of the School of Medical Sciences – University of Campinas/Brazil (Protocol number:

1.100.978). All experiments were performed in accordance with relevant guidelines and regulations regarding samples of human origin.

Sample collection and preparation

Plates of silica gel 60G, suitable for thin-layer chromatography (Merck, Darmstadt, Germany) were cut into small squares of 1 cm^2 and were superposed on the back of the individuals of both groups for 1 minute. Then, silica plates were extracted with 1000 µL of Methanol: Water (1:1) and 1 µL ammonium hydroxide was added to each sample to facilitate ionization.

Mass spectrometry analysis

Samples were directly injected in an ESI-LTQ-XL Orbitrap Discovery instrument (Thermo Scientific, Bremen, Germany) with nominal resolution of 30,000 (FWHM) under the following conditions: flow rate of 10 $\mu L.min^{-1}$, sheath gas at 10 arbitrary units, spray voltage of 5 kV and capillary temperature of 280°C. AnalysesSamples were directly injected in an ESI-LTQ-XL Orbitrap Discovery instrument (Thermo Scientific, Bremen, Germany) with nominal resolution of 30,000 (FWHM) under the following conditions: flow rate of 10 $\mu L.min^{-1}$, sheath gas at 10 arbitrary units, spray voltage of 5 kV and capillary temperature of 280°C. Analyses were performed in triplicates and all data were acquired in the negative ion mode at the mass range of 150-700 *m/z*.

Statistical analysis and biomarker election

An Orthogonal Partial Least Squares Discriminant Analysis (O-PLS-DA) using the online software MetaboAnalyst 3.0 (www.metaboanalyst.ca) [16] was performed to select characteristic ions from each group; the choice considered the VIP score list (Variable Importance in Projection) generated by the software. To verify the accuracy of the biomarkers candidates chosen by the VIP score list, a Receiver Operating

Characteristic (ROC) Curve was performed.

METLIN (Scripps Center for Metabolomics, La Jolla, CA), HMDB version 3.6 (Human Metabolome database - www.hmdb.ca), Lipid MAPS online database (University of California, San Diego, CA – www.lipidmaps.org) and KEGG Pathway Database (Kyoto Encyclopedia of Genes and Genomes – www.genome.jp/keeg/pathway.html) were consulted to elect potential biomarkers with mass accuracy no greater than 2 ppm.

RESULTS

In 4 months, 16 CF patients were selected for this study, with ages ranging from 5 to 19 years old (mean: 12 years; median: 13 years), with 56% males and 44% females. The sweat test presented an average concentration of chloride of 111 mEq/L (minimum: 76 mEq/L; maximum: 166 mEq/L). All patients presented deficits in lung function and 69% presented two or more different microorganism isolated from sputum within the past 12 months. Regarding further clinical conditions, 100% of patients presented pancreatic insufficiency, 67% have evidence of liver disease, 44% make use of ursodeoxycholic acid and 19% developed diabetes. The control group was composed of 16 healthy individuals within the same sex and age range as the CF group and collected at the same period.

HRMS analysis on negative ion mode was performed, and Figura 9 shows representative mass spectra from each group.

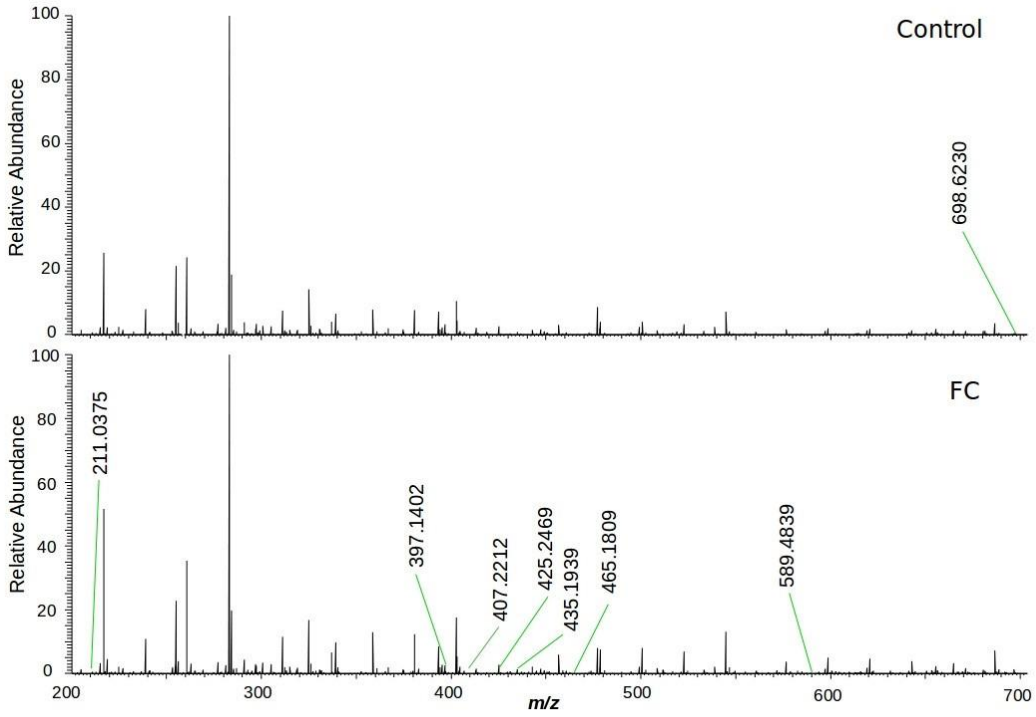

Figura 9: Representative fingerprinting spectra on negative ion mode (150-700 *m/z*) of the groups: Control and Cystic Fibrosis. The mass of each group's biomarkers are highlighted, and full information on these molecules can be seen in Tabela 4.

Information extracted from spectral data served as basis for the statistical analyses using O-PLS-DA, which compared the control and CF groups to elect biomarkers for CF. O-PLS-DA plot (Figure 10) shows complete separation of control and CF groups; from these analyses, 1 biomarkers were selected for control group and 7 biomarkers for the CF group, all with VIP scores above 2.

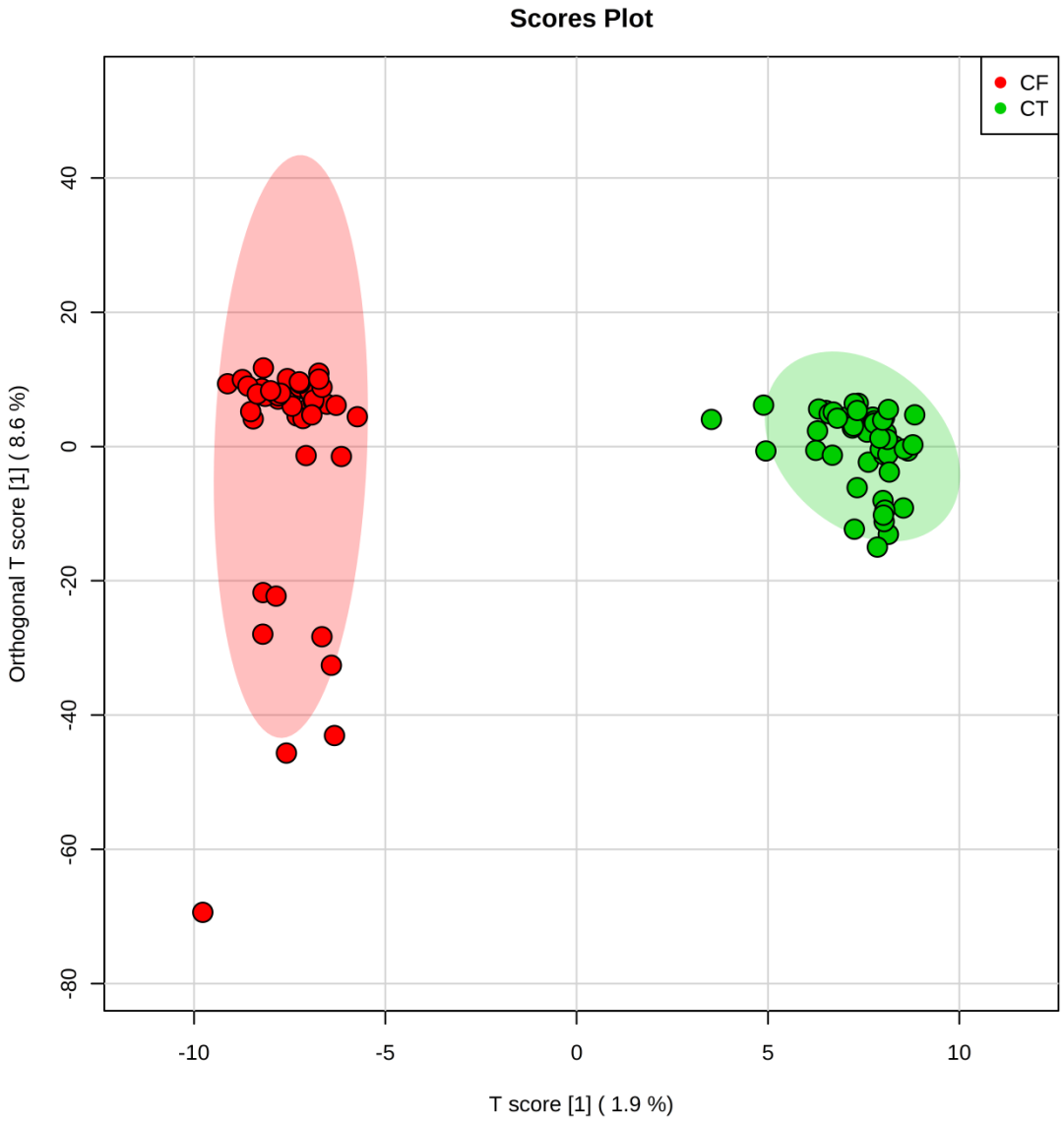

Figura 10: Partial Least Square Discrimination Analysis (PLS-DA), with the comparison between the Control (green) and Cystic Fibrosis (red) groups. The 95% confidence interval of the model is displayed as the shaded colors among samples within each group.

Detailed information on the biomarkers elected for control and CF groups can be seen in Table 1. To evaluate the relevance of these biomarkers, a ROC curve was built, presenting 90% of specificity and 73% of sensitivity (Figure 11).

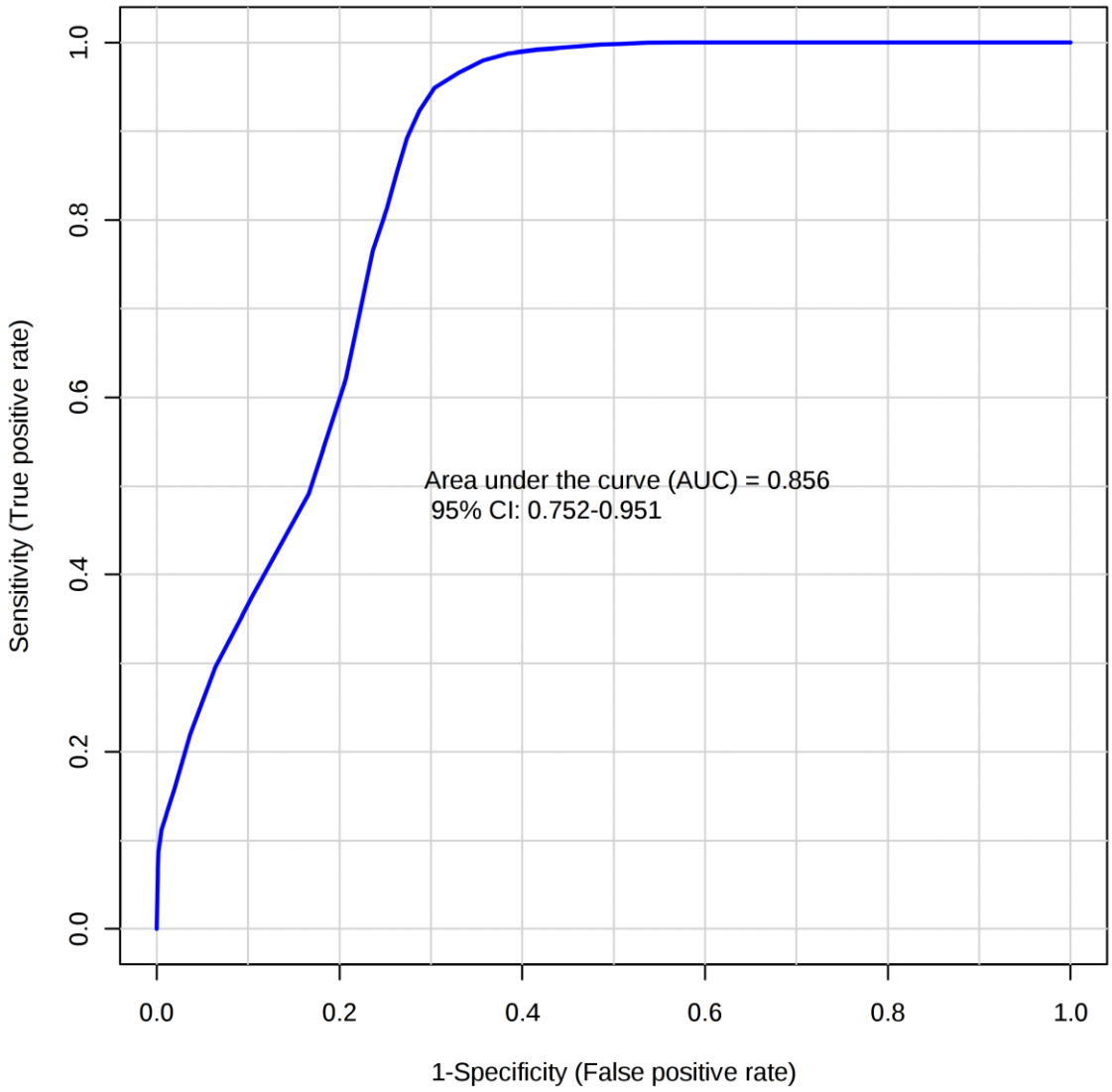

Figura 11: Receiving Operating Characteristic (ROC) between Control and Cystic Fibrosis groups. Calculated results led to 90% of specificity and 73% of sensitivity.

Tabela 4: Biomarkers identified via ESI-MS (negative ion mode) for Control, Cystic Fibrosis groups. Identification is based on the comparison between the exact and theoretical masses of each compound in Lipid Maps, METLIN and HMDB.

Groups	Molecule	Adduct	ID	Experimental Mass	Theoretical Mass	Error (PPM)
Control	Cer(d18:1/25:0)	[M+Cl]-	MID83713 / MID 7210/ HMDB04957	698.6230	698.6223	-1.0020
Cystic Fibrosis	2,3-dimethyl-3-hydroxy-glutaric acid	$[M+Cl]^-$	MID45921/LMFA01170081/HMDB02025	211.0375	211.0379	1.8954
	Thyrotropin releasing hormone	[M+Cl]-	MID44807/HMDB05763	397.1402	397.1397	-1.2590
	19(R)-hydroxy-PGF1α	[M+Cl]-	MID36115/LMFA03010038	407.2212	407.2206	-1.4734
	7beta-Hydroxy-3-oxo-5beta-cholan-24-oic Acid	[M+Cl]-	MID84475/LMST04010161/HMDB00503	425.2469	425.2464	-1.1758
	7alpha-Hydroxy-3,12-dioxochola-1,4-dien-24-oic Acid and/or 3,7,12-Trioxochol-5-en-24-oic Acid and/or 3,7,12-Trioxochol-4-en-24-oic Acid and/or 3,7,12-Trioxo-5beta-chol-1-en-24-oic Acid	[M+Cl]-	MID84697/LMST04010391 MID84671/LMST04010364 MID84670/LMST04010363 MID84669/ LMST04010362	435.1939	435.1944	1.1489
	PA(18:4(6Z,9Z,12Z,15Z)/0:0)	[M+Cl]-	MID82343/LMGP10050024	465.1809	465.1814	1.0748
	DG(20:3(8Z,11Z,14Z)/14:0/0:0)and/or DG(16:0/18:3(6Z,9Z,12Z)/0:0)and/or DG(14:0/20:3(8Z,11Z,14Z)/0:0)and/or DG(18:3(6Z,9Z,12Z)/16:0/0:0)and/or DG(18:3(9Z,12Z,15Z)/16:0/0:0)	[M-H]-	MID58977/HMDB07472 MID58733/HMDB07104 MID58659/HMDB07024 MID58824/HMDB07272 MID58853/HMDB07301	589.4839	589.4837	-0.3393

Abbreviations: Cer: Ceramide; DG: Diacylglycerol; PA: Phosphatidic Acid; PGF1α: Prostaglandin F1α.

DISCUSSION

Development of HRMS method

Sweat test remains the gold-standard method for CF diagnosis; however, this test lacks sensitivity and specificity at the concentration range of 40 – 60 mmol/L of chloride in sweat. Additionally, this test often needs to be performed more than once to present confirmatory results. Sweat collection is not only time consuming, it is also uncomfortable for the patient, and the method for chloride determination requires specific protocols; therefore, methodological errors are common (Mattar et al., 2014). On the other hand, the method based on mass spectrometry and metabolomics, described in this contribution, presents a simple and quick sample collection, data acquiring and processing, and does not bring discomfort to the patient.

Silica plates have a useful particularity for sampling purposes: the adsorption and stabilization of molecules, such as those present in the skin surface. Thus, employing silica plates as tools for in-field collection of samples that will be forwarded to referral analyses centers seems an obvious advantage of this technique, dispensing need for additional apparatus for sample preserving (Lima et al., 2014). This relatively simple sample preparation, coupled with direct-infusion mass spectrometry, provides a more straightforward analysis protocol for biomarkers monitoring. With less sample preparation steps, the errors associated to the methodology decrease, hence making the method more reproducible.

The seven markers chosen and statistically validated for the CF group, when monitored together, provide 73% of sensitivity and 90% of specificity; these values may be considered adequate when compared to the current methods for diagnosis of the disease, especially when considering that the method encompasses sweat test-borderline cases in the result. The chosen markers are not only involved with the ionic imbalance caused by the disease in the sweat gland, but with the pathophysiology and progression of the disease, and therefore may be considered specific markers when monitored together for the F508del mutation. Although this contribution has evaluated

only an F508del scenario, it is possible to infer that these markers may also be identified in CF patients with other mutations, since the method is sufficiently sensitive to detect small levels of altered components associated with skin physiological changes caused by other mutations. Nonetheless, further investigation is required, with a heterogeneous CF group, for conclusive results.

Skin control biomarker

Epidermis is very active in the synthesis of fatty acids, cholesterol and ceramides (Cer). These lipids provide fundamental limitation to water and electrolyte movement, and act as a barrier against microorganisms' invasion (Pappas et al., 2009; Khara et. al., 2016). Epidermal Cer have unique structures that cannot be found in other cell types of human body (Khara et. al., 2016). Cer containing odd carbon chains are not common to be found in the body but represent about 30% of the total Cer present in the epidermis (Khara et. al., 2016), this corroborates the election of Cer (d18:1/25:0) (698 *m/z*) as a control group marker. In fact, control markers are only elected to demonstrate the capability of the method in differentiating both groups by providing molecules that describe the normal composition of the skin, thereby suggesting that there are changes in the composition of skin lipids in CF due to electrolytic imbalance. Therefore, this class of Cer stands out in this comparison, defining the controls group skin, as determined by O-PLS-DA.

Cystic fibrosis biomarkers

CFTR channel gating is predominantly regulated by protein kinase A (PKA)-dependent phosphorylation, which mediates gene expression, conformal changes, and the trafficking of the protein from the endoplasmic reticulum (ER) to Golgi apparatus and cell surface (Chin et al., 2016). However, the phenylalanine deletion at position 508 (F508del) of the CFTR gene reduces the rates of phosphorylation and may contribute to improper folding, defective trafficking, and slower rates of channel

activation in some tissues (Chin et al., 2016; McClure et al., 2016). The F508del-CFTR is produced and retained in the ER, and undergoes ER-associated degradation through the proteasome pathway. A limited number of misfolded protein reaches the Golgi apparatus for glycosylation; however, they are redirected back to ER for degradation. When they reach cell surface, these altered proteins are unstable and are targeted to lysosomal degradation (Chin et al., 2016).

The export from ER depends of the interaction between the properly folded CFTR and the coat complex II (COPII) budding machinery (Chin et al., 2016: Hashimoto et al., 2008). The COPII coated vesicles formation depends of the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) by phospholipase D (PLD) (Hashimoto et al., 2008). The relationship between PA and CFTR trafficking is largely unknown but some studies performed by Hashimoto and coworkers (Hasnhimoto et al., 2016) showed that PLD-mediated PA formation is required for CFTR transport form ER exit site to the Golgi (Hashimoto et. al., 2008) that corroborates the election of PA (465 m/z) as CF biomarker. Regarding the process of translocation and ER- associated degradation (ERAD) of CFTR, the conversion of PA to diacylglycerol (DG) (Hashimoto et. al., 2008) also corroborates with the elections of DG isomers (589 m/z) as CF markers. It may be postulated that the organism is trying to maintain a compensatory mechanism of PA synthesis to enhance the concentration of COPII and the transport of F508del-CFTR to maturation in the Golgi apparatus. However, the excess of PA is possibly converted in DG, which also helps in the ERAD translocation process of misfolded F508del-CFTR.

The F508del-CFTR and the altered ion transportation impair several physiological process such as transepithelial transport, membrane conductance, cell volume and pH regulation, and the GSH-related detoxification and antioxidant activity in the extracellular medium. The redox imbalance causes abnormal generation of reactive oxygen species, leading to exacerbation of oxidative stress and development of inflammatory and degenerative lesions in target issues (Galli et al.,

2012). Thereby, the increase of inflammatory response corroborates the election of 19(R)-hydroxy-PGF1α (407 m/z) as a CF biomarker.

The CRTF channels in sweat ducts are reported to be independent of PKA and cyclic adenosine monophosphate (cAMP) phosphorylation activity (Reddy et al., 2003). Reddy et al., 2003 showed that glutamate and related metabolites are demonstrated to activate CFTR channels for Cl^- conductance in the absence of cAMP and ATP, and may induce the conductance of HCO_3^- in the presence of ATP, evidencing that the selectivity of an anion channel in a native epithelium may be altered by a physiological mediator. The same study evaluated the response of F508del-CFTR to glutamate, and showed that there is no activation of the channels, since a low amount of misfolded proteins reach the cell surface and are quickly degraded (Reddy et al., 2003; Chin et al., 2016). Assuming there is a compensatory mechanism for enhancing the glutamate production in an attempt to activate the F508del-CFTR channels, which remain nonfunctional on the cell surface, the Krebs cycle may be shifted for producing of oxalacetate and 2-oxo-glutarate, which could enter via glutamate synthesis. Thus, other intermediates of the Krebs cycle would be produced and moved to other pathways, including the lysine and tryptophan biosynthesis. The elevated concentration of these amino acids by both the Krebs cycle and the intense degradation of F508del-CFTR misfolded proteins may activate their conventional degradation pathways by using glutaryl-CoA dehydrogenase and, alternatively, by glutaric acid production and its derivates, which justifies the election of 2,3-dimethyl- 3-hydroxy- glutaric acid (211 *m/z*) as a CF biomarker.

The skin is also a place of synthesis and metabolism of several hormones, neurotransmitters and biological regulators, including hypothalamic-pituitary-tyroid (HPT) axis (Harvey et al., 2012). Thyroid-stimulating hormone (TSH) expression in the skin is increased by thyrotropin-releasing hormone (TRH) and reduced by thyroid hormones (Harvey et al., 2012; Bodo et al., 2010). CF population is reported to present subclinical hypothyroidism and iodine deficiency (Naehrlich et al., 2013). Thus, it is expected that high levels of TRH (397 *m/z*) be found in skin samples of CF

patients. Interestingly, these results brought to our attention that a more frequent follow-up regarding the levels of thyroid hormones is necessary, since these tests are not routinely performed at our center.

Another physiological important alteration is CF liver disease (CFLD), a serious complication responsible for 2-4% of total mortality. The diagnosis of CFLD lack specific and sensitive markers; however, the prevalence rate is 2-37% in children and adults (Kobelska-Dubiel et al., 2014; Van der Fenn et al, 2016). Given that bile alkalization occurs, and viscosity increases in CF, these events result in higher levels of free radicals and accumulation of hydrophilic bile acids, which may provide damage to the hepatocytes and bile ducts (Kobelska-Dubiel et al., 2014).When in high concentration in the bloodstream, bile acids may be deposited on the skin, which corroborates the election of primary bile acids (425 *m/z* and 435 *m/z*) as CF markers, since there are 67% of patients with suspected CFLD in the population selected for the study. Considering the treatment for these patients, only 44% use ursodeoxycholic acid, a drug that is used to reduce the viscosity of bile and increase its flow, although its effectiveness is yet to be proven.

CONCLUSION

The prospected biomarkers elected by O-PLS-DA, when observed in a cystic fibrosis pathological condition, gain context, as discussed above, and assist in the description and discrimination of individuals with the disease when compared to a control group. Interestingly, this contribution has evidenced the choice of primary bile acids as biomarkers for CF; this demonstrates that the concept of this approach may be useful not only for prospecting molecules that render a more specific diagnosis of CF in patients with the ΔF508 mutation, but may also be used for monitoring the progression of the disease in other mutations.

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CONCLUSÃO

Os métodos baseados em espectrometria de massas desenvolvidos apresentaram valores consideráveis de sensibilidade e especificidade e se mostraram eficientes para selecionar biomarcadores que podem ser potencialmente aplicados ao diagnóstico de doenças com etiologias distintas.

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