

Sttephany Silva Bernardino

Plataforma não invasiva para detecção de Transtorno do Espectro Autista baseada em Lipidômica Salivar acoplada à Inteligência Artificial.

Non-invasive platform for Autism Spectrum Disorder detection based on Salivary Lipidomics coupled with Artificial Intelligence.

Dissertação apresentada à Faculdade
de Odontologia da Universidade de
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RESUMO

O diagnóstico oportuno e preciso do Transtorno do Espectro Autista (TEA) em ambientes clínicos permanece um desafio devido à falta de marcadores diagnósticos. A aplicação da lipidômica não direcionada emergiu como uma análise poderosa para detectar novos biomarcadores. Nesse contexto, a detecção de lipídios salivares pode oferecer detecção não-invasiva em larga escala para o TEA. Neste estudo, utilizamos a lipidômica não direcionada baseada em cromatografia gasosa acoplada a espectrometria de massa, juntamente com algoritmos de inteligência artificial, para identificar alterações no perfil lipídico salivar como alternativa para a detecção de TEA. Foram analisadas 33 amostras de saliva de crianças, 18 neurotípicas e 15 com diagnóstico de TEA sem uso de medicamentos. Foram identificados 180 lipídios salivares, 27 foram expressas com filtro de frequência de 75% e alguns lipídios salivares foram identificados de forma pioneira na saliva. Três lipídios salivares 1-Naftalenamina, N-fenil-; ácido hexadecanóico, éster 2-hidroxi-1-(hidroximetil)etílico; e 13-Docosenamida, (Z)- tiveram sua expressão reduzida ($p < 0,05$) no TEA comparada aos neurotípicos. Também testamos 6 algoritmos de inteligência artificial baseados em Python, e o algoritmo de Redes Neurais teve o melhor desempenho alcançando 84% de acurácia, 80% de especificidade e 88% de sensibilidade com o escore SHAP mais alto para o lipídeo 13-Docosenamida. Em resumo, estes dados destacam o potencial do perfil lipídico salivar acoplado por algoritmos de inteligência artificial como uma ferramenta não invasiva para triagem de crianças com TEA, permitindo avanços na medicina de precisão e odontologia personalizada.

Palavras-chave: biomarcadores salivares, lipídios, TEA, análise GC-MS, aprendizado de máquina

ABSTRACT

Timely and accurate diagnosis of Autism Spectrum Disorder (ASD) in clinical settings remains a challenge due to the lack of diagnostic markers. The application of untargeted lipidomics has emerged as a powerful analysis for detecting novel biomarkers. In this context, the detection of salivary lipids may offer large-scale, non-invasive detection for ASD. In this study, we utilized untargeted lipidomics based on gas chromatography-mass spectrometry, coupled with artificial intelligence algorithms, to identify alterations in the salivary lipid profile as an alternative for ASD detection. We analyzed 33 saliva samples from children, including 18 neurotypical and 15 with a diagnosis of ASD who were not using medication. We identified 180 salivary lipids, with 27 being expressed with a frequency filter of 75%, and some salivary lipids were identified for the first time in saliva. Three salivary lipids—1-Naphthalenamine, N-phenyl-; hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; and 13-Docosenamide, (Z)—had reduced expression ($p < 0.05$) in ASD compared to neurotypical individuals. We also tested 6 artificial intelligence algorithms based on Python, and the Neural Network algorithm had the best performance, achieving 84% accuracy, 80% specificity, and 88% sensitivity, with the highest SHAP score for the lipid 13-Docosenamide. In summary, these data highlight the potential of salivary lipid profiling coupled with artificial intelligence algorithms as a non-invasive tool for screening children with ASD, enabling advances in precision medicine and personalized dentistry.

Keywords: salivary biomarkers, lipids, ASD, GC-MS analysis, machine learning

INTRODUÇÃO E REFERENCIAL TEÓRICO

Nos últimos anos, o Transtorno do Espectro Autista (TEA) tem recebido crescente atenção da comunidade científica e da sociedade em geral, devido à sua prevalência crescente e ao impacto significativo que o diagnóstico tem na vida dos pacientes e de seus familiares. O TEA é uma condição de neurodesenvolvimento caracterizada por déficits persistentes na comunicação social e interação social, juntamente com padrões restritos e repetitivos de comportamento, interesses ou atividades conforme descrito nos critérios diagnósticos do Manual diagnóstico e estatístico de transtornos mentais (DSM-5)(1).

O TEA pode ser dividido em três níveis de suporte que são determinados com base na necessidade de amparo, e isso se traduz na quantidade de ajuda ou adequações que o indivíduo precisa na sua rotina diária(2). O Centro de Controle e Prevenção de Doenças (CDC) dos Estados Unidos mostra que uma em cada 36 crianças tem recebido diagnóstico de TEA. A estimativa encontrou uma prevalência de 2,8% de crianças de 8 anos com TEA e também foram identificados 2,2% de adultos, destacando a importância de estratégias de diagnóstico preciso e intervenções precoces(3)(4).

Quanto antes o diagnóstico for realizado maiores serão as chances do indivíduo conseguir conviver melhor com as muitas comorbidades e dificuldades que podem surgir, o não acesso ao diagnóstico leva ao desenvolvimento e também piora das inúmeras comorbidades que causam diversos prejuízos a vida do indivíduo(5). Entre as dificuldades na comunicação e interação social, assim como o enfrentamento de estigmas e discriminação podem contribuir para sentimentos de isolamento e dificuldades de subsistência(6). Além disso, a presença de comorbidades psiquiátricas, como depressão e ansiedade, transtorno de déficit de atenção e hiperatividade (TDAH) são comuns em pessoas com TEA, todos estes fatores associados a baixa empregabilidade resultam em danos significativos ao indivíduo sem diagnóstico ao longo da vida(7).

O diagnóstico do TEA é baseado em uma avaliação abrangente do desenvolvimento do indivíduo, incluindo observação direta do comportamento e histórico clínico detalhado por meio de questionários diagnósticos, aplicação de

instrumentos de triagem e questionários específicos(8). Até o momento, não há um teste único de diagnóstico para o TEA e o processo diagnóstico geralmente envolve uma equipe multidisciplinar experiente composta de profissionais de saúde, como médicos, pediatras, psiquiatras, psicólogos, neuropsicólogos e terapeutas especializados(9). O diagnóstico de autismo pode ser desafiador devido a várias razões, incluindo: a complexidade do espectro autista pois cada indivíduo manifesta as características atípicas de forma única, a diferença entre as manifestações entre homens e mulheres(10), a sobreposição de sintomas com outras condições e mesmo a falta de capacidade técnica de identificar traços de TEA por profissionais(11).

Frequentemente o diagnóstico do TEA é realizado seguindo os critérios do DSM-5 (Manual Diagnóstico e Estatístico de Transtornos Mentais – 5ªed - 2013) . O DSM-5 foi desenvolvido com apoio de mais de 200 especialistas no assunto e contém extenso texto descritivo com critérios atualizados para o diagnóstico de transtornos mentais, o que permite uma abordagem similar para a comunicação entre profissionais da saúde e a população afetada pelo TEA. O DSM-5 e o classificador internacional de doenças (CID) devem ser considerados como publicações complementares. O CID contém os números de código usados para o monitoramento de estatísticas de morbidade e mortalidade por agências de saúde nacionais e internacionais, incluindo a Organização Mundial de Saúde (OMS). O DSM-5 é o manual usado globalmente por profissionais de saúde como balizador do diagnóstico do TEA e de outros transtornos mentais. Neste contexto, o DSM-5 contém descrições, sintomas e outros critérios para o diagnóstico consistente do TEA(1).

A necessidade de uma abordagem multidisciplinar é desafiadora para ser implementada em serviços de saúde. Isto ocorre pois o número de profissionais capacitados nos setores público e privado não são capazes de atender parte significativa da população que deveria ter acesso aos serviços de saúde mental, em especial no Brasil, devido à grande parte da população não conseguir arcar com o alto custo do diagnóstico e o sistema único de saúde não suportar o volume desta demanda de triagem e diagnóstico (12–14) como resultado, uma parcela

significativa da população pode passar por toda infância, adolescência e parte da vida adulta sem diagnóstico. Sabe-se que o diagnóstico tardio do TEA pode atrasar o acesso a intervenções e serviços especializados, e assim, esta população é afetada por inúmeras comorbidades que causam diversos prejuízos a vida do indivíduo ou podem receber diagnósticos equivocados com tratamentos desnecessários e ineficientes para outras condições (7,15) portanto, o desenvolvimento de uma plataforma de diagnóstico complementar que permita rápida liberação de resultados, de forma não-invasiva e que possa facilitar este processo complexo, de difícil acesso pode ter papel muito relevante no enfrentamento da triagem diagnóstica do TEA.

Neste cenário o uso da saliva como biofluido diagnóstico se faz promissor, pois sua utilização já é vastamente comprovada para diversas doenças e condições sistêmicas(16). A saliva é um biofluido secretado principalmente pelas glândulas salivares maiores, parótidas, submandibulares e sublinguais, em conjunto com milhares de glândulas salivares menores distribuídas por toda a cavidade oral. A saliva tem seu volume e sua composição regulada principalmente pelo sistema nervoso autônomo, com o sistema parassimpático envolvido principalmente na regulação do volume salivar e o sistema nervoso simpático na secreção de proteínas. A saliva é um biofluido complexo composto por uma variedade de componentes, incluindo proteínas, peptídeos, lipídeos, carboidratos, RNA mensageiro, metabólitos entre outros, que refletem o estado de saúde do indivíduo (17).

Como um biofluido diagnóstico a saliva apresenta inúmeras vantagens como: coleta não-invasiva, coleta que oferece mais conforto e segurança para o paciente e para o profissional que realiza a coleta comparada com sangue ou fluído de nasofaringe, coleta é indolor com menor risco de contaminação, oferece meio de coleta conveniente com custo de armazenamento e transporte reduzidos quando comparada com outros fluidos diagnósticos como o sangue, permite múltiplas coletas de forma sequencial de um mesmo indivíduo, o que pode ser essencial em análises complementares (18). Sendo assim, a análise do perfil de lipídeos

salivares associada com algoritmos de inteligência artificial podem ser considerados uma área de pesquisa promissora.

Os lipídios estão presentes na saliva humana e desempenham várias funções importantes, são componentes essenciais das membranas celulares e desempenham papéis importantes na sinalização celular, na regulação do metabolismo e na resposta imunológica(19). Eles são essenciais para a formação do filme lipídico na superfície da saliva, que ajuda na lubrificação e proteção da mucosa oral contra danos físicos, infecções e desidratação(16). Além disso, os lipídios na saliva podem estar envolvidos na manutenção do equilíbrio microbiológico na cavidade oral, influenciando a composição da microbiota bucal e, consequentemente, afetando a saúde oral. A composição específica dos lipídios na saliva pode variar dependendo de vários fatores, incluindo dieta, estado de saúde e fatores genéticos(19)

A abordagem lipidômica engloba uma compreensão abrangente das alterações metabólicas associadas à saúde e à doença, a diversidade química das espécies lipídicas, seu papel biológico(20). O lipidoma, que representa o conjunto completo de lipídios presentes em um organismo, desempenha um papel relevante na manifestação de condições patológicas, refletindo as mudanças metabólicas que podem ser decorrentes dessas doenças. Essas variações nas moléculas lipídicas têm sido consideradas como potenciais marcadores para o rastreamento e detecção precoce de distúrbios metabólicos e outras condições(21). Além disso, oferece a capacidade de analisar a composição lipídica em nível sistêmico, fornecendo informações abrangentes sobre as alterações metabólicas que ocorrem durante o curso de uma doença. Essa abordagem pode levar à identificação de biomarcadores específicos, permitindo o diagnóstico precoce e o monitoramento de doenças, o que é crucial para melhorar os resultados clínicos e a qualidade de vida dos pacientes.(22)

A lipidômica de saliva pode ter implicações clínicas importantes, como identificação de biomarcadores para diagnóstico, prognóstico ou monitoramento de doenças, incluindo câncer, doenças metabólicas e distúrbios inflamatórios(22). Além disso, os lipídios identificados como alterados podem servir como alvos

terapêuticos potenciais ou indicar mudanças no estilo de vida ou tratamentos farmacológicos para modificar os perfis lipidômicos e melhorar a saúde(23). A vantagem de usar a lipidômica para diagnósticos está na capacidade de identificar e quantificar mudanças no lipidoma de células, tecidos, órgãos ou biofluidos com alta resolução por espectrometria de massa, o que permite o monitoramento das mudanças metabólicas durante o curso da doença, proporcionando dados clínicos valiosos com mínimo ou nenhum desconforto para o paciente(22).

Entretanto, a lipidômica enfrenta desafios significativos tanto na realização das análises experimentais quanto na interpretação dos dados obtidos(23). A complexidade das amostras biológicas, a necessidade de metodologias sensíveis e específicas, bem como a análise e interpretação adequadas dos dados são aspectos críticos que demandam constante desenvolvimento e aprimoramento na área da lipidômica(24).

A extração de lipídios apresenta desafios devido à natureza hidrofóbica ou anfipática dos lipídios, à diversidade em propriedades estruturais e físico-químicas entre centenas de milhares de lipídios, e a falta de um método único que resulte no repertório completo de lipídios(25). Além disso, a identificação inequívoca das espécies de lipídios é um desafio, a falta de dados estruturais corretos em bancos de dados de lipídios, gerados por outros meios que não a espectrometria de massa, também pode resultar em falsas identificações(24). As variações nas moléculas lipídicas podem servir como marcadores potenciais para triagem e detecção precoce de doenças(26).

Neste contexto a inteligência artificial entra como uma excelente ferramenta no auxílio da categorização e análise dos dados. A utilização de algoritmos de inteligência artificial (IA) direcionadas para análises ômicas têm auxiliado no diagnóstico de doenças, proporcionando avanços significativos na melhoria da acurácia de novas plataformas em desenvolvimento(27). Estas tecnologias permitem a integração e análise de grandes volumes de dados biológicos, identificando padrões complexos que seriam impossíveis de detectar manualmente(28). Algoritmos de aprendizado de máquina, incluindo Support Vector Machine, Random Forest, Adaboost, e Neural Network, são capazes de descobrir

biomarcadores específicos e perfis moleculares associados a diferentes patologias, facilitando a detecção precoce e o desenvolvimento de tratamentos personalizados(29). Além disso, a IA contribui para reduzir erros de diagnóstico e melhorar a capacidade preditiva, resultando numa abordagem mais eficaz e eficiente no combate a diversas doenças(30).

Neste contexto, esta dissertação de mestrado tem como objetivo investigar a utilização da análise lipidômica da saliva como uma ferramenta alternativa para detecção do transtorno do espectro autista.

2 PROPOSIÇÃO

Esta pesquisa visa contribuir para o desenvolvimento de métodos de diagnóstico mais precisos e eficazes para o transtorno do espectro autista (TEA), o que pode possibilitar intervenções mais oportunas e direcionadas para os indivíduos dentro deste espectro.

O presente estudo buscou realizar uma análise lipidômica não-direcionada comparando o perfil de lipídeos salivares de crianças com TEA em comparação com crianças neurotípicas. O objetivo central deste estudo foi desenvolver uma plataforma inovadora para detecção do TEA baseada no perfil de lipídeos salivares acoplados em algoritmos de inteligência artificial incluindo os algoritmos Support Vector Machine, Random Forest, Adabost e Neural Network.

Artigo a ser enviado para publicação

Non-invasive platform for Autism Spectrum Disorder detection based on Salivary Lipidomics coupled with Artificial Intelligence

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Abstract

The timely and accurate diagnosis of Autism Spectrum Disorder (ASD) in clinical settings remains challenging due to the lack of diagnostic markers. The application of untargeted lipidomics has emerged as a powerful analysis to detect novel biomarkers. In this context, the non-invasive detection of salivary lipids can offer large-scale detection of ASD. In this study, we used Gas chromatography-mass spectrometry-based untargeted lipidomics coupled with artificial intelligence algorithms to identify changes in salivary lipid profile to be used as an alternative for ASD detection. Saliva samples from 33 ASD and matched-control children were analyzed. A total of 180 salivary lipids were identified, 27 had a frequency filter of 75% and some salivary lipids were pioneeringly identified. Three salivary lipids 1-Naphthalenamine, N-phenyl-; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; and 13-Docosenamide, (Z)- were higher ($p < 0.05$) expressed in ASD than matched-controls. We also tested 4 state-of-the-art Python-based artificial intelligence algorithms, the Neural Networks had the best performance achieving 84% of accuracy, 80% of specificity, and 88% of sensitivity with the highest SHAP score of 13-Docosenamide. In summary, these data highlight the potential of salivary lipidomics profile supported by artificial intelligence algorithms as a non-invasive tool for screening of ASD children, enabling advances in precision medicine and personalized dentistry.

Keywords: human saliva, salivary biomarkers, lipids, Biophotonic, GC-MS analysis, machine learning

INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by persistent deficits in social communication and social interaction, alongside restricted and repetitive patterns of behavior, interests, or activities, as outlined in the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders (1). The U.S. Centers for Disease Control and Prevention (CDC) estimates that one in 36 children is diagnosed with ASD. The estimative found a prevalence of 2.8% of 8-year-old children with ASD and also have been identified as 2.2% of adults (3,4). The disparities and underreporting of diagnosis for children with ASD are multifactorial, and highlight the critical importance of accurate screening and diagnostic strategies to provide early therapeutic interventions (31).

The diagnosis of ASD is based on a multifaceted assessment encompassing direct behavioral observation and a detailed clinical history. This history is typically gathered through diagnostic questionnaires, standardized screening tools, and specific instruments (3). A multidisciplinary team of healthcare professionals conducts a comprehensive evaluation, employing partially structured direct observation of the child's behavior and standardized measures obtained via caregiver interviews related to the development trajectory and behaviors(32). This assessment should include the use of standardized and validated tools, such as the Autism Diagnostic Interview-Revised (ADI-R), the Autism Diagnostic Observation Program - 2nd edition (ADOS-2), and the Childhood Autism Rating Scale (CARS)(16). However, it is important to recognize the current limitations in obtaining a specific diagnosis, since ASD presents a wide heterogeneity of clinical manifestations, and there is still no definitive biological marker(3). The combination of these approaches aims to minimize these limitations and provide the most accurate diagnosis possible, although challenges remain in ASD identification.

In this context, the use of saliva as a diagnostic fluid for detecting physiological changes and systemic diseases is an emerging method for accurate disease detection. Saliva is a complex fluid primarily composed of water, containing organic and inorganic components that change its concentration reflecting oral and systemic diseases. Saliva contains electrolytes, proteins (peptides, enzymes), carbohydrates, nucleic acids(DNA and RNA), and lipids that can access saliva through paracellular and transcellular flow with close

relation with serum concentrations (16,20,33,34).

Saliva contains a variety of lipids, including phospholipids, fatty acids, and cholesterol, which can reflect metabolic changes associated with pathological conditions (35,36). These salivary lipids are generally insoluble in water and can be involved in enzyme activities and stabilization of DNA helix(37). Salivary lipids are essential for the formation of a lipid film on the saliva surface, which aids in lubricating and protecting the oral mucosa and maintaining microbial balance in the oral cavity(38). Studies indicate that changes in salivary lipid levels are related to diseases such as diabetes, cardiovascular disease, and neurodegenerative disorders (17,39). Salivary lipids are primarily secreted by major salivary glands by passive diffusion directly from serum (35,40). The development of saliva-based diagnostic platforms offers numerous advantages, such as allowing for self-collection, painless and non-invasive sampling, lower collection costs, and simplicity in storage and transportation compared to blood. However, the application of saliva in conjunction with omics analyses and artificial intelligence algorithms is still in its early stages(38).

Lipidomic platforms are analytical tools that allow for the identification and quantification of lipids in a biological sample. Detecting changes in lipid composition can be used for monitoring and early detection of metabolic disorders and pathologies (17,39). The use of artificial intelligence (AI) algorithms in omics analysis has been applied in the diagnostic of several diseases, providing significant advances in accuracy and acceleration in the release of outcomes (30). These AI analyses allow the integration of large volumes of biological data, identifying complex patterns that would be impossible to detect manually using(41). Machine learning algorithms are enable to discover specific biomarkers and molecular profiles associated with different pathologies to improve early detection (42). Furthermore, AI contributes to reducing diagnostic errors and improving predictive capacity, resulting in a more effective and efficient approach to combating various (43) diseases. However, to our knowledge, this lipidomic approach with AI algorithms has not yet been applied to ASD using saliva samples.

Here, we tested the hypothesis that salivary lipids detected in a platform based on gas chromatography-mass spectrometry-based untargeted lipidomics coupled to learning-machine algorithms could be used to discriminate ASD from matched-control subjects. The present study aimed to compare salivary lipid profiles between ASD patients and matched control subjects by using a

lipidomic platform coupled to Support Vector Machine, Random Forest, Adabost, and Neural Network algorithms.

MATERIALS AND METHODS

Study design

All experimental procedures were carried out in compliance with the Declaration of Helsinki. This protocol was authorized by the Ethical Board of CEUMA University (#3.597.769). Written informed consent was acquired from all participants of this study, including both ASD and matched-control subjects. Each ASD or matched-control subject or their legal guardian signed the approved written informed consent voluntarily. The 33 participants were categorized as follows: 18 neurotypical matched-controls and 15 ASD subjects without medication. This ASD classification was conducted according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) by a medical expert. The presence of oral or systemic infection, or any other disease 30 days before sample collection, was considered an exclusion criterion for both neurotypical and ASD children included and the samples were collected from patients in the health system in the state of Maranhão - Brazil.

Saliva collection

The collection of salivary samples was performed following the child's preference and it was supported by the caregiver. Saliva was collected by negative pressure using a 3 ml syringe in the sublingual region of the mouth. Subsequently, the saliva was recovered by centrifugation for 3,000 rpm at 4°C for 15 minutes, and the supernatants were kept frozen at -80°C until analysis. Saliva was collected in the morning, and participants were also requested to abstain from feeding 1 hour before the saliva collection.

Processing and analysis of salivary lipidomic

To understand the changes in the lipidomic profile of patients that occur due to ASD, an assessment of lipids differentially synthesized between different conditions was carried out. To do this, 100 µL of saliva was aliquoted from each person in each group to which 200 µL of dichloromethane and 100 µL of methanol were added. The material was homogenized, kept at room temperature for 30 minutes, and

centrifuged for 10 minutes at 13000xg. The organic phase was collected, transferred to a new tube, and subjected to a vacuum concentrator for 60 min at 40°C. Samples were resuspended in 50 µL of dichloromethane and transferred to vials.

The analyses were performed on an Agilent Technologies model 7890B instrument coupled to a mass detector (MSD 5977B) with the automatic injector. The column used was the DB-5HT capillary chromatographic column (30 m x 250 µm x 0.25 µm), electronic impact ionization (EI) mode, 70 eV ion source, with a high vacuum pump and quadrupole analyzer. The chromatographic conditions used were: injector temperature, 290 °C. The carrier gas used was high-purity helium at a constant flow rate of 1.2 mL min⁻¹. Injections of 6 µL were made. The initial temperature was 80°C; this temperature was held for 0 min, increased to 300°C at a rate of 6°C/min, and held for 12 min. Then, the temperature was changed to 350°C at 25 °C/min, totaling 50.67 min of analysis. The following mass spectrometry conditions were used: the MS and MS quad source temperatures were 150°C and 280°C, respectively. The acquisition mode occurred in the range of m/z 50–550, with a count threshold of 150 and solvent delay of 4 min.

Statistical Data Analysis

The statistical analysis of the children's demographic characteristics was performed using GraphPad Prism 8. The Shapiro–Wilk test was used to assess the normality of the data. For normally distributed data, the t-test was used, and the results are presented as the means (standard deviation). A p-value less than 0.05 was considered with statistical significance.

The statistical analyses of the lipidomic analysis data were performed with log₂-transformed values. The unpaired t-test was applied when the p-value was < 0.05 and

the fold change was equal to or greater than 2.00. The lipids were identified using the METLIN 2019 database and the public databases PubChem and The Human Metabolome Database (HMDB). The mass samples acquired in the lipidomic analysis were extracted into the TXT format and submitted to the multi-paradigm numerical computing MATLAB program (MATrix LABoratory) to evaluate principal component analysis (PCA) Volcano plots and heat maps were generated to highlight and identify the differentially expressed metabolites between the groups. Lipidomic expression was analyzed by differential expression with up- or down-regulation based on the Log₂ fold-change. PCA, volcano plot, and heatmap were generated with MPP. The data were processed in MassHunter Qualitative v. 07, followed by data alignment using MPP. These data were subsequently exported to a CSV extension.

Artificial Intelligence Algorithms

The classifications with each pre-processing parameter were tested with machine learning algorithms associated with Support Vector Machine, Random Forest, Adabost, and Neural Network algorithms. The predictive performance of these artificial intelligence algorithms was indicated after cross-validation with ten times stratifications. Furthermore, this strategy was repeated three times with changes in the sample configurations in subsets to estimate the real performance. The sensitivity or true positive rate is the proportion of positives (ASD subjects) correctly classified, and the specificity or true negative rate is the proportion of negatives (Matched-controls subjects) that were correctly classified. The accuracy is defined as the total number of samples correctly classified considering true and false negatives.

RESULTS

The mean age was similar ($p > 0.05$) in ASD and matched-control subjects (7.4 ± 5.1 and 8.3 ± 5.13 years old, respectively). The gender was 73% of males and 13% of females in ASD and 66% of males and 33% of females in matched-control subjects (Table 1).

Table 1. Demographic characteristics ASD and age- and gender-matched neurotypical control subjects.

Parameter	Control	ASD	p
Age			
Mean	8.3 years old	7,4 years old	p > 0.05
Gender			
Male	12 (66%)	13 (77%)	p > 0.05
Female	6 (33%)	2 (13%)	

Salivary lipidomic analysis

A total of 180 salivary lipids were detected in saliva samples. After applying a 75% frequency filter in at least one group, 27 lipids were considered for statistical analysis. 3 lipids detected in saliva were considered down-regulated based on the Log₂ fold-change. in ASD compared to matched-controls.

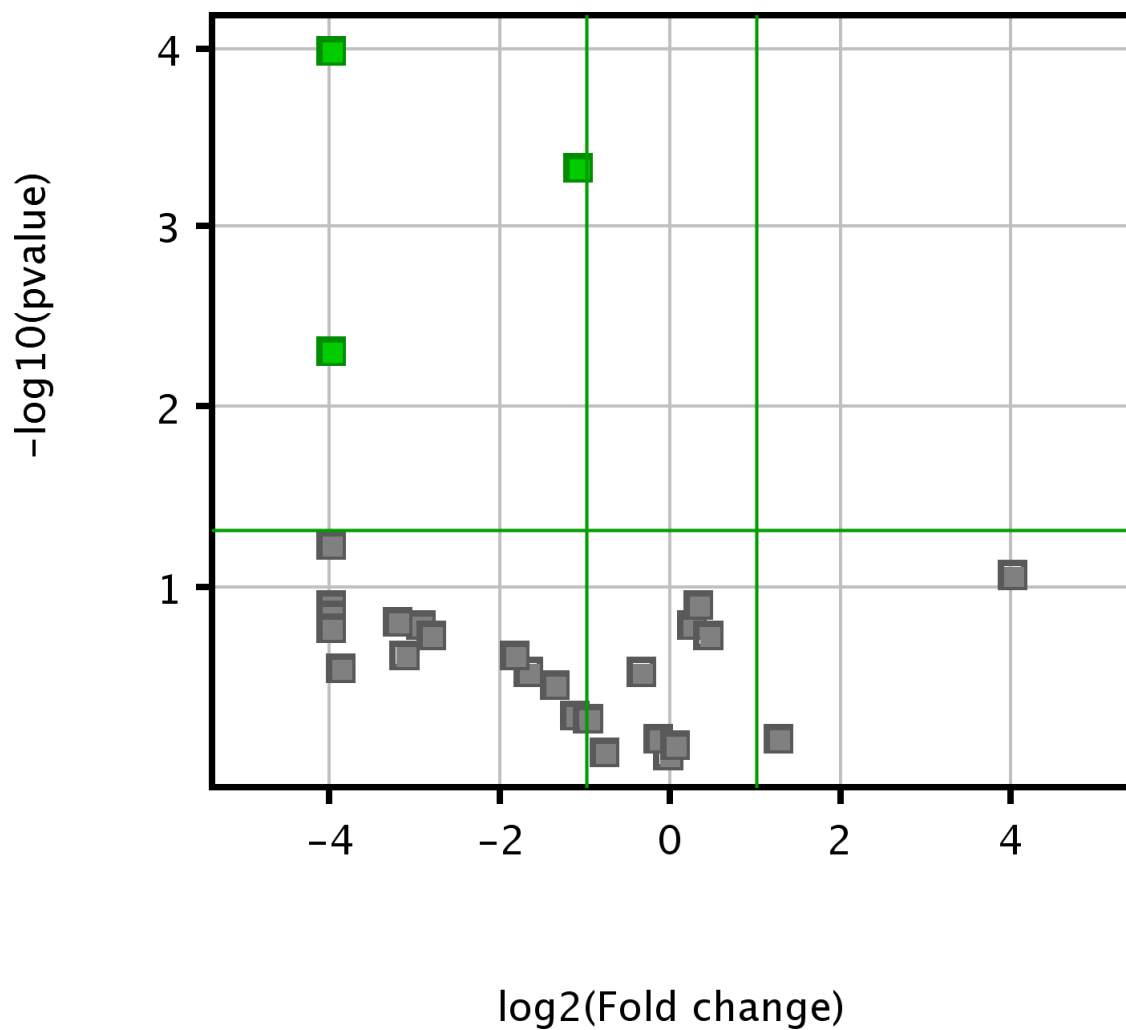


Figure 1. Volcano plot of significant lipids expression affected between ASD and matched-controls subjects.

The PCA model was based on three principal components (PCs) that explained 77.41% of the variance, with 70.97% using the X- and Y-axis and 6.44% in the Z-axis within the dataset (Figure 2).

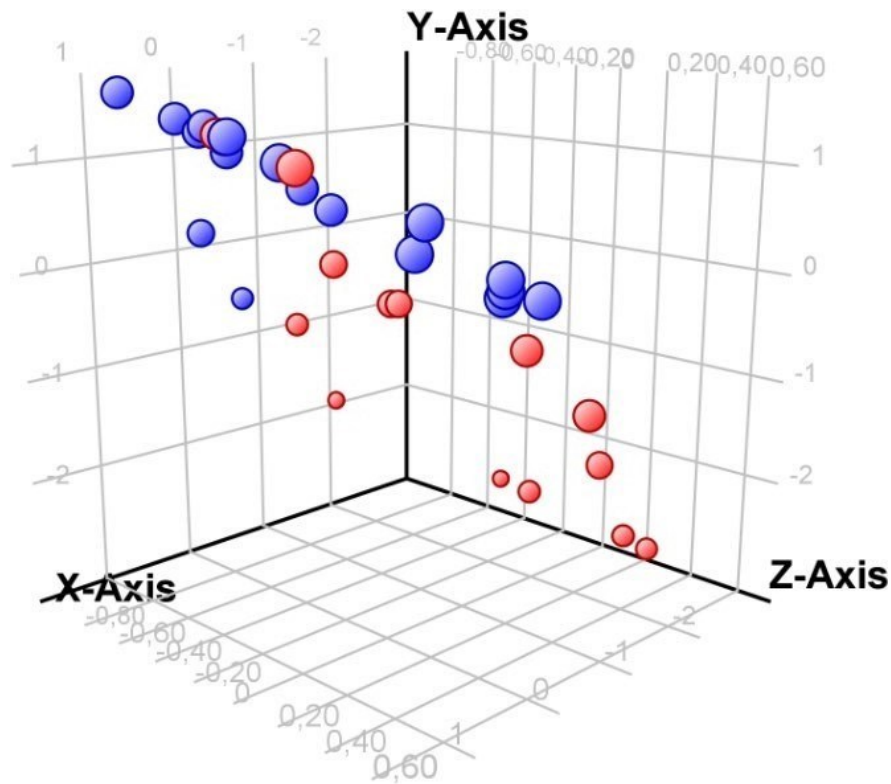


Figure 2. Principal component analysis score plot for X-, Y- and Z-Axis between ASD and matched-control subjects.

Figure 3 shows the average changes in the expression of the 3 salivary lipids, as represented by the heatmap (differences expressed in the volcano plot graph). Three salivary lipids 1-Naphthalenamine, N-phenyl-; Hexadecanoic acid, 2-hydroxy-1- (hydroxymethyl)ethyl ester; and 13-Docosenamide, (Z)- were lower ($p < 0.05$) expressed in ASD than matched-controls.

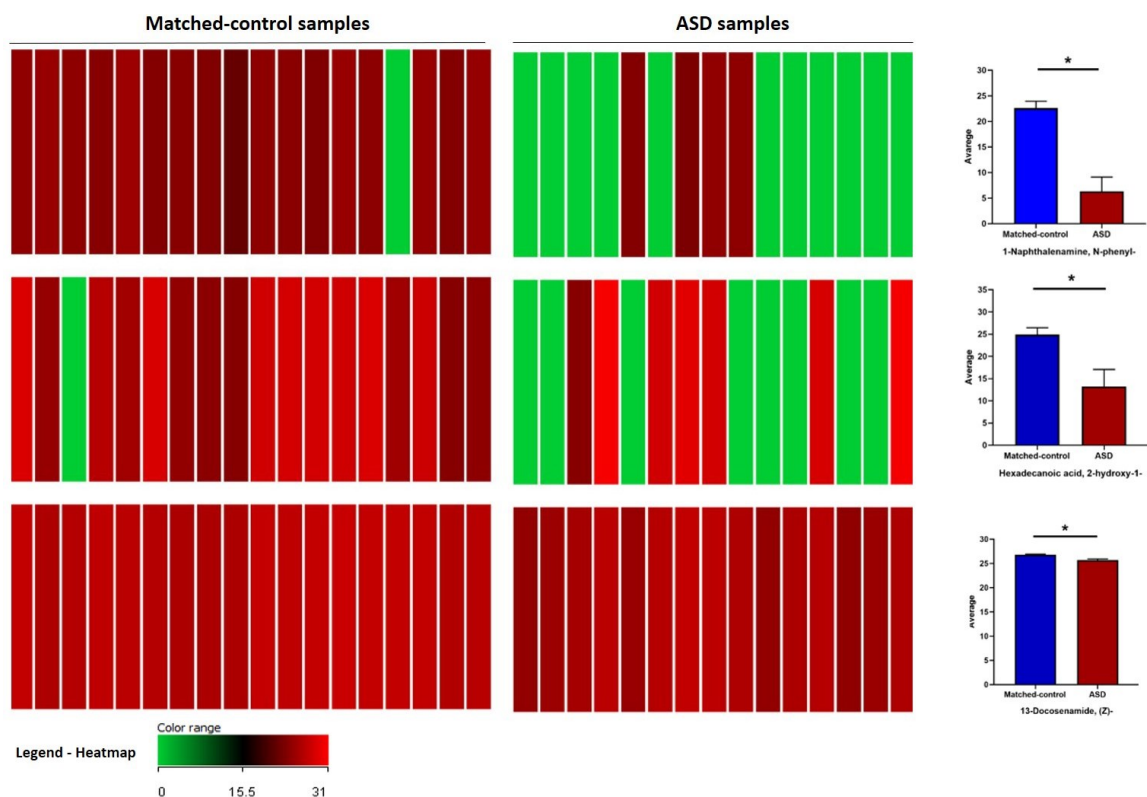


Figure 3. Individual heatmap chart and profile of 3 salivary lipids (1-Naphthalenamine, N-phenyl-; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; and 13-Docosenamide) in ASD and matched-controls subjects.

The classification of Support Vector Machine, Random Forest, Adabost, and Neural Network algorithms showed the discrimination of salivary lipidomic spectra applied in ASD and matched-control samples. The best discrimination using the expression of 180 salivary lipids achieved an accuracy of 78%, sensitivity of 73%, and specificity of 83% with the Random Forest algorithm. When a filter on 27 salivary lipids was applied with a differential expression using the scoring methods T-test, the best discrimination of ASD than matched-control samples was obtained with the Neural Network algorithm achieving an accuracy of 84%, sensitivity of 80%, and specificity of 88% (Table 2).

Table 2. Machine learning algorithms are applied in salivary spectra to classify AI and control samples.

Algorithm Model	Based on 180 filtered salivary lipids			Based on 27 salivary lipids		
	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity
Support vector machine	60%	13%	100%	78%	66%	88%
Random Forest	78%	73%	83%	81%	73%	88%
Adabost	72%	73%	72%	72%	73%	72%
Neural Network	75%	73%	77%	84%	80%	88%

The SHAP plot with salivary lipids in the best discriminating algorithm indicates 13-Docosenamide, (Z); 1- Naphthalenamine, N-phenyl; Octyl tetracosyl ether; Phthalic acid, propyl nonyl ester; tert-Hexadecanethiol; Pentadecane; Hexadecane, 2,6,10,14-tetramethyl; Docosyl octyl ether; cis-13-Octadecenoic acid; and Nonadecane are the main responsible discrimination between ASD and matched-control samples performed by the Neural Network algorithm (Figure 4).

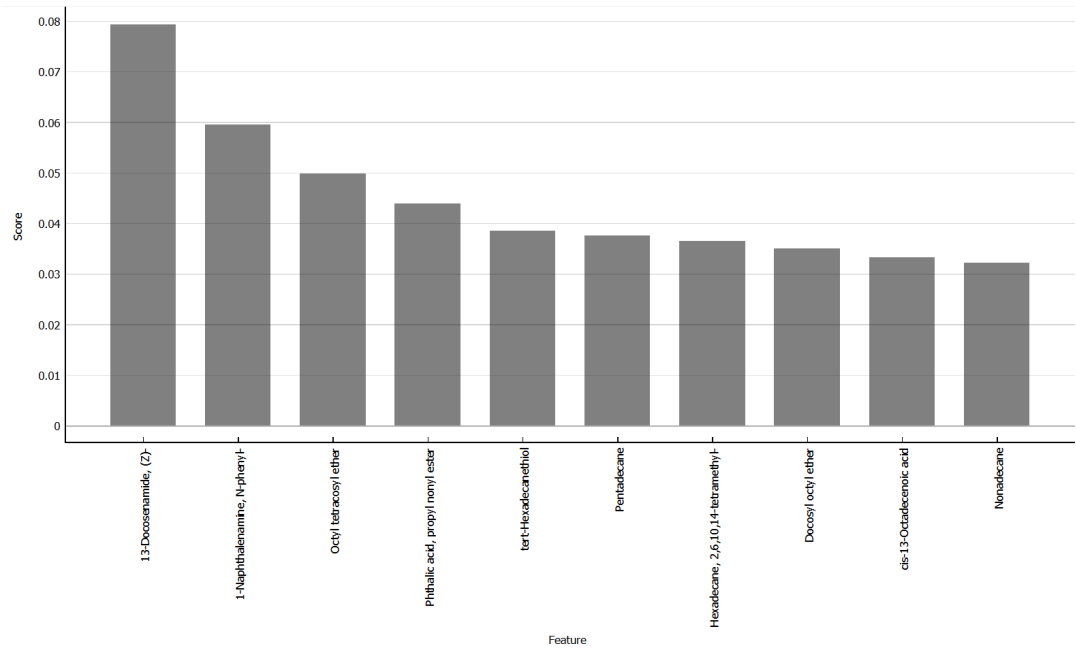


Figure 4. SHAP characteristics to discriminate ASD and matched-control samples were performed by Neural Network algorithm using salivary lipids.

DISCUSSION

The present study provides a pioneering analysis indicating that salivary lipids present the potential to be used as a novel platform for ASD detection. Additionally, we presented here that a state-of-the-art Python-based artificial intelligence algorithm can be used in the salivary lipids as a screening platform for ASD.

Currently, the application of questionnaires for ASD diagnosis presents some limitations regarding the difficulty of respondents in correctly interpreting the questions, especially due to the abstract and subjective nature of many of the behaviors and feelings assessed. This can lead to responses that do not accurately reflect the reality of the individual assessed. Furthermore, the complexity and heterogeneity involved in describing behaviors and subjective characteristics can result in vague or inconsistent answers, compromising diagnostic accuracy. Therefore, these questionnaires must be used in conjunction with other assessment methodologies, such as direct observations and clinical interviews, but mainly with potential biological biomarkers for ASD to ensure a comprehensive and effective diagnosis. The American Academy of Pediatrics recommended the application of standardized screening for ASD for all children at 18 and 24 months of age(3,8). A screening questionnaire with 20 questions, named Modified-Checklist for Autism in Toddlers, Revised (M-CHAT-R), is commonly applied as a screening tool for ASD in clinical settings for children aged between 16 to 30 months. When the score of M-CHAT-R is higher than 2, it is considered a risk for ASD, which demands additional analysis with clinicians and a multidisciplinary team(3,44). In the United Kingdom, The National Institute for Health and Care Excellence requires the application of Autism-Spectrum Quotient – 10 items (AQ-10) as a screening tool for adults based on clinical evaluation(3,5). Altogether, it indicates limitations in the application of questionnaires for ASD screening. This is due to the difficulty in correctly interpreting the questions and the complexity of providing clear responses based on subjective aspects. Therefore, the use of biomarkers or standardized spectral analyses for ASD could serve as alternative methods for ASD screening.

Some of the most prevalent tools for ASD diagnosis include the Autism

Diagnostic Interview, Revised (ADI-R) and Autism Diagnostic Observation Schedule, Second Edition (ADOS-2) with diagnostic conclusion based on DSM-5(1,3). A recent systematic review and meta-analysis indicate that the ADI-R presents a general sensitivity of 75% and specificity of 82% (and with 72% specificity in a subset of clinical settings) and the ADOS-2 presents a sensitivity of 90% and specificity of 83%(45). However, despite an apparent accuracy, it is still based on clinical judgment and the assessment of communication skills and speech capabilities can be influenced by behavioral and emotional issues. Furthermore, sensitivity and specificity values may vary depending on the clinical setting and the experience of the professionals administering the tests, affecting diagnostic accuracy. Another factor to consider is that the tests rely on reports from parents or caregivers, which can be influenced by their own perceptions and memories, potentially introducing biases. Additionally, these tests require considerable time to administer and interpret, which can be challenging in resource-limited settings. Although there are currently some genetic tests, in addition to being expensive, they are recommended after ASD diagnostics to provide data about recurrence risks and prognostic for specific genetic conditions(3).

Salivary proteomic analysis revealed significant differences in the expression of proteins including salivary neutrophil elastase, lactotransferrin, statherin, MCP-1, RANTES and histatins between individuals with ASD and neurotypical controls(46–48). These proteins are involved in inflammatory and immune response processes, suggesting a possible correlation with the immunological and neurodevelopmental disorders related to ASD. The transcriptomics of saliva from ASD subjects compared with neurotypical children showed changes in the expression of specific microRNAs, including up-regulation of MiR-29a-3p and miR-141-3p in saliva of ASD subjects, while down-regulation of miR-16-5p, let-7b-5p, and miR-451a in those ASD subjects. These microRNAs were related with neural signaling and inflammation pathways, representing potential biomarkers for diagnosis(49). Furthermore, analysis of the gut microbiome has shown significant differences in individuals with ASD(50). A panel of serum biomarkers related to the immune and inflammatory system have also been evaluated and identified(51). To our knowledge, this study is a pioneer in evaluating salivary lipid biomarkers using the lipidomics tools.

Three salivary lipids 1-Naphthalenamine, N-phenyl-; Hexadecanoic acid, 2-hydroxy-1- (hydroxymethyl)ethyl ester; and 13-Docosenamide, (Z)- were lower ($p < 0.05$) expressed in ASD than matched-controls. These three salivary lipids for ASD can be pointed out as trustworthy molecular markers in saliva as complementary biomarkers for the diagnosis. Although this discrimination should be validated in large-scale multicenter studies, from the screening application perspective, the accuracy of 84%, sensitivity 80%, and specificity of 80% using the Neural Network algorithm for 27 selected salivary lipids can be considered a remarkable potential for translational application in ASD detection using the salivary platform to ASD.

1-Naphthalenamine, Nphenyl-, an aromatic amine, shows potential neurotoxic interactions with proteins and brain receptors. For instance, modifications in N-(1,2,3,4-tetrahydronaphthalen-1-yl)-4-aryl-1-piperazinehexanamides, a derivative compound, have been studied for their affinity to different receptors, showing significant interactions with 5-HT₇ receptors, which are implicated in cognitive and memory functions(52). 1,2-Diacetylbenzene, a metabolite of naphthalene, has been shown to cause oxidative stress, thereby impairing neural progenitor cells and hippocampal neurogenesis. Although specific investigations related to this organic compound and autism are scarce, it is known that similar compounds can adversely influence neural development and signaling. It also was showed that 1-Naphthalenamine, N-phenyl- (NPNA) bound with a stable hydrophobic binding in a non-glycosylated domain of a high-molecular-weight mucin-glycoprotein (MGI) isolated from human submandibular/sublingual saliva. This binding can occur in MG1 due to the reduced presence of disulfide bridges, which do not occur in smaller mucin (MG2). The binding of NPNA with salivary mucin was independent of pH. Alternatively, this lipid can be detected with an excitation wavelength of 342 nm and an emission wavelength of 420 nm(53) or with fluorescent emission spectra of 440nm with maximum fluorescent changes by pH and some competitors(54).

Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, a monoester formed from glycerol and stearic acid, is relevant due to its role in the structure and functionality of neuronal membranes(55,56). Exogenous stearic acid is incorporated into the lipids of neurons and astrocytes, contributing to the synthesis and stability of neuronal membranes. This process involves the incorporation of stearic acid into phospholipids and other lipid structures in the

brain, indicating its essential role in membrane composition(55). The ester and ether linkages in phospholipids, affect the physical properties of membranes, such as their fluidity and flexibility, which are crucial for proper neuronal function(56). Besides, glycerol monostearate forms closely packed monolayers and is indicative of its role in maintaining membrane integrity and functionality(57). In that regard, monoesters formed from glycerol and stearic acid, such as hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, are integral to neuronal membranes' structure, stability, and functionality. Although there are no specific studies on its relationship with ASD, the roles in membrane composition, dynamics, and enzymatic processes are critical for maintaining neuronal health and function and may be related to ASD.

13-Docosenamide, derived from erucic acid, is a fatty acid amide that can affect neurotransmission, neuroprotection and helps in reducing oxidative stress and inflammation, which are crucial factors in neurodegenerative diseases(58–60) and preventing memory deficits. Erucic acid is capable of crossing the blood-brain barrier and is incorporated into specific lipid pools in the brain. This ability is crucial for its neuroprotective effects as it allows direct action on neural tissues(61). In addition, 13-Docosenamide and its derivatives can inhibit acetylcholinesterase, an enzyme involved in the breakdown of acetylcholine. This inhibition can enhance cholinergic neurotransmission, thereby improving cognitive function and providing neuroprotection(61). Studies have indicated abnormal fatty acid compositions in the red blood cells of children with ASD, including increased levels of certain fatty acids such as eicosenoic acid and erucic acid in those with developmental regression compared to typically developing controls(62,63). Moreover, imbalances in the levels of fatty acid amides are observed in individuals with ASD, implicating these molecules in the modulation of synaptic plasticity and neural communication, crucial aspects for brain development and functionality(64,65).

To the best of our knowledge, it is the pioneer detection of Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; and 13-Docosenamide in salivary

lipidomic analysis. This detection could be related to the lower expression of these salivary lipids in healthy subjects. Novel multicenter clinical studies can confirm the higher expression of both salivary lipids in ASD conditions, but these results are interesting to explore new avenues in the salivary screening of ASD using state-of-the-art Python-based artificial intelligence algorithms in salivary lipidomic spectra.

Overall, we observed changes in the expression of some salivary lipids in ASD subjects. Despite the restricted study setting, the present findings strongly suggest that some salivary lipids and a novel artificial intelligence algorithm based on salivary lipidomic spectra can be alternatively used as a screening platform for non-invasive ASD detection.

Therefore, lipid signaling dysfunction, which is crucial for neural development, has been observed in individuals with ASD and studies indicate that these individuals often exhibit altered metabolic profiles which may reflect or contribute to dysfunctions in energy metabolism and neurotransmitter production(66–68). Additionally, certain lipids are implicated in modulating inflammatory processes and oxidative stress responses, both recognized as contributing factors to ASD development(69). As fundamental components of cellular membranes, lipids are also essential for neurogenesis and synaptic plasticity(70). In this context, its reduction can affect several lipidomic pathways. Although research into the role of lipids in ASD is still ongoing, accumulating evidence suggests that abnormalities in their levels and functions may be associated with autism.

This study, although innovative in its application of salivary lipids as a potential platform for ASD detection, has limitations that must be considered. First, the representativeness of the sample may not encompass broader genetic and environmental diversity, potentially limiting the generalizability of the results. Additionally, interactions between biological variables not considered in this study, such as the influence of medications and other health conditions, may introduce additional variability to the results, requiring additional studies to validate and refine the proposed model.

The present cohort study shows three salivary lipids 1-Naphthalenamine, N- phenyl-; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; and 13- Docosenamide, (Z) with lower expression in ASD than neurotypical subjects. Besides, one state-of-the-art Python-based artificial intelligence discrimination using Neural Networks algorithms presents its best performance achieving 84%

of accuracy, 80% of specificity, and 88% of sensitivity with the highest SHAP score based in 13-Docosenamide, (Z). In summary, these data emphasize the potential of salivary lipidomics profile supported by artificial intelligence algorithms as an innovative non-invasive alternative for screening of ASD children, enabling advances in precision medicine and personalized dentistry. Overall, we observed changes in the expression of some salivary lipids in ASD subjects. Despite the restricted study setting, the present findings strongly suggest that some salivary lipids and a novel artificial intelligence algorithm based on salivary lipidomic spectra can be alternatively used as a screening platform for non-invasive ASD detection and enabling advances in precision personalized medicine. Future research is needed to elucidate the exact mechanisms involved and explore potential diagnostic or therapeutic applications based on these lipid findings.

CONFLICTS OF INTEREST

The authors declare interest in filling patent with this data.

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ANEXO 1 - COMITÊ DE ÉTICA



CENTRO UNIVERSITÁRIO DO
MARANHÃO - UNICEUMA



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ANÁLISE DA ESPECTROSCOPIA ATR-FTIR DE COMPONENTES DA SALIVA COMO FERRAMENTA DIAGNÓSTICA PARA O TRANSTORNO DO ESPECTRO AUTISTA

Pesquisador: Marjorie Adriane da Costa Nunes

Área Temática:

Versão: 2

CAAE: 15979719.7.0000.5084

Instituição Proponente: Centro Universitário do Maranhão - UniCEUMA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.597.769

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1293135.pdf	15/09/2019 21:55:39		Aceito
Cronograma	cronograma.pdf	15/09/2019 21:54:26	Marjorie Adriane da Costa Nunes	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termo_de_consentimento.pdf	15/09/2019 21:53:05	Marjorie Adriane da Costa Nunes	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termo_de_assentimento.pdf	15/09/2019 21:50:59	Marjorie Adriane da Costa Nunes	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_detalhado.pdf	15/09/2019 21:45:59	Marjorie Adriane da Costa Nunes	Aceito
Declaração de Pesquisadores	Declaracao_de_Pesquisadores2.pdf	15/09/2019 21:41:26	Marjorie Adriane da Costa Nunes	Aceito
Declaração de Pesquisadores	Declaracao_de_Pesquisadores.pdf	15/09/2019 21:40:39	Marjorie Adriane da Costa Nunes	Aceito
Recurso Anexado pelo Pesquisador	carta_resposta.pdf	15/09/2019 21:04:16	Marjorie Adriane da Costa Nunes	Aceito
Folha de Rosto	folha_De_rosto.pdf	14/06/2019 20:46:50	Marjorie Adriane da Costa Nunes	Aceito
Orçamento	18.pdf	31/03/2019 20:19:00	Marjorie Adriane da Costa Nunes	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_de_Instituicao_e_Infraestrutura.pdf	31/03/2019 20:17:43	Marjorie Adriane da Costa Nunes	Aceito

Situação do Parecer:

Aprovado

Necessita Avaliação da CONEP:

Não

SAO LUIS, 25 de Setembro de 2019