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Espectroscopia ATR-FTIR da saliva como ferramenta de triagem diagnóstica e avaliação do estresse oxidativo no transtorno do espectro autista

ATR-FTIR spectroscopy of saliva as a diagnostic screening tool and assessment of oxidative stress in autism spectrum disorder

Tese apresentada ao Programa de Pós-Graduação em Odontologia da Universidade CEUMA em associação com a Universidade Federal de Uberlândia para obtenção do título de Doutora em Odontologia

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Área de concentração: Odontologia Integrada

Orientador: Prof. Dr. Etevaldo Matos Maia Filho (Universidade Ceuma- MA)

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Tese em Odontologia

FOLHA DE APROVAÇÃO

Tese apresentada e defendida por Marjorie Adriane da Costa Nunes

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DEDICATÓRIA

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RESUMO

O Transtorno do Espectro Autista (TEA) pode ser definido como uma síndrome comportamental que compromete o desenvolvimento motor e psiconeurológico dificultando a cognição, a linguagem e a interação social da criança. Devido à heterogeneidade genética do TEA, os testes genéticos apresentam acurácia reduzida para estabelecer este diagnóstico. Desta forma, o diagnóstico do TEA é estabelecido com base em uma série de critérios comportamentais, de acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5), frequentemente aplicado após 5 anos. Nesta fase, as crianças com TEA já enfrentam severos problemas de convivência social e linguagem. Sabe-se que o aumento do estresse oxidativo salivar pode impactar na saúde oral, no entanto, esta abordagem não está estabelecida para o TEA. Neste contexto, esta pesquisa buscou investigar a aplicação da espectroscopia de reflexão total atenuada no infravermelho com transformada de Fourier (ATR-FTIR) em amostras de saliva como uma alternativa para o desenvolvimento de uma plataforma de diagnóstico de TEA baseada na composição molecular da saliva em protocolo sem utilização de reagentes, de forma rápida e custo reduzido. Adicionalmente, buscamos avaliar alterações da atividade de enzimas oxidativas e anti-oxidativas na saliva de indivíduos com TEA. Para tanto, realizou-se um Estudo Clínico Transversal e Analítico dos constituintes salivares de pacientes com TEA atendidos na cidade de São Luís (MA). Foram utilizados três grupos de pacientes: sujeitos neurotípicos, sujeitos com diagnóstico comprovado de TEA por laudo médico sem utilização de medicação controlada e sujeitos com diagnóstico de TEA comprovado por laudo médico com utilização de medicação controlada. O perfil salivar foi analisado por espectroscopia ATR-FTIR e os modos vibracionais foram avaliados quanto à capacidade diagnóstica por inteligência artificial. Neste estudo, utilizou-se a ferramenta ATR-FTIR associada aos algoritmos *Support Vector Machine* (SVM) e *Linear Discriminant Analysis* (LDA) para identificar o potencial da saliva como

método de triagem para detecção de TEA na saúde e indivíduos com TEA com e sem medicação. A melhor classificação dos espectros de infravermelho salivar pela máquina de vetor de suporte (SVM) mostrou uma sensibilidade de 70%, especificidade de 87% e acurácia de 82% entre indivíduos saudáveis e com TEA sem medicação. A melhor classificação dos espectros de infravermelho salivar pela Análise Discriminante Linear (LDA) mostrou sensibilidade de 61%, especificidade de 80% e acurácia de 72% entre indivíduos saudáveis e TEA com medicação. A concentração de proteínas totais e a atividade da enzima superóxido dismutase (SOD) foi, respectivamente, diminuída e aumentada na saliva de sujeitos com TEA em comparação com neurotípicos. Estes parâmetros foram semelhantes aos neurotípicos em sujeitos com TEA com utilização de medicação. Em resumo, esses dados destacam o potencial das plataformas ATR-FTIR, juntamente com o aprendizado de máquina, como uma ferramenta sustentável, livre de reagentes, não invasiva e altamente sensível para triagem de indivíduos com TEA usando um volume ultrabaixo de saliva e preparação mínima de amostras. As alterações de composição salivar e atividade da SOD na saliva indicam que mais estudos clínicos devem ser realizados na população TEA para avaliar o impacto destas alterações na saúde oral.

Palavras chave: Autismo; saliva; ATR-FTIR; biomarcador e diagnóstico, estresse oxidativo

Nunes, M.A.C. ATR-FTIR spectroscopy of saliva as a diagnostic screening tool and assessment of oxidative stress in autism spectrum disorder [Tese de Doutorado em Odontologia]. São Luís. Universidade CEUMA em associação com a Universidade Federal de Uberlândia; 2022.

ABSTRACT

Autism Spectrum Disorder (ASD) can be defined as a behavioral syndrome that compromises motor and psychoneurological development, hindering the child's cognition, language and social interaction. Due to the genetic heterogeneity of ASD, genetic tests have reduced accuracy in establishing this diagnosis. In this way, the diagnosis of ASD is established based on a series of behavioral criteria, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), often applied after 5 years. At this stage, children with ASD already face severe problems with social coexistence and language. It is known that increased salivary oxidative stress can impact oral health, however, this approach is not established for ASD. In this context, this research sought to investigate the application of Fourier transform infrared attenuated total reflection spectroscopy (ATR-FTIR) in saliva samples as an alternative for the development of an ASD diagnostic platform based on the molecular composition of saliva in protocol without the use of reagents, quickly and at reduced cost. Additionally, we sought to evaluate changes in the activity of oxidative and anti-oxidative enzymes in the saliva of individuals with ASD. Therefore, a Cross-sectional and Analytical Clinical Study was carried out on the salivary constituents of patients with ASD treated in the city of São Luís (MA). Three groups of patients were used: neurotypical subjects, subjects with a confirmed diagnosis of ASD by medical report without the use of controlled medication and subjects with a diagnosis of ASD confirmed by a medical report with the use of controlled medication. The salivary profile was analyzed by ATR-FTIR spectroscopy and the vibrational modes were evaluated for diagnostic capacity by artificial intelligence. In this study, the ATR-FTIR tool associated with the Support Vector Machine (SVM) and Linear Discriminant Analysis (LDA) algorithms was used to identify the potential of saliva as a screening method for the detection of ASD in health and individuals with ASD with and without medication. The best classification of salivary infrared spectra by the support vector machine (SVM) showed a sensitivity of 70%, specificity of 87% and accuracy of 82%

among healthy subjects and those with ASD without medication. The best classification of salivary infrared spectra by Linear Discriminant Analysis (LDA) showed sensitivity of 61%, specificity of 80% and accuracy of 72% between healthy individuals and ASD with medication. Total protein concentration and superoxide dismutase (SOD) enzyme activity were respectively decreased and increased in the saliva of subjects with ASD compared to neurotypicals. These parameters were similar to the neurotypical ones in subjects with ASD using medication. In summary, these data highlight the potential of ATR-FTIR platforms, together with machine learning, as a sustainable, reagent-free, non-invasive, and highly sensitive tool for screening individuals with ASD using an ultra-low volume of saliva and minimal preparation. of samples. Changes in salivary composition and SOD activity in saliva indicate that further clinical studies should be performed in the ASD population to assess the impact of these changes on oral health.

Key words: Autism; saliva; ATR-FTIR; biomarker and diagnosis

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1 INTRODUÇÃO

O Transtorno do Espectro Autista (TEA) pode ser definido como uma síndrome comportamental que compromete o desenvolvimento motor e psiconeurológico dificultando a cognição, a linguagem e a interação social da criança, podendo ser resultante de efeitos de diversos fatores biológicos e/ou fatores psicológicos, incluindo fatores genéticos, fatores ambientais e interações gene/ambiente[1,2].

O transtorno agora é amplamente aceito como uma condição complexa, abrangente e heterogênea com múltiplas etiologias, subtipos e trajetórias de desenvolvimento. Desta forma, a identificação da etiologia em cada paciente e, por consequência, o aconselhamento genético das famílias é complexo[3]. Estudos recentes pontuam como fatores de risco: aumento da idade materna e paterna; prematuridade e baixo peso ao nascer e estresse materno gestacional [2,4].

A prevalência de pessoas com TEA vem aumentando progressivamente ao longo dos anos. Segundo o Centro de Controle e Prevenção de Doenças (CDC). Em 2004, o número de crianças com TEA aos oito anos de idade era de 1 a cada 166. Em 2012, esse número era de 1 para 88. Já em 2018, passou a 1 em 59. Na última publicação do CDC americano de 2021, a prevalência de TEA é de 1 a cada 44 crianças[5].

Atualmente, o diagnóstico do TEA tem permanecido como uma tarefa desafiadora. Para melhorar a precisão e confiabilidade dos diagnósticos de autismo, muitos estudiosos têm desenvolvido métodos de triagem pré-diagnóstico para ajudar a identificar comportamentos autistas em um estágio inicial[6].

Desta forma, o diagnóstico é estabelecido com base em uma lista de critérios comportamentais, de acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5). Além disso, considera-se que os sintomas devem estar presentes no início da infância, mas podem não se manifestar completamente até que as demandas sociais excedam o limite de suas capacidades[7].

Sendo assim, o TEA é, de maneira geral, diagnosticado apenas após a idade média de cinco anos. Entretanto, nessa fase, as crianças dentro do espectro autista já enfrentam severos problemas de convivência social e linguagem, estabelecimento de comportamentos repetitivos e rotinas não funcionais [8].

O Diagnóstico precoce de crianças com TEA é importante para garantir que estas recebam os serviços e terapias de suporte necessárias para atingir todo o seu potencial [9]. E, além disso, melhorar a compreensão do TEA para as diferentes partes interessadas envolvidas, como pais, cuidadores, professores e familiares[6].

Uma variedade de escalas e instrumentos de triagem e avaliação padronizados vêm se mostrando ferramentas úteis e necessárias, que podem contribuir para encaminhamentos para diagnóstico no mundo todo. Cada uma com uma aplicação específica, dependendo da finalidade e da idade da criança[10]. Questões relacionadas ao processo de tradução e validação para uso no Brasil e o pagamento de direitos autorais aos autores e editores responsáveis, restringem o uso destes instrumentos aqui no Brasil[11].

O uso da saliva como método diagnóstico, em detrimento de outros que impliquem técnicas invasivas, torna-se ainda mais relevante no TEA devido sua ampla reatividade sensorial e comportamental que podem tornar problemática a coleta de amostra. Desta forma, a saliva tem emergido como uma alternativa viável para obter informações biológicas e pode ter um papel ainda mais relevante na triagem diagnóstica de crianças com TEA[12].

A saliva pode ser coletada de forma fácil, indolor, com desconforto reduzido, de forma não-invasiva, é de fácil manipulação e armazenamento, menor risco de contaminação quando comparado ao sangue, não necessita de equipamentos e profissionais especializados para a sua coleta, pode ser coletada com mais frequência, além de ser possível realizar a coleta em todas faixas etárias[13].

A espectroscopia de infravermelho (IR) vem despontando como uma técnica quantitativa e qualitativa, potencial, para caracterização de compostos biológicos, podendo ser utilizada como alternativa sustentável para o diagnóstico de doenças orais e sistêmicas[14]. Esta tecnologia é considerada uma ferramenta valiosa para a determinação da estrutura de moléculas, devido à sua sensibilidade, à composição química e arquitetura das moléculas; pode ser aplicada na investigação da estrutura da proteína e do mecanismo molecular das reações proteicas, e ainda em sistemas biológicos maiores que proteínas, como exemplo temos a possibilidade de identificar cepas bacterianas do espectro infravermelho e diferenciar e classificar microrganismos[15].

Dentre as técnicas atualmente estudadas, as análises de espectrometria no infravermelho com transformada de Fourier (ATR-FTIR) são de especial interesse devido à sua alta sensibilidade na detecção de alterações bioquímicas e moleculares em amostras biológicas[16]. Possui ainda como vantagens a capacidade de diagnosticar de forma rápida, não-invasiva, livre de reagentes, volume muito reduzido de amostra e alta especificidade[17].

As análises dos espectros no infravermelho podem ser realizadas de diferentes maneiras, sendo as mais utilizadas a de transmissão, transflexão e reflexão total atenuada (ATR). Medidas realizadas no ATR não necessitam de substratos especiais e nenhuma ou pouca preparação da amostra. Desta forma, o material estudado (sólido ou líquido) deve ser colocado em contato direto com o elemento de reflexão interna, tornando o processo de análise mais rápido e simples, tendo ampla aplicabilidade para o diagnóstico de desordens patológicas[18,19].

O TEA também tem sido associado ao aumento da produção de Espécies Reativas de Oxigênio (ROS) e menor capacidade antioxidante. As ROS constituem os radicais livres ou ânions e moléculas reativas que contém átomos de oxigênio, tais como o radical hidroxila (OH), ânion superóxido ($O2^{-}$), peróxido de hidrogênio (H2O2) e peroxinitrito ($ONOO^{-}$)[20,21]. Em organismos aeróbios saudáveis, existe um balanço entre a produção de espécies reativas e as defesas antioxidantes. Entretanto, o excesso de ROS pode alterar este equilíbrio, levando ao processo conhecido como estresse oxidativo[22].

Fatores de risco ambientais e genéticos podem intensificar a vulnerabilidade ao estresse oxidativo no autismo. O aumento do estresse oxidativo no autismo pode contribuir para o desenvolvimento dessa alteração tanto em termos de patogênese quanto de sintomas clínicos[20].

Dentre as consequências mais importantes do estresse oxidativo, está a peroxidação dos lipídios das membranas celulares, que pode resultar em alterações na fluidez da membrana e ainda interferir na permeabilidade de íons, comprometendo assim a capacidade de seletividade celular para a entrada e saída de diferentes compostos [23].

O processo de oxidação proteica também é relatado como uma importante consequência do estresse oxidativo, e pode ser mensurado por meio da quantificação de proteínas carboniladas [24]. As ROS podem danificar as proteínas através da reação direta com elas ou por meio indireto, através de seus produtos secundários gerados por reações com outras moléculas, como os lipídeos e açúcares. Essas reações de oxidação promovem modificações que podem acarretar na perda da função estrutural ou catalítica da proteína, além de torná-las mais susceptíveis a degradação proteolítica [24,25].

Nossa hipótese é de que o ATR-FTIR é capaz de identificar alterações nos componentes salivares para ser usado como ferramenta de triagem diagnóstica para

o TEA e que a atividade de enzimas salivares relacionadas ao estresse oxidativo também estejam alteradas. O presente estudo teve como objetivo analisar o perfil do espectro infravermelho da saliva em indivíduos neurotípicos saudáveis comparando com indivíduos com TEA não tratados e tratados para desenvolver uma plataforma sustentável, livre de reagentes e de forma não-invasiva para triagem diagnóstica do TEA e adicionalmente avaliar alterações da atividade de enzimas oxidativas e anti-oxidativas na saliva de indivíduos com TEA.

2 CAPÍTULO 1

SALIVARY ATR-FTIR SPECTROSCOPY COUPLED WITH LEARNING MACHINE ALGHORITMS FOR SCREENING OF AUTISM SPECTRUM DISORDER

ABSTRACT

The Autism spectrum disorder (ASD) diagnostic process is based on informed current symptomatology, a developmental history provided by a caregiver of children associated with an expert clinical review of behavioral symptoms. We hypothesize that the application of saliva in ATR-FTIR technology coupled to machine learning techniques could be used as a screening method for ASD detection. In this study, we used the ATR-FTIR tool associated with the support vector machine (SVM) and Linear Discriminant Analysis (LDA) algorithms in order to identify the potential of saliva as a screening method for ASD detection in health and ASD subjects with and without medication. The best classification of salivary infrared spectra by support vector machine (SVM) showed a sensitivity of 70%, specificity of 87%, and accuracy of 82% between healthy and ASD subjects without medication. The best classification of salivary infrared spectra by Linear Discriminant Analysis (LDA) showed a sensitivity of 61%, specificity of 80%, and accuracy of 72% between healthy and ASD subjects with medication. In summary, these data highlight the potential of ATR-FTIR platforms coupled with machine learning as a sustainable, reagent free, non-invasive, and highly sensitive tool to screening ASD subjects using an ultra-low volume of saliva and minimal sample preparation.

Keywords: ASD, ATR-FTIR, SVM, salivary biomarkers, diagnosis

1. Introduction

Autism spectrum disorder (ASD) is characterized by early-appearing deficits in communication and socialization frequently accompanied by uncommon and/or repetitive social-sensory–motor behaviors associated with a strong genetic component as well as other causes[1]. ASD presents several genetic and non-genetic causes, and the prevalence is about 1 in 44 children based on the diagnostic and statistical manual of mental disorders, 5th Edition (DSM-5)[2]. It described some disparities in access to the ASD diagnostic services for communities. It points out the importance of the development of novel strategies to provide equitable access to ASD detection, allowing rapid support and therapy to improve neural development and quality of life [3].

Actually, the ASD diagnostic process is based on informed current symptomatology, a developmental history provided by a caregiver of children associated with an expert clinical review of behavioral symptoms[4]. Several factors can explain the delay in ASD diagnosis, frequently it occurs due to inadequate parental perception, and delayed medical appointments[5]. The severity of autistic behavior such as social, sensory, or motor behaviors was related to late ASD diagnosis improvement [5].

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy has been applied to discriminate control and pathological samples[6]. This label-free technology can detect the molecular fingerprint related to lipids, peptides, proteins, carbohydrates, and nucleic acids[7]. Although the analysis of saliva in ATR-FTIR platform is incipient in ASD children, the application of saliva in ATR-FTIR permits a protocol with several advantages as non-invasive collection, reagent free, sustainable and fast anlysis[8]. Some studies described changes in saliva of AS children. In this context, salivary nitrite and immunoglobulin G4 were higher and in ASD children compared to controls[9]. On the other hand, salivary alpha-synuclein concentration was lower in ASD children[10].

Here, we hypothesized that ATR-FTIR is capable to identify changes in salivary components to be used as a screening tool for ASD. The present study aimed to analyze infrared salivary components in healthy neurotypical subjects comparing with non-treated and treated ASD subjects to develop a sustainable, label-free, and non-invasive platform for ASD screening.

2. Materials and methods

2.1 Ethical Aspects and Study Subjects

All experimental procedures were conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the Ethical Board of the CEUMA University (protocol number 3.597.769). Written informed consent was obtained from all the participants of this study including healthy and ASD subjects.

The studied population in this traversal study were recruited from the health centers in Sao Luiz, Maranhão, Brazil. The diagnostic of ASD were analyzed and confirmed by clinical experts according to the DSM-5 [2,3]. The present study was performed in 92 subjects: 42 healthy controls, 20 ASD subjects without medication and 30 ASD subjects with medication. The neurotypical subjects present typically developed children without signs of other neurodevelopmental disorders. The neurotypical children were recruited in a similar geographic area to minimize environmental influences. The exclusion criteria for both neurotypical and ASD children included the presence of oral or systemic infection or presence of other disease 30 days previous the sample collection.

2.2 Saliva Sample Collection and Preparation

Saliva collection was performed standing or sitting, according to the preference of each child, supported by the caregiver responsible for the child. Saliva was collected by means of negative pressure performed with a 3 ml syringe in the sublingual region. Then, the saliva was transferred to eppendorf tubes for storage at -20ºC. removal from the sublingual region or else, many of them threw the saliva to the tip of the tongue and in both cases 3ml disposable syringes were used to aspirate the saliva and then it was transferred to the eppendorf tube. The saliva was recovered by centrifugation for 3000 rpm at 4ºC for 15 minutes, and the supernatant was collected and aliquoted. All samples were kept frozen at -80ºC until analysis.

2.3 ATR-FTIR Spectroscopy

To obtain the salivary spectra this biofluid was analyzed in 4000-400cm^{-1} using ATR-FTR spectrophotometer coupled to attenuated total reflectance (ATR)

component (Agilent Cary 630 FTIR, Agilent Technologies, Santa Clara, CA, USA). The crystal in ATR unit is a diamond disc as an internal reflection element. The salivary pellicle penetration depth ranges between 0.1 and 2 μm depending on the wavelength, incidence angle of the beam, and the refractive index of ATR-crystal material. Saliva (1μl) was directly dried at room temperature on ATR-crystal for 5 minutes before spectra collection. Previous to each sample analysis, the atmospheric air spectrum was used as a background. Each spectrum was obtained in a room with a temperature around 22–23°C, 4 cm^{-1} of resolution, and 32 scans were performed.

2.4 Discrimination Analysis Method

The infrared spectral data analysis was divided into two stages: pre-processing and classification. Pre-processing consisted of aggregation, attribute selection, and data transformation. The arithmetic mean of the three spectral readings of each patient was performed in aggregation. The spectral data were truncated with the lipidic region (3050-2800) associated with the biofingerprint region (1800-900 cm^{-1}). Then, the Savitzky-Golay smoothing filter was applied to each spectrum followed by a first order derivative and pre-processed by vector normalization.

The classification was tested with state-of-the-art machine learning algorithms of feature extraction coupled to discriminant analysis tools. The Support Vector Machine (SVM) was selected based on better results during model training. To analyze the predictive performance of the machine learning algorithms, ten times stratified cross-validation was used. The samples were divided into ten subsets, with each iteration; nine of them were used to train the algorithm and one exclusively to test it, so that each subset was part of the test once. In addition, the procedure was repeated three times with changes in the samples configurations in these subsets to achieve a closer estimate of the real performance of the model, thus totaling thirty executions. To measure the results obtained, three performance measures consolidated in the literature were used: sensitivity, specificity, and accuracy. The sensitivity or true positive rate is the proportion of ASD subjects that were correctly classified, and the specificity or true negative rate is the proportion of healthy neurotypical subjects that were correctly classified. The accuracy is defined as the total number of samples correctly classified in sensitivity and specificity

3. Results

Demographic and metabolic laboratorial data of neurotypical and ASD subjects with or without medication for your treatment are described in table 1 and table 2.

Table 1. Gender and mean age distribution of healthy, ASD subjects with or without medication.

	ASD without medication	ASD with medication	Neurotypical
Female	(15,0%)	(36, 7%)	(33,3%)
Male	(85,0%)	(63,3%)	(66,7%)
Age (Mean±SD)	8,85 (±8,14)	8,56 (±2,89)	8,02 (±2,04)

Table 2. Racial distribution of healthy, ASD subjects with or without medication.

	ASD without medication	ASD with medication	Neurotypical
Brown	14 (50,0%)	21 (70,0%)	30 (71,4%)
Black	4 (20%)	6 (20,0%)	7 (16,7%)
White	1 (5,0%)	3 (10,0%)	3 (7,1%)
Indigenous	1 (5,0%)	0 (0,0%)	2 (4,8%)

The mean infrared salivary spectra of neurotypical subjects, ASD subjects with medication and ASD subjects without medication were shown in Figure 1A and Figure 1B, respectively.

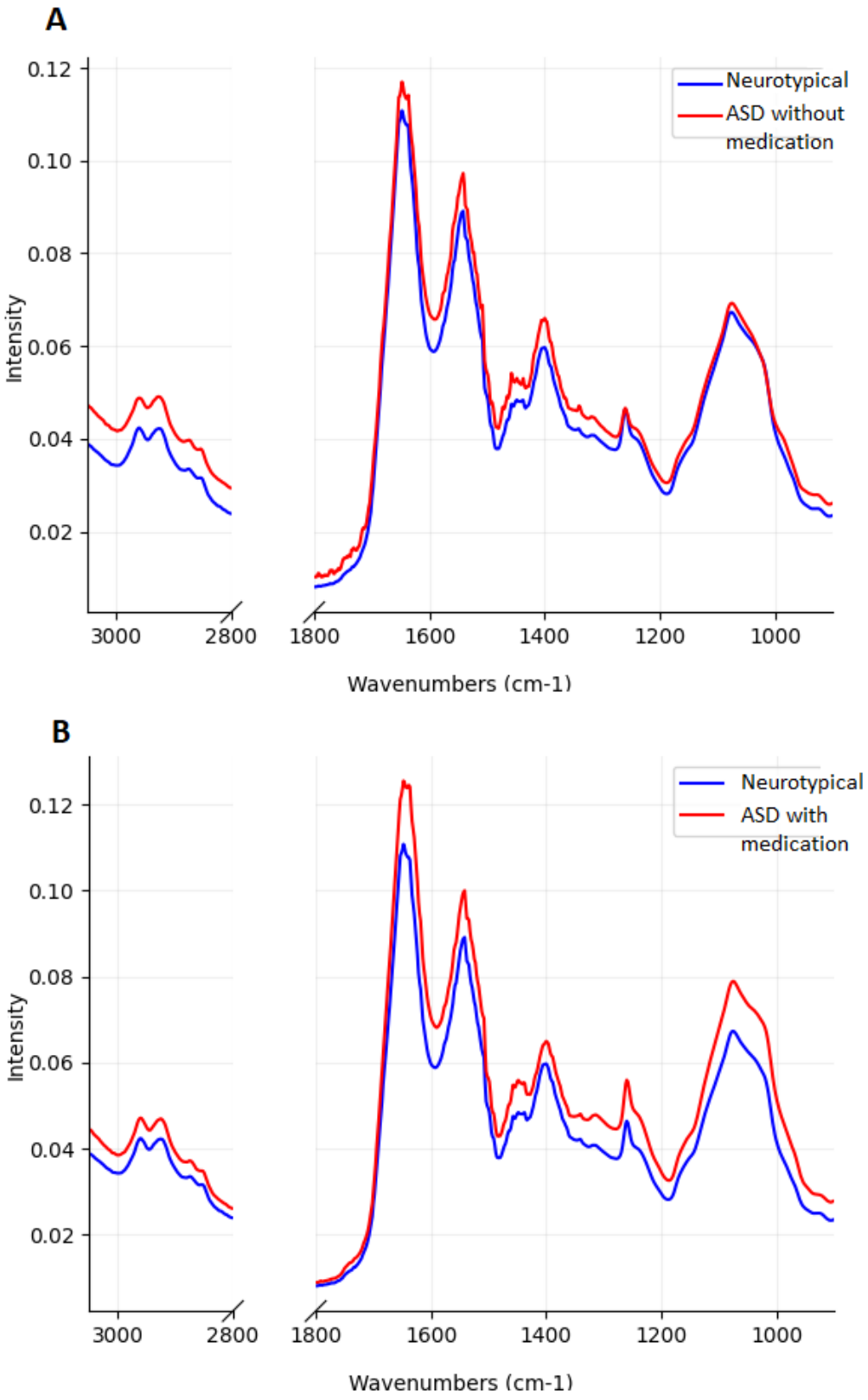

Figure 1. Representative average ATR-FTIR spectra (4000–400 cm-1) in saliva of neurotypical, ASD subjects without medication (A) or with medication (B).

Table 3. Learning Machine algorithms classification to discriminate ASD subjects with or without medication from neurotypical subjects.

Algorithm	Dataset	Pre-processing (band)	Sensitivity	Specificity	Accuracy
Linear Discriminant Analysis	Neurotypical x ASD without medication	Raw data (1800-900cm-1; 3050-2800cm-1)	0,59	0,91	0,81
		Rubberband+amida1 (1800-900cm-1; 3050-2800cm-1)	0,57	0,84	0,75
		1st deriv, Savgolay (1800-900cm-1; 3050-2800cm-1)	0,48	0,71	0,64
	Neurotypical x ASD with medication	Raw data (1800-900cm-1; 3050-2800cm-1)	**0,61**	**0,80**	**0,72**
		Rubberband+amida1 (1800-900cm-1; 3050-2800cm-1)	0,55	0,69	0,63
		1st deriv, Savgolay (1800-900cm-1; 3050-2800cm-1)	0,55	0,56	0,55
Support Vector Machine	Neurotypical x ASD without medication	Raw data (1800-900cm-1; 3050-2800cm-1)	**0,70**	**0,87**	**0,82**
		Rubberband+amida1 (1800-900cm-1; 3050-2800cm-1)	0,54	0,77	0,70
		1st deriv, Savgolay (1800-900cm-1; 3050-2800cm-1)	0,63	0,85	0,78
	Neurotypical x ASD with medication	Raw data (1800-900cm-1; 3050-2800cm-1)	0,62	0,61	0,62
		Rubberband+amida1 (1800-900cm-1; 3050-2800cm-1)	0,64	0,73	0,69
		1st deriv, Savgolay (1800-900cm-1; 3050-2800cm-1)	0,55	0,69	0,63

As described in table 3, the best classification of salivary infrared spectra by support vector machine (SVM) showed a sensitivity of 70%, specificity of 87%, and accuracy of 82% between healthy and ASD subjects without medication. The best classification of salivary infrared spectra by Linear Discriminant Analysis (LDA) showed a sensitivity of 61%, specificity of 80%, and accuracy of 72% between healthy and ASD subjects with medication.

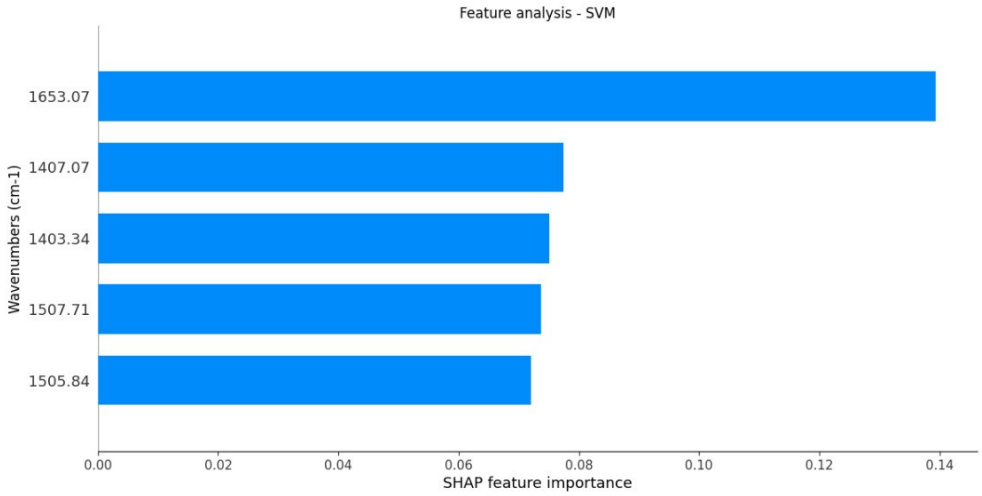

Figure 2. Selected wavenumbers used in the Learning Machine algorithms classification.

As described in figure 2, the vibrational modes at 1653 cm^{-1}, 1407 cm^{-1}, 1403 cm^{-1}, 1507 cm^{-1} and 1505 cm^{-1} were the main vibrational modes used in the best classification by support vector machine (SVM) algorithm to discriminate salivary infrared spectra from neurotypical and ASD children.

4. Discussion

Based in the present sample, the results support the tested hypothesis. The ASD diagnosis should be improved when a valid biomarker can be applied to substitute the laborious diagnostic process based on behavioral analysis. The biophotonic analysis could be an alternative to analyze several types of components as proteins, carbohydrates, lipids, and DNA/RNA in biofluids, which can contribute to the development of a universal and more precise diagnostic or screening platform [11, 12, 13]. This biophotonic salivary test can reduce the anguish of the lengthy process of releasing results and facilitate the early intervention program improving long-term positive effects on neurological behavior into adulthood[14]. The reduced size of ATR-FTIR platforms in recent years using non-invasive saliva samples can be an outstanding opportunity to increase the tests in general medical practice, decentralized laboratorial settings [13,15] and dental clinics using a reagent-free, sustainable, non-invasive, and fast platform to detect changes in salivary components of ASD population.

The salivary discriminatory vibrational modes of ASD population compared to neurotypical subjects were related to: α-helix amide I (1653 cm^{-1})[16], stretching symmetric COO^- in fatty acids (1407 cm^{-1})[16], Symmetric CH3 bending modes of the methyl groups of proteins (1403 cm^{-1}) [17]; CH bending vibration from the phenyl rings (1507 cm^{-1} and 1505 cm^{-1})[17] In this context, the unsupervised PCA-based model in blood samples in ASD and neurotypical population indicates changes in protein profile using ATR-FTIR platform without a in-depth indication of specific vibrational modes used in the discrimination[18].

A briefly overview about these tentative assignments indicates that Amide I is frequently detected in salivary proteins and this vibrational was related to salivary α-Amylase, Cystatins, Mucins, Proline-rich proteins and albumin[19, 20, 21]. Several esterified and non-esterified fatty acids were detected in human saliva [22,23]. The symmetric CH3 bending were also with unspecified salivary proteins[24]. A phenyl rings attached to alanine is related to phenylalanine, which is a an amino acid detected in saliva with concentration of 61 mol/L [25].

Here, the best classification of the support vector machine (SVM) algorithm presents a sensitivity of 70%, specificity of 87%, and accuracy of 82% between neurotypical and ASD subjects without medication. From the perspective of the screening application, the sensitivity of 70% with a specificity of 87% can serve as an expressive translational impact to develop a non-invasive screening platform to ASD. The application of the SVM in ASD subjects with medication showed a reduction in the accuracy, indicating the potential role of drugs to change the salivary spectra. However, an LDA algorithm was more effective to discriminate ASD subjects with medication from neurotypical subjects presenting a sensitivity of 61%, specificity of 80%, and accuracy of 72%

The development of novel screening platforms with suitable accuracy can be relevant to permits early intervention services and/or early treatment in very young children[26]. It is also expected that this screening platforms can improve the slow diagnostic process based on behavioral analysis and reduce medical and psychologist costs[26]. In this context, an ATR-FTIR is a eco-friendly platform from the perspective of sustainable development using non-invasive with only 1 uL of saliva and minimal sample preparation, allowing the release of the result quickly.

This preliminary clinical study suggests a potential of ATR-FTIR towards salivary screening platform for ASD. Although the higher incidence of ASD recorded

diagnoses were also related to a improvement in the provision for ASD diagnosis in several healthcare systems worldwide, novel platforms are critical to improve several barriers to improve public health issues related with this disorder[27]. Novel studies with large population are necessary to validate these encouraging results, in this sense it is establish that frequently machine learning algorithms improve the accuracy with large number of samples. Thus, the salivary biophotonic infrared spectroscopy coupled with learning machine discrimination could provide a promising alternative for novel screening to ASD.

5. Conclusion

Here, we suggested the vibrational modes at 1653 cm^{-1}, 1407 cm^{-1}, 1403 cm^{-1}, 1507 cm^{-1}, and 1505 cm^{-1} with higher potential to discriminate ASD from neurotypical subjects. the best classification of salivary infrared spectra by support vector machine (SVM) showed a sensitivity of 70%, specificity of 87%, and accuracy of 82% between healthy and ASD subjects without medication. The best classification of salivary infrared spectra by Linear Discriminant Analysis (LDA) showed a sensitivity of 61%, specificity of 80%, and accuracy of 72% between healthy and ASD subjects with medication. In summary, these data suggest ATR-FTIR platforms coupled with machine learning algorithms as a emergent tool to screening ASD population in a manner which permits reagent free, non-invasive, eco-friendly analysis and allowing the release of the result quickly.

6. Conflicts of interest

The authors declare that they have no conflicts of interest.

7. Acknowledgements

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2 CAPÍTULO 2

OXIDATIVE STRESS PARAMETERS IN SALIVA OF AUTISM SPECTRUM DISORDER

ABSTRACT

The detection of autism spectrum disorder (ASD) has been increased worldwide. The salivary oxidative status of redox balance was not evaluated in ASD subjects. In this context, the aim of this study was to evaluate salivary changes in the redox status and biomarkers of oxidative stress of ASD children without or on drug therapy comparing with neurotypical children. Saliva was collected from 79 participants divided in: 30 neurotypical controls, 19 ASD subjects without medication and 30 ASD subjects with medication. Our results indicate that occur an increase in the total antioxidant capacity of saliva related to parallel increase in salivary SOD activity in ASD children on drug therapy comparing with neurotypical children. Interestingly, only the salivary SOD activity was higher in ASD children without drug therapy compared with neurotypical children, suggesting an antioxidant response related to the ASD condition. Besides, salivary proteins in ASD children without drug therapy were lower compared with neurotypical controls, this effect was reversed in ASD children with drug therapy. Although this study was carried out with an intermediate number of samples with innovative data on the balance of oxidant and antioxidant system, the present findings should be confirmed in larger-scale studies.

1. Introduction

Autism Spectrum Disorder (ASD) has been defined as a heterogeneous neurodevelopmental syndrome clinically characterized by impaired social communication, difficulty in interacting with other people, presence of restricted and repetitive behaviors [1,2]. Taken together, its symptoms represent in most cases a lifelong disorder[3]. The incidence of ASD has shown a significant increase in recent decades. Recent studies indicate that the prevalence is 1 in 44 children in the United States[4] and it has been detected in 1 in 110 children worldwide, suggesting problems with the adequate diagnostic. ASD can occur in all racial, ethnic and socioeconomic

groups. The frequency of diagnosis is higher in boys, with a ratio of four boys to one girl[4] . However, it is important to highlight that this prevalence may have its limitations and differences due to the low diagnostic capacity and complexity, especially in developing countries[5].

Currently, according to the World Health Organization (WHO), ASD is now included as a unified diagnosis by the new International Classification of Diseases and Related Health Problems, ICD-11. The new version of the classification unites all diagnoses that were previously classified as Pervasive Developmental Disorders (PDD) as ASD[6]. In addition, the subdivisions became related to impairments in functional language and intellectual disability[6]. Thus, it allows therapies to be more effective in reducing disabilities in ASD[7].

Despite the efforts and advances, the etiology of ASD still remains poorly understood and, therefore, there are currently no clinical biomarkers for ASD[8]. Several genetic, epigenetic, and environmental factors have been associated with an increased risk of developing ASD[9,10]. Among the evidences associated with the pathophysiology of ASD, immune dysregulation and inflammation, mitochondrial dysfunction and oxidative stress have been highlighted[8,11]. Endogenous oxidative stress in response to exposure to environmental factors plays an essential role in several human diseases[12] and it is also presumed to be involved in the clinical manifestations of ASD[13-15].

The development of ASD by oxidative stress may be due to post-translational alterations of proteins, abnormal metabolism and toxic accumulation of reactive oxygen species (ROS)[8]. Several oxidative stress biomarkers presents in urine, blood were higher in ASD. Besides, the oxidative stress were also observed in specific regions in brains of ASD population [15-18]. Brain inflammation and behavioral changes involved in ASD were related to decreased cellular antioxidant defense mechanisms and increased oxidative stress as a contribution to [8,18]. Levels of ROS scavenging enzymes in the blood such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) are at lower concentrations in subjects with ASD compared with neutypical controls[19]. Increased oxidation of DNA, lipids and proteins were also observed in brain tissue, plasma and urine, with a decrease in redox homeostasis [15-18].

To the best of our knowledge, the salivary oxidative status of redox balance were not evaluated in ASD subjects. Saliva is a potential fluid for the evaluation of

biomarkers in the medical area, due to the presence of a wide range of molecular components with a non-invasive and painless collection [20]. Furthermore, the activity levels of salivary enzymes involved in redox balance could be related with changes in oral health [21]. In this context, the aim of the present study was to evaluate changes in the redox status and biomarkers of oxidative stress in the saliva of ASD children without or on drug therapy comparing with neurotypical children.

2. Methods

2.1 Ethical Aspects and Study Subjects

All experimental procedures were conducted in accordance with the Declaration of Helsinki. This protocol was approved by the Ethical Board of the CEUMA University (#3.597.769). Written informed consent was obtained from all the participants of this study including healthy and ASD subjects. Each neurotypical and ASD subject or the person who is legally responsible signed the approved written informed consent voluntarily. The 79 participants were divided in: 30 neurotypical controls, 19 ASD subjects without medication and 30 ASD subjects with medication. This ASD classification was performed according to the DSM-5 by a medical expert. The description of oral or systemic infection or yet the presence of other disease 30 days previous the sample collection was considered an exclusion criterion for both neurotypical and ASD children included

2.2 Saliva Sample Collection and Preparation

Saliva collection was performed in accordance with preference of each child and supported by the caregiver standing or sitting. Saliva was collected using a 3 ml syringe in the sublingual region by negative pressure. After that, the saliva was recovered by centrifugation for 3000 rpm at 4ºC for 15 minutes, and the supernatants were kept frozen at -80ºC until analysis.

2.3 *Oxidative stress biomarker analysis*

Total protein was performed using the Bradford method and a bovine serum albumin curve [22].

Total antioxidant capacity due to ferric-reducing antioxidant power (FRAP): Total antioxidant capacity was evaluated by the capacity of the samples to reduce Fe^{+3} to Fe^{+2}, which was then chelated by TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) in order to form the deep-blue colored Fe^{+2}-TPTZ complex [23]. This complex was measured in a spectrophotometer at 593 nm.

Superoxide dismutase (SOD) activity: SOD activity was measured by the inhibition autoxidative capacity of pyrogallol. The SOD activity was evaluated using a spectrophotometer at 420 nm. A calibration curve was constructed using SOD as standard. A 50% inhibition of autoxidation of pyrogallol was defined as one SOD unit[23].

Catalase (CAT) activity: CAT activity evaluation was based upon hydrogen peroxide decomposition by CAT present in the samples. Samples were mixed with 10 mmol L-1 potassium phosphate buffer (pH 7.0) containing 0.2% hydrogen peroxide. The hydrogen peroxide decomposition was monitored at 240 nm for 10 min[23].

Advanced oxidation protein products (AOPP): Saliva samples were diluted in citric acid (0.20 mol/L) and after incubation for 5 min the fluorescence was measured in a spectrophotometer at 340 nm. Water was used as the blank and a chloramine-T standard curve. AOPP concentrations were expressed in μmol/L equivalents of chloramine-T [24].

Reactive oxygen species production: Reactive species were determined in samples by 20 ,70 -dichlorofluorescein diacetate (DCFDA) oxidation as a general index of ROS production following. A mix containing the samples, 10 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) and 5 mM Tris-HCl buffer (pH 7.4) was incubated for 3 min at 37 ◦C and the fluorescence emission of the DCF resulting from DCF-DA oxidation was measured an excitation wavelength of 485 nm and an emission wavelength of 530 nm[25].

2.4 Statistical analysis

The Kolmogorov-Smirnov test was used to test the normality of each data. The results were compared using Kruskal-Wallis test followed by Dunn's multiple comparisons test. To perform the statistical analyses were used the software GraphPad Prism (GraphPad Prism version 7.00 for Windows, GraphPad Software, San Diego, CA, USA). Only values of $p < 0.05$ were considered significant and the results were expressed as mean ± S.D.

3. Results

Demographic and metabolic laboratorial data of neurotypical, ASD without drug therapy or ASD on drug therapy are described in table 1 and table 2.

Tabela 1. Gender and mean age distribution of healthy, ASD subjects with or without medication.

	ASD without medication	ASD with medication	Neurotypical
Female	11 (36,7%)	3 (15,8%)	12 (40,0%)
Male	19 (63,3%)	16 (84,2%)	18 (60,0%)
Age (Mean±SD)	8,56 (±2,89)	7,10 (±2,40)	8,03 (±1,87)

Tabela 2. Racial distribution of healthy, ASD subjects with or without medication.

	ASD without medication	ASD with medication	Neurotypical	Total
Brown	3 (10,0%)	1 (5,3%)	2 (6,7%)	6 (7,6%)
Black	6 (20,0%)	4 (21,1%)	7 (23,3%)	17 (21,5%)
White	21 (68,4%)	13 (68,4%)	20 (66,7%)	54 (68,4%)
Indigenous	0 (0,0%)	1 (5,3%)	1 (3,3%)	2 (2,5%)

Oxidative stress biomarkers were evaluated in saliva samples of neurotypical, ASD without drug therapy and ASD with drug therapy children (Figure 1). The total protein was reduced ($p < 0.05$) in saliva of non-medication ASD compared to neurotypical individuals, whereas the presence of medication in ASD children increased the total protein ($p < 0.05$) in saliva compared to non-medication ASD group, returning for level of neurotypical children (1.A.). The advanced oxidation protein products (AOPP) and cellular ROS assessed by the DCFA method were similar in both groups (1.B. and 1.C.). Furthermore, the total antioxidant capacity assessed by the FRAP method in saliva was higher ($p < 0.05$) in medication ASD children compared to neurotypical individuals (1.D.)

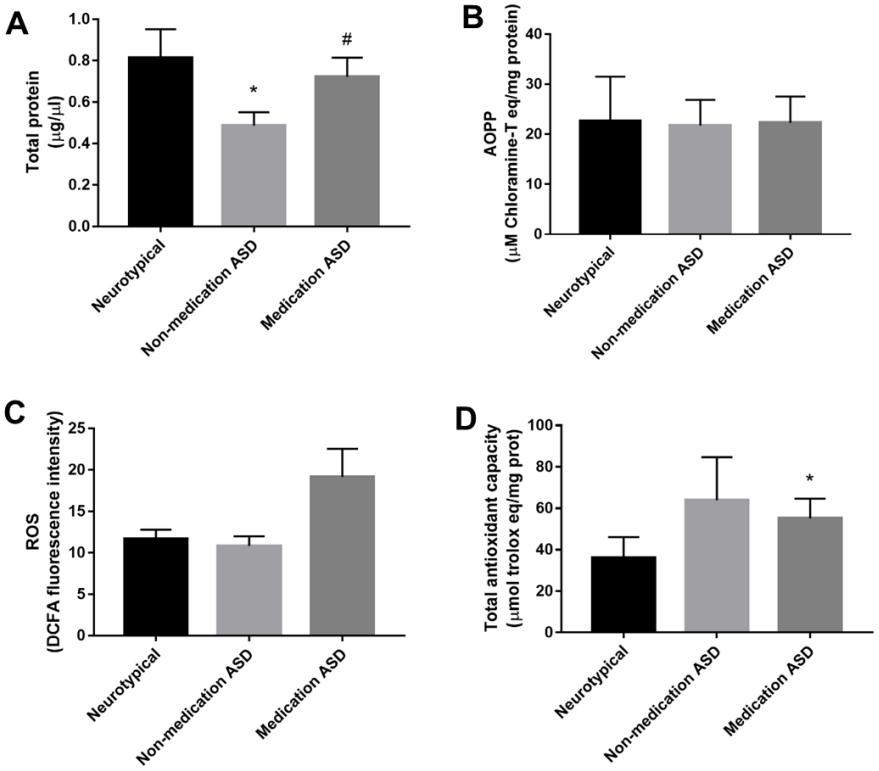

Figure 1. Redox status and biomarkers of oxidative stress in saliva of individuals with ASD (non-medication and with medication). Data are reported as mean ± SEM. (A) Total protein. (B) Advanced oxidation protein products (AOPP). (C) ROS using DCFA assay. (D) Total antioxidant capacity using FRAP assay. Kruskal-Wallis test followed by Dunn's multiple comparisons test. *Significantly different compared to neurotypical; #Significantly different compared to non-medication ASD. Outliers were detected by performing ROUT test using GraphPad Prism.

SOD and CAT antioxidant enzymes activities in saliva are shown in Figure 2. The non-medication ASD children presented increase ($p < 0.05$) in salivary SOD activity compared to neurotypical individuals, whereas medication ASD individuals do not present statistical differences. No difference was observed in the CAT activity in the groups analyzed.

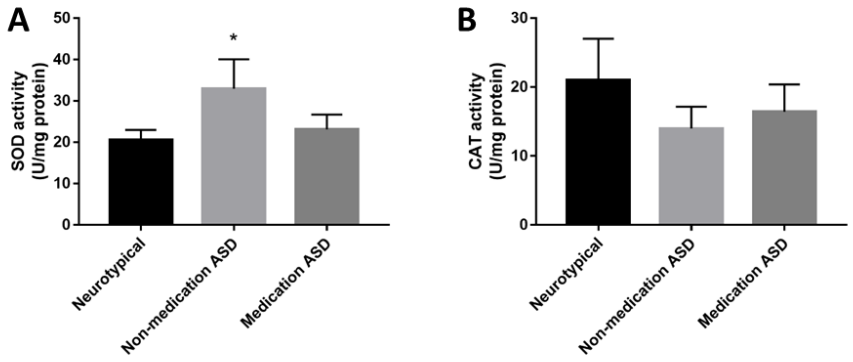

Figure 2. (A) Superoxide dismutase (SOD) activity. (B) Catalase (CAT) activity. Data are reported as mean ± SEM. Kruskal-Wallis test followed by Dunn's multiple comparisons test. *Significantly different compared to neurotypical. Outliers were detected by performing ROUT test using GraphPad Prism.

4. Discussion

The results of our study indicates increase in total antioxidant capacity of saliva in parallel with a higher salivary SOD activity in ASD children on drug therapy comparing with neurotypical children. Although, the mean value of total antioxidant capacity of saliva was higher in ASD children without drug therapy compared with ASD children on drug therapy, this tendency of increase was not statistically significant when compared with neurotypical children. Besides, the SOD activity in saliva of ASD children without drug therapy was also higher compared with neurotypical children, suggesting an antioxidant response related to the ASD condition and not a drug therapy effect. Furthermore, it is important point out the reduction of salivary proteins in ASD children without drug therapy compared with neurotypical controls, which was reversed when the ASD children use medication. To the best of our knowledge, this is a pioneer study addressing oxidative stress and antioxidant levels in saliva of ASD children.

Saliva is a complex biofluid composed of components from salivary glands, gingival crevicular fluid and oropharyngeal/respiratory secretions. In this context, molecules from blood, desquamated epithelial cells, bacteria, viruses and other pathogens can be detected in saliva[26]. The salivary molecules from salivary glands such as proteins, lipids, metabolites generally are transported to the mouth revealing physiological states [27, 28]. In addition, saliva contains several biomarkers that exhibit strong correlations with plasma or serum components, including redox status and

oxidative biomarkers[23,29]. Therefore, saliva is rich in antioxidants, as uric acid, albumin, ascorbic acid, glutathione and antioxidant enzymes[30].

The higher level of salivary SOD activity was detected in ASD compared with neurotypical subjects. We also analyzed catalase, other important antioxidant enzyme, which was unchanged in ASD subjects. Although it is a complex mechanism, we can suggest that SOD activity in saliva promotes an increase in water conversion due to its action in dismuting the superoxide anion. The accumulation of hydrogen peroxide can be neutralized in two enzymatic pathways of action, one of which would be catalase activity, but we did not observe changes in its activity in this study. In this sense, we believe that it is being directed towards the glutathione system pathway of action, therefore, additional analyzes are necessary to confirm this hypothesis, evaluating the activity of other enzymes as GPx, GSH, GR and GSSG[31].

Interestingly, we detected a reduction in total protein concentration of saliva in ASD children without drug therapy compared with neurotypical children. The drug therapy in ASD children was effective to maintain the total protein concentration in a similar level with neurotypical children. These findings can be related with dysfunctional proteostasis, a common consequence of several genetic mutations linked to ASD[32].

Oxidative stress is deeply involved in ASD molecular mechanism, however the changes in oxidative stress and antioxidant system of ASD are variable[32]. These changes were related with increased production of pro-oxidants, or damages of antioxidants system or both [14, 33]. The ability of ROS to be eliminated by the enzymatic system by cells and to maintain the redox balance depends on a considerable action of proteins, mostly superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The blood levels of transferrin (iron-binding protein) and ceruloplasmin (copper-binding protein) were found at reduced concentrations in serum of children diagnosed with ASD, suggesting changes in antioxidant activity[33]. Transferrin is an antioxidant enzyme that can reduce freee ferrous ion levels by catalyzes of the hydrogen peroxide to highly toxic hydroxyl radical[33]. In saliva, no alterations were found in the total antioxidant capacity evaluated by the ferric reduction capacity by the FRAP method in individuals with ASD without medication. In contrast, the use of medication in subjects with ASD increased salivary total antioxidant capacity, suggesting from our findings that pharmacological treatment may form a potential therapeutic pathway for this redox imbalance observed in saliva. Although an

increase in reactive oxygen species and a dysfunction in protein proteostasis in serum, plasma and urine has been described [8, 15, 18, 34], in our study we did not observe changes in ROS and AOPP in saliva.

5. Conclusion

Our results indicate that occur an increase in the total antioxidant capacity of saliva and a parallel increase in salivary SOD activity in ASD children on drug therapy comparing with neurotypical children. Interestingly, only the salivary SOD activity was higher in ASD children without drug therapy compared with neurotypical children, suggesting an antioxidant response related to the ASD condition. Besides, salivary proteins in ASD children without drug therapy were lower compared with neurotypical controls, this effect was reversed in ASD children with drug therapy. Although this study was carried out with an intermediate number of samples with innovative data on the balance of oxidant and antioxidant system, the present findings should be confirmed in larger-scale studies.

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5 *APÊNDICES*

APÊNDICE 1

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(Conselho Nacional de Saúde, Resolução 466/12)

Seu filho está sendo convidado (a) a participar da pesquisa intitulada ANÁLISE DA ESPECTROSCOPIA ATR-FTIR DE COMPONENTES DA SALIVA COMO FERRAMENTA DIAGNÓSTICA PARA O TRANSTORNO DO ESPECTRO AUTISTA, sob a responsabilidade dos pesquisadores Marjorie Adriane da Costa Nunes, Etevaldo Matos Maia Filho e Robinson Sabino da Silva. Nesta pesquisa nós estamos buscando contribuir para melhorar o diagnóstico e o monitoramento do Transtorno do Espectro Autista, por meio da identificação de substâncias salivares. Para tanto utilizaremos cerca de 5 mL de sua saliva.

O Termo de Consentimento Livre e Esclarecido será obtido pela pesquisadora Marjorie Adriane da Costa Nunes na Clínica de Odontologia do Instituto Florence de Ensino Superior (IFES), na cidade de São Luís (MA), antes da coleta da saliva. Todos os dados serão coletados no mesmo dia em que você trouxer seu filho (a) à clínica para tratamento que ele já está sendo submetido. Na sua participação você será submetido a responder um questionário sobre a condição de saúde geral de seu filho. Após isso, coletaremos sua saliva em tubos específicos e mediremos seu fluxo salivar sem estimulação e a saliva será guardada e refrigerada até que sejam feitas as análises, após isso serão descartadas. A utilização das amostras atenderá a Resolução CNS 441/2011; -In 001 de 2013 e portaria 2.201 de 14 de setembro de 2011. Ele também será submetido a uma avaliação geral de sua saúde oral. Serão feitos levantamentos de: Índice de Placa (IP), no Índice de Dentes Obturados, Perdidos e Cariados (CPOD) e periograma. Isso demorará cerca de 30 minutos. Esta avaliação será feita por um pesquisador responsável numa cadeira odontológica confortável e serão procedimentos indolores. Coletaremos e armazenaremos a saliva de seu filho (a) até que sejam feitas análises de glicose, proteínas, ureia, amilase, composição iônica e também mediremos o pH de sua saliva (acidez) e por fim, correlacionaremos o fluxo salivar com os diversos constituintes salivares e com os valores de sua pressão arterial. Em nenhum momento ele será identificado, pois usaremos código numérico de identificação. Os resultados da pesquisa serão publicados e ainda assim a identidade dele será preservada. Você não terá nenhum gasto e ganho financeiro por participar na pesquisa.

Nenhum destes procedimentos será invasivo e, portanto, não causarão dor e nem consequências para a saúde oral de seu filho (a).

Os riscos consistem na sua identificação, no desconforto em responder os questionários, no desconforto de ter sua saúde oral avaliada e tempo despendido para tal avaliação, riscos de contaminações cruzadas no consultório odontológico. Estes riscos serão minimizados pelo uso de numeração para identificação, efetuaremos a avaliação de sua saúde bucal de uma

forma menos incômoda e por um menor tempo possível. Utilizaremos superfícies com assepsia anterior e materiais devidamente esterilizados.

Os benefícios serão contribuir para a melhoria da saúde bucal dos pacientes com Transtorno do Espectro Autista, através do tratamento odontológico que se fizer necessário.

Seu filho (a) é livre para deixar de participar da pesquisa a qualquer momento sem nenhum prejuízo ou coação.

Uma via original deste Termo de Consentimento Livre e Esclarecido ficará com você.

Qualquer dúvida a respeito da pesquisa, você poderá entrar em contato a qualquer momento com: Marjorie Adriane da Costa Nunes. Rua Munin, Bloco 09, apto 201, Condomínio Vitrê, Bairro Recanto dos vinhais, São Luís, Maranhão. fone: (98) 98822-3321

Poderá também entrar em contato com o Comitê de Ética na Pesquisa com Seres-Humanos –Comitê de Ética em Pesquisa do UNICEUMA Rua Josué Montello, Nº 01 - Renascença II

São Luís, ... de .....de 20.....

_____

Assinatura dos pesquisadores

Eu aceito participar do projeto citado acima, voluntariamente, após ter sido devidamente esclarecido.

_____

Responsável

APÊNDICE 2

FICHA PARA COLETA DE DADOS ANTROPOMÉTRICOS, DE SAÚDE GERAL, SOCIOECONÔMICOS E DE HIGIENE ORAL

Pesquisador:_____ Data:____/____/_____

1. Número de Identificação:

_____

2. Dados Antropométricos e hábitos gerais:

2.1 Peso: _____ 2.2 Altura:_____ 2.3 IMC:_____ 2.4Circunferência abdominal:_____
2.5 Circunferência do braço direito:_____
2.6Consulta Odontológica : ( ) urgência ( ) tratamento ( ) manutenção
2.7Queixa Principal/Motivo da Consulta: _____
2.8Tratamento (s) Odontológico (s) Realizado(s) : _____
2.10Retorno à Clínica: SIM NÃO DATA: ___/___/___

2.11Consome bebida alcoólica? Sim, quanto? _____ Não
2.12Tem o hábito de fumar? Sim, quanto? _____ Não
2.13Tem o hábito de praticar atividade física? sim, quanto? _____ Não
2.14Consome Drogas? Sim, quais/quanto? _____ Não
2.15Toma café/ chás com cafeína/ estimulantes? Sim, quais? _____ Não

3. Inquérito de Saúde:
3.1 Como está a sua saúde?
Ótima Boa Regular Péssima

3.2 Qual a última vez que foi ao médico e qual motivo?

_____
3.3 Já fez alguma cirurgia? Sim Não Onde?_____ Há quanto tempo?_____

3.4 Tem exames de laboratoriais recentes? Quais?

_____
_____

3.5 Possui alergia a alimento ou medicamento? Sim. Qual(is)? _____Desde quando?_____ Não

3.6Tem ou já teve Anemia? Sim, quando?_____ Não Não sei
3.7Tem ou já teve Pressão alta? Sim, quando?_____ Não Não sei
3.8Tem ou já teve Diabetes? Sim, quando?_____ Não Não sei
3.9 Tem ou já teve Asma? Sim, quando?_____ Não Não sei
3.10Tem ou já teve Desmaios? Sim, quando?_____ NãoNão sei
3.11Tem ou já teve problemas no fígado? Sim, quando?_____ Não Não sei
3.12Tem ou já teve problemas no rim? Sim, quando?_____ Não Não sei
3.13Tem ou já teve problemas no coração? Sim, quando?_____ Não Não sei
3.14Tem ou já teve tumores de cabeça/pescoço? Sim, quando?_____ Não Não sei
3.15 Tem ou já teve problemas na coagulação? Sim, quando?_____ Não Não sei
3.16 Tem ou já teve infecções? Sim, quando?_____ Não Não sei
3.17Tem ou já teve distúrbios psicológicos? Sim, quando?_____ Não Não sei
3.18 Faz uso de hormônios? Sim, qual?_____ Não Não sei
3.19Tem ou já teve infecções nas glândulas salivares? Sim, quando?_____ Não Não sei
3.18Tem Síndrome de *Sjögren*? Sim, quando?_____ Não Não sei

3.19. Alterações no sistema digestivo?
3.19.1Azia 3.19.2 Refluxo gastroesofágico 3.19.3 Hérnia de hiato 3.19.4 Gastrite nervosa
3.19.5 Gastrite *H. pylori* 3.19.6 Úlcera.

3.20 Alterações no **intestino**?
3.20.1 Funcionamento normal 3.20.2 Preso 3.20.3Diarreias 3.20.4 Gases

3.21 Alterações nasais ou no **sistema respiratório superior**?
3.21.1 Sinusite 3.21.2 Rinite 3.21.3 Respiração pela boca
3.21.4 Respiração pelo nariz 3.21.5 Diminuição do olfato ou paladar
3.21.6 Sensação de pigarro ou cisco na garganta

3.22 Como é o seu **sono**?
3.22.1 Horário de dormir _____ 3.22.2 Insônia 3.22.3 Acorda durante a noite
3.22.4 Acorda a noite para beber água 3.22.5 Acorda cansado
3.22.6 Ronca 3.22.7 Horário de acordar _____

3.23 Você sente alguns desses sintomas? (**qualidade de vida**)
3.23.1 Fadiga 3.23.2 Tensão muscular 3.23.3 Estresse
3.23.4 Ansiedade 3.23.5 Depressão

Possui algumas dessas condições? 3.24. Paralisia Cerebral; 3.25 Síndrome de *Down*; 3.26 *Alzheimer;*3.27Mal de *Parkinson;* 3.28 Psoríase 3.29Artrite reumatoide; 3.30 Esclerose múltipla 3.31Doença de *gravis* 3.32Hipotireoidismo 3.33 Hipotireoidismo

3.33 Você possui alguma doença/ problema significativo não descrito?
Sim, qual (is) ?_____ Não.

4. Medicamentos em uso (ocasional ou contínuo)?

Medicamento	**Dosagem**	**Posologia e modo de uso**	**Desde quando?**
4.1			
4.2			
4.3			
4.4			
4.5			
4.6			
4.7			
4.8			
4.9			
4.10			

5. Caracterização Socioeconômica
5.1 Quantas pessoas, incluindo o sr.(a) residem em sua casa?_____
5.2 Quantos cômodos estão servindo permanentemente de dormitório para os moradores de seu domicílio?_____
5.3 Quantos bens tem em sua residência? (considerar: televisão, geladeira, aparelho de som, telefone, telefone celular, máquina de lavar roupa, computador etc.)_____

5.4 No mês passado, quanto receberam, em reais, juntas, todas as pessoas que moram em sua casa incluindo salários, bolsa-família, pensão, aluguel, aposentadoria ou outros rendimentos?_____
5.5 Até que série o sr.(a) estudou? (anotar anos estudados)_____
5.6 O sr. (a) considera que sua cor/raça é?
5.7.1 Branca 5.7.2 Negra 5.7.3 Parda 5.7.4 Indígena

6. Higiene Oral

6.1 Você acha que a sua higiene bucal do seu filho (a) é:
6.1.1 Excelente 6.1.2 Muito boa 6.1.3 Boa 6.1.4 Ruim 6.1.5 Muito ruim

6.2 Quantas vezes seu filho (a) escova os dentes?
6.2.1 1 vez ao dia 6.2.2 2 vezes ao dia ou mais
6.2.3 3 vezes por semana 6.2.4 1 vez por semana ou menos

6.3 Qual é o tipo de cerdas da sua escova?
6.3.1 macia 6.3.2 média 6.3.3 dura

6.4 Seu filho (a) usa fio dental?
6.4.1 não uso 6.4.2 sim, diariamente
6.4.3 3 vezes por semana 6.4.4 1 vez por semana ou menos

6.5 Considera que seu filho (a) necessita de avaliação com dentista atualmente?
6.5.1 Não 6.5.2 Sim

6.6 Usa de soluções antissépticas para bochecho?
6.6.1 Sim 6.6.2 Não 6.6.3 Às vezes

6.7 Quantas vezes seu filho (a) se alimenta no dia?
6.7.1 Até 3/dia 6.7.2 Entre 3 e 5/dia 6.7.3 6/dia 6.7.4 Mais de 6/dia

6.8 Qual o tipo de alimento predominante entre as refeições?
6.8.1 Salgadinhos 6.8.2 Doces 6.8.3 Frutas/Sucos 6.8.4 Biscoitos
6.8.5 Iogurtes 6.8.6 Outros ( ) Qual:_____

6 *ANEXOS*

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 Apreciação da CONEP:
Não

SAO LUIS, 25 de Setembro de 2019

ANEXO 2 – ARTIGO PUBLICADO

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ORIGINAL ARTICLE

Association of salivary alpha-2-macroglobulin with glycemia and glycated hemoglobin in type 2 diabetes mellitus: a systematic review and meta-analysis study

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KEYWORDS (MeSH terms):

Saliva.

Biomarkers.

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Blood glucose.

Salivary proteins and peptides.

AUTHORS' KEYWORDS:

HbA1C.

Glycemia.

Salivary protein.

ABSTRACT

BACKGROUND: Chronically elevated alpha-2-macroglobulin (A2MG) in the blood has been correlated with diabetes and the HbA1c profile; however, no systematic review has been conducted to evaluate the association of A2MG salivary levels and glycemia or HbA1c levels in diabetes mellitus type 2 (DM2) patients.

OBJECTIVE: To evaluate whether A2MG salivary levels are related to the glycemia or HbA1c levels in DM2 patients.

DESIGN AND SETTING: Systematic review developed at Universidade Federal de Uberlândia (UFU), Brazil.

METHODS: Eight databases were used as research sources. The eligibility criteria included studies that reported data regarding mean salivary A2MG and the correlation between glycemia and/or HbA1c levels of DM2 subjects (uncontrolled and well-controlled) and non-diabetic subjects. The risk of bias of the studies selected was assessed using the Joanna Briggs Institute (JBI) critical appraisal tools for use in JBI systematic reviews. Pooled correlation coefficients were estimated using the Hunter-Schmidt method. Study estimates were

weighted according to their sample size, and heterogeneity was calculated using the chi-square statistic.

RESULTS: Four studies on DM2 patients were included in this systematic review after careful analysis of 1482 studies. Three studies compared A2MG with HbA1c and glycemia. Overall, the correlation between A2MG and HbA1c was strong (r = 0.838). In contrast, the correlation between A2MG and glycemia was low (r = 0.354).

CONCLUSION: The strong association between HbA1C and salivary A2MG suggests that this salivary protein has the potential to be a surrogate for HbA1C, if corroboratory further evidence is obtained through large-scale studies.

SYSTEMATIC REVIEW REGISTRATION: CRD42020183831.

INTRODUCTION

Type 2 diabetes mellitus (DM2) is a metabolic disorder caused by a combination of decreased insulin secretion and decreased insulin sensitivity in peripheral tissues, primarily in the liver, muscles and adipose tissue as target organs.[1] Currently, glycemia levels and glycated hemoglobin-A1c (HbA1c) are the gold-standard parameters for diagnosing and monitoring DM2. HbA1c is suitable for reflecting glycemic control from the previous 2-3 months, in accordance with the half-life of red blood cells.[2]

Different diagnostic tools, such as glycemia, HbA1C and the oral glucose tolerance test (OGTT), are used in the diagnosis of diabetes. According to the American Diabetes Association (ADA) guidelines, individuals with glycemia concentration ≥ 126 mg/dl, HbA1C level ≥ 6.5% or two-hour plasma glucose value after 75-gram OGTT ≥ 200 mg/dl are considered to be people with diabetes.[3] The blood tests are invasive and painful[4] and may lead to development of finger calluses, poor peripheral finger circulation and risk of infection.[4]

However, the classical HbA1c tests require several reagents with relatively high cost, and need some laboratory platforms.[5] This reduces the availability of HbA1c tests in low and middle-income countries, despite their well-recognized capability for diabetes surveillance.[6] Consequently, other types of biological samples for evaluating glycemic control, such as salivary biomarkers, might be an attractive alternative for early detection and monitoring of DM2.

The major salivary glands secrete saliva in response to the autonomic nervous system, which regulates the salivation process, including the flow and concentration of some salivary components such as α-amylase, which provides a reliable measurement of the sympathetic response.[7] We previously showed that diabetes promotes changes in the autonomic activity of

salivary glands, affecting both acinar and ductal cells, which are reflected in salivary composition.[8,9]

Human saliva contains a wide variety of proteins, including enzymes derived from salivary glands, blood, microorganisms and gingival crevicular fluid.[10] In this context, saliva may contain potential biomarkers for DM2, which could be used as alternative non-invasive biofluids for diagnosing and monitoring DM2. Diabetes mellitus affects both salivary composition and salivary flow, due to microvascular alterations, neuropathies and hormonal imbalances.[11] In this regard, both salivary sugars and glycosylated proteins have been found to be capable of distinguishing between hyperglycemic and normoglycemic conditions.[12]

Alpha-2-macroglobulin (A2MG) is a glycoprotein produced by the liver that can be present in human blood plasma, cerebral spinal fluid and saliva fluid.[13] The molecular structure of A2MG (720 kDa) consists of an assembly of four 180 kDa subunits into two disulfide-linked dimers, which form a noncovalent association that completes the tetrameric quaternary structure of the protein.[14] A2MG is a glycoprotein capable of inhibiting a broad spectrum of proteases, and it also regulates the activity of cytokines, hormones, growth factors and other proteins.[15] It can be stimulated by several factors, including by cytokines related to activation of the NF-kB, C/EBPb and C/EBPd pathways.[16] Thus, patients with diabetes with positively regulated acute-phase proteins frequently express higher concentration of A2MG synthesis. Therefore, the clearance of tetrameric α2-macroglobulin-protease complexes is higher and, in compensation, there is enhanced synthesis of entire A2MG molecules, thus resulting in a net increase in the non-tetrameric circulating complex.[17] Furthermore, the condition of proteinuria in patients with diabetes also can induces greater protein synthesis in the liver, thereby increasing the concentration and activity of plasma A2MG.[18]

Chronically elevated A2MG in the blood has been correlated with diabetes.[19,20] Moreover, plasma A2MG levels have been correlated with the HbA1c profile.[21] High serum A2MG levels could decrease the bioavailability of insulin and lead to impairment of blood sugar control.[4,22] Salivary proteomic analysis on DM2 cases has indicated that A2MG was increased in subjects with uncontrolled diabetes, compared with prediabetic subjects.[23,24] Furthermore, Aitken et al. (2015) and Chung et al. (2016) suggested that the level of salivary A2MG could be used as a surrogate for glycemic control in diabetic patients and that this protein represents a potential non-invasive alternative method for evaluating metabolic control.[22,25] In this way, A2MG salivary levels could be useful as an alternative auxiliary tool for diagnosing DM2.

OBJECTIVE

The aim of the present systematic review was to answer the following guiding question: "Are A2MG salivary levels related to glycemia or HbA1c levels in DM2 patients?" We tested the following hypothesis: salivary A2MG concentrations are correlated with HbA1c and glycemia levels in uncontrolled DM2 patients, compared with well-controlled DM2 patients or normoglycemic subjects.

METHODS

Protocol and registration

The protocol for this study was reported in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P)[26] and was submitted to the International Prospective Register of Systematic Reviews (PROSPERO) database, under the number CRD42020183831 (registration date: July 5, 2020), available from: https://www.crd.york.ac.uk/prospero/. This systematic review was reported following the guidelines for the Preferred Reporting Items of Systematic Review and Meta-Analysis (PRISMA)[27] and was conducted in accordance with the Joanna Briggs Institute (JBI) Manual.[28]

Eligibly and exclusion criteria of the study

Studies were included if they were observational studies (cross-sectional) among patients with uncontrolled type 2 diabetes mellitus and if they also assessed the correlation between salivary A2MG concentration and blood sugar level and/or serum HbA1c, compared with well-controlled DM2 patients or normoglycemic subjects. Studies were selected without restriction regarding their year and publication status (published or accepted/ahead of print articles).

The exclusion criteria consisted of the following situations: I) the study was unrelated to the objective; II) the study was a review article; III) the study was a follow-up or it assessed participants with other comorbid diseases, like patients with rheumatic diseases, terminal illnesses, chronic liver disease, chronic inflammatory processes in the oral cavity, chronic kidney disease in stages IV and V and autoimmune diseases; IV) the study did not report the procedures in accordance with the ethical standards.

Sources of information and search

We searched for studies that evaluated salivary A2MG levels and serum glycemia and glycated hemoglobin (HbA1c) in type 2 diabetes mellitus cases. The MEDLINE (via PubMed), Scopus, LILACS, Web of Science, Embase and SciELO electronic databases were used as the primary study sources. In addition, OpenGrey and OpenThesis were used to partially capture the "gray literature". MeSH (Medical Subject Headings), DeCS (Health Sciences Descriptors) and Emtree (Embase Subject Headings) were used to search the descriptors. The Boolean operators "and" and "or" were combined with the descriptors to improve the search strategy (**Table 1**). The bibliographic search was conducted up to a cutoff point of November 2020. In addition, we also manually checked the reference sections of the eligible studies and any indications by expert researchers, for the possibility of any additional studies that might have been missed by the electronic search. E-mails were sent out to three referral specialists for articles potentially eligible for this review.

Study selection

Studies were selected in four stages. Initially, a calibration exercise was performed to fit pre-specified eligibility criteria and apply them to a small sample of the studies (20%) that had been retrieved, in order to determine inter-examiner agreement. After achieving an appropriate level of concordance (kappa ≥ 0.81), the reviewers (DCC and PRCP) performed a methodical analysis on all the study titles independently. Any disagreements between these examiners were discussed with a third reviewer (LRP), so as to reach a consensus.

In the first stage, the studies obtained from the databases were identified. The data were exported to the EndNote Web™ software (Thomson Reuters, Toronto, Canada), in which duplicates were removed. The remaining results were exported to Microsoft Word™ 2016 (Microsoft™ Ltd, Washington, United States), in which any remaining duplicates were manually removed.

In the second stage, all the titles were analyzed independently by the two reviewers, in order to determine their relevance. The reviewers were not blinded to the names of authors and journals. Titles that were not related to the topic were eliminated in this phase.

Then, in the third stage, the abstracts were reviewed in order to apply the exclusion criteria mentioned above. Titles in accordance with the aims of the present study but without abstracts available were fully analyzed in the fourth stage. In addition, expert investigators and potentially eligible studies found in the reference lists were included for subsequent analyses.

In the fourth stage, the full texts of the preliminarily eligible studies were obtained and evaluated to verify whether they did indeed fulfill the eligibility criteria, including expert investigators and potentially eligible studies found in the reference lists.

Data collection

The two reviewers (DCC and PRCP) then independently accessed full-text copies of all eligible articles and collected data from each study using a pre-prepared spreadsheet. The following data were extracted from the studies: author, year, country, DM2 population, average age, average age range, gender ratio, diagnosis and collection period. In addition, information on the characteristics, preparation and measurement of the samples in the eligible studies was collected (saliva collection, saliva collection criteria, saliva preparation, blood collection, A2MG measurement, glycemia measurement and HbA1c measurement), along with the main results from the studies included (mean glycemia, mean HbA1c, mean A2MG, correlation of salivary A2MG with glycemia and correlation of salivary A2MG with HbA1c).

In order to ensure consistency between the reviewers, a calibration exercise was performed with both reviewers (DCC and PRCP), in which information was extracted jointly from an eligible study. Any disagreement between the reviewers was resolved through discussions, and if the disagreement continued, a third reviewer (LRP) was consulted to make a final decision.

Risk of individual bias of the studies

The Joanna Briggs Institute Critical Appraisal Tools for use in JBI systematic reviews on observational (cross-sectional) studies[28] were used to assess the risk of bias and the individual quality of the studies selected. Two authors (DCC and RSS) independently assessed each domain regarding its potential risk of bias, as recommended in the PRISMA statement.[27]

Each study was categorized according to the percentage of positive responses to the questions of the assessment tool. The risk of bias was considered high when 49% of the responses relating to the study in question were "yes" answers, moderate when 50% to 69% of the responses were "yes" and low when more than 70% of the responses were "yes".[29]

Statistical analyses

The correlations between the A2MG and DM2 biomarkers (glycemia or HbA1c) were considered in the meta-analysis. Correlation coefficients were pooled using the Hunter-Schmidt method[30,31] and stratified according to the DM2 biomarker, for comparison with

A2MG. Estimates using this method are weighted according to the sample size of each study. The correlation was considered perfect if the coefficients were equal to 1 or -1; strong if the coefficients ranged between |0.7| and |0.9|; moderate if the coefficients ranged between |0.4| and |0.6|; weak if the coefficients ranged between |0.1| and |0.3|; and zero if the coefficients were 0.[32]

The presence or absence of between-study heterogeneity was also assessed through the Hunter-Schmidt method using the chi-square statistic.[30,31] The significance level was taken to be 5% in all analyses, which were all conducted using the Stata 16.1 software (StataCorp LLC, Lakeway Dr., Texas, United States).

Certainty of evidence

Quality of evidence and strength of recommendation were assessed using the Grading of Recommendation, Assessment, Development and Evaluation (GRADE) tool. The GRADE pro GDT software (http://gdt.guidelinedevelopment.org) was used for summarizing the results. This assessment was based on study design, methodological limitations, inconsistencies, indirect evidence, imprecision and other considerations. The quality of evidence was characterized as high, moderate, low or very low.[33]

RESULTS

Study selection

During the first phase of study selection, 1,581 results were found distributed in eight electronic databases, including the "gray literature". After removing duplicate results, 1,482 articles remained for analysis of titles and abstracts.

In this phase, after a detailed analysis of titles and abstracts, only seven studies were found to be eligible for full-text analysis. The references of these seven potentially eligible studies were also carefully evaluated and one additional article was selected. Besides that, one article was indicated by an expert investigator, thus resulting in nine studies for full-text reading.

After reading the full text, five studies were found not to fulfil the inclusion criteria and were eliminated. Among these excluded studies, one[34] was not related to the objective of this systematic review, two[23,24] were proteomic analysis studies, one[35] was a review study and another one[25] was a follow-up study. Therefore, for these reasons, they were removed from further consideration.

Thus, four studies[22,36-38] were selected for qualitative evaluation and meta-analysis. **Figure 1** depicts the search, identification, inclusion and exclusion process for article selection.

Study characteristics of eligible studies

The studies selected were published between 2015 and 2019 and were performed in Chile,[22] China,[36] Egypt[37] and India.[38] All studies[22,36-38] had been approved by the ethics committee of their respective institution or hospital and also reported that informed consent had been obtained from the subjects prior to the start of the study. None of the articles used the STROBE checklist for cross-sectional studies.

Three studies included the sources of funding: Fondo Investigación Facultad de Odontología, Universidad de Chile (FIOUCH 13-002),[22] ICMR Short Term Studentship Funding[38] and nil (no funding).[37] Other information regarding demographics and characteristics of the populations are presented in **Table 2**.

Risk of bias within studies

All the studies presented a low risk of bias or high methodological quality. However, one study[38] did not describe any specific information about the population and the parameters that assisted in making the diagnosis of diabetes. Therefore, this was indicated as unclear in the risk-of-bias table (**Table 3**).

Summary measurements and synthesis of results

Table 4 describes the correlation of salivary A2MG with glycemia and/or HbA1c and the respective means/standard deviations for glycemia, HbA1c and A2MG in the selected studies that were included in the quantitative analysis. All of these four studies were also included in the meta-analysis. However, only three studies compared A2MG with HbA1c,[22,36,37] and only three studies compared A2MG with glycemia.[36-38]

The correlation between A2MG and HbA1c ranged from 0.722 to 0.977 in the three studies analyzed. Overall, the pooled correlation between these biomarkers was strong ($r = 0.838$; 95% confidence interval, CI: 0.719 to 0.956; $P < 0.001$) (**Figure 2**). In contrast, the pooled correlation between A2MG and glycemia was low ($r = 0.354$; 95% CI: 0.077 to 0.630; $P = 0.006$). Both meta-analyses presented significant heterogeneity between study results ($P < 0.001$); however, the heterogeneity levels were higher for glycemia analysis than for the HbA1c analysis.

Certainty of evidence

The GRADE tool[33] assessed two outcomes. Both outcomes (correlation between A2MG and HbA1c and correlation between A2MG and glycemia) were categorized as very low level of certainty, which means that the true effect is likely to be substantially different from the estimated effect. **Table 5** shows more details regarding each outcome.

DISCUSSION

We conducted a systematic review to evaluate whether the increase in salivary A2MG concentration was correlated with HbA1c and glycemia levels in blood, in DM2 patients. We showed that there was a strong correlation between salivary A2MG and HbA1c, but with a low level of certainty. Hence, further studies are needed in order to determine the potential for application of A2MG in salivary platforms. However, the low association between A2MG and glycemia levels suggests that A2MG is not an accurate salivary protein that can act as a surrogate in glycemia tests.

Considering that glycemia reflects the blood glucose levels at the moment of the analysis, this test presents limitations with regard to reflecting glucose control over prolonged periods.[38] The HbA1c test has been recommended as a means for assessing variations in glucose tolerance in type 2 diabetic patients, for long-term monitoring of diabetes.[6] In addition, HbA1c tests can be performed at any time of the day without concerns about the fasting and it can indicate the average plasma glucose concentration over two to three months.[40,41]

However, the classical HbA1c test is performed in laboratory settings and only have limited use in point-of-care (POC) devices.[5] This reduces the availability of HbA1c tests in low and middle-income countries.[6] Moreover, several biological factors such as clinical conditions that alter erythropoiesis, glycation rate and erythrocyte destruction, and analytical interferences such as hyperbilirubinemia, carbamylated hemoglobin, certain medications and hemoglobin variants, affect the alteration cutoff values of the HbA1C test.[42] Our findings from this meta-analysis confirm the hypothesis that A2MG presents a strong correlation with HbA1c test.

In this context, the higher correlation between salivary A2MG and HbA1C levels indicates that saliva is a promising alternative biofluid for diagnosing and monitoring diabetes. Among the advantages, saliva is simple and non-invasive to collect; it is convenient to store; and, compared with blood, it requires less handling during clinical procedures. Hence, further studies should be carried out in order to investigate the clinical applicability of salivary A2MG as a surrogate for HbA1C in diagnosing and monitoring DM2.

This systematic review had some limitations. The absence of a control group in some studies included[22,38] could be considered a limitation, but their analysis on uncontrolled hyperglycemic subjects and subjects with type 2 diabetes presenting suboptimal control is also clinically relevant. In addition, the GRADE evaluation found that there were high levels of inconsistency and imprecision in the results obtained through the meta-analysis, which means that the evidence obtained was of very low level and that, possibly, the effect estimate found may differ from the real effect. Further studies with larger populations should be carried out in order to minimize imprecisions: these should include normoglycemic subjects, uncontrolled diabetic subjects and well-controlled diabetic subjects. Although HbA1c levels reflect the average blood glucose levels during approximately the previous 75 days, the mean duration of diabetes was not included in these studies.

On the other hand, lastly, the absence of systematic reviews and meta-analyses in this field gives added importance and timeliness to the meta-analysis of the present study. In the future, it will be important to define the predictive power of salivary A2MG for estimating HbA1c levels.

CONCLUSION

The present study described a strong association between HbA1C and A2MG levels in saliva, in uncontrolled DM2 subjects, compared with well-controlled DM2 patients or normoglycemic subjects. On the other hand, the meta-analysis suggests that there was a very low correlation between glycemia and salivary A2MG. Further large-scale studies are needed in order to be able to recommend salivary A2MG levels as alternative surrogate for HbA1c. Nonetheless, the present study suggests that this has a potential role in providing a clinically valuable advance towards salivary monitoring of diabetes.

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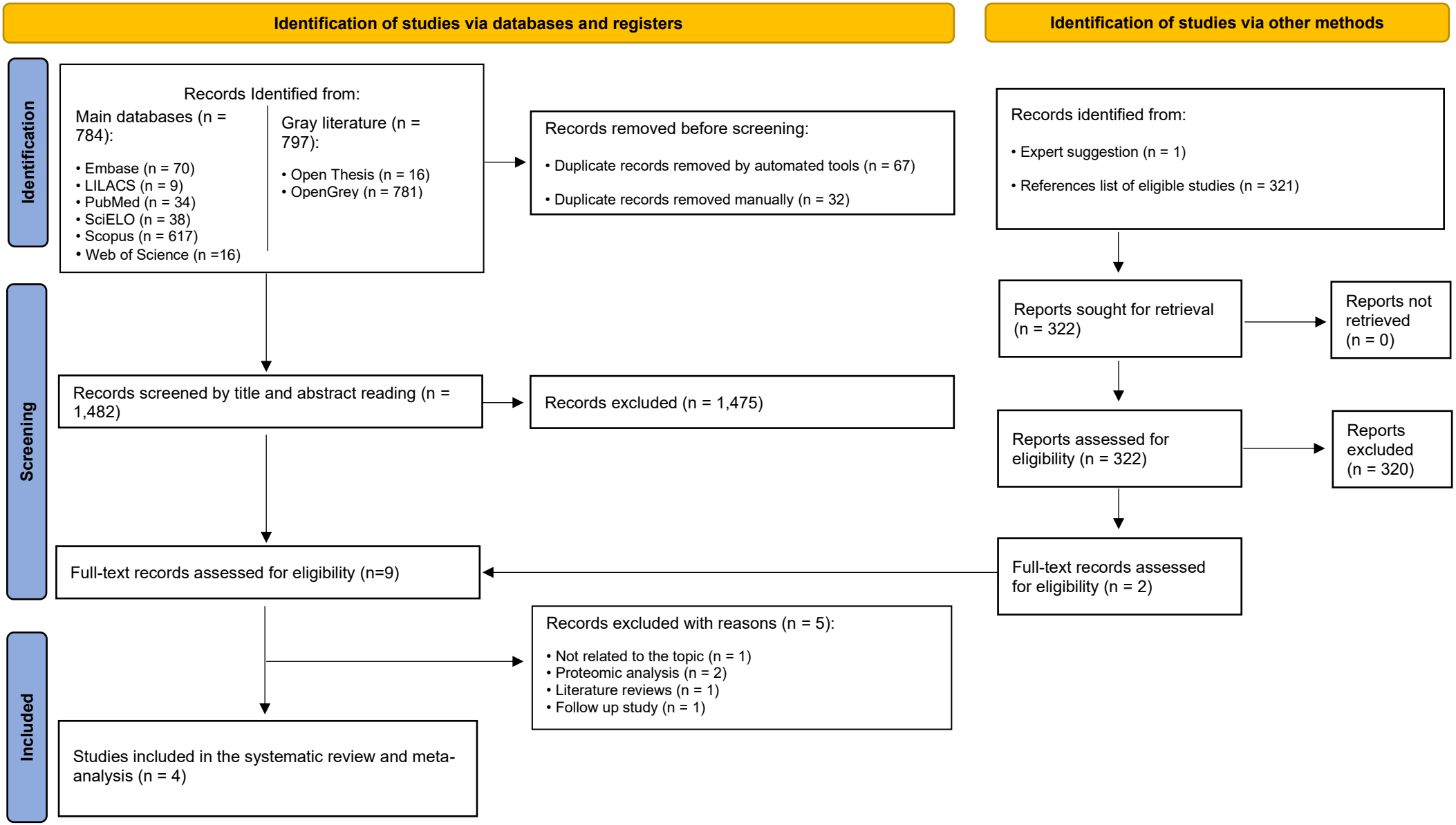

Figure 1. Flow-chart showing the search strategy, identification and inclusion/exclusion criteria used in the systematic review and meta-analysis.

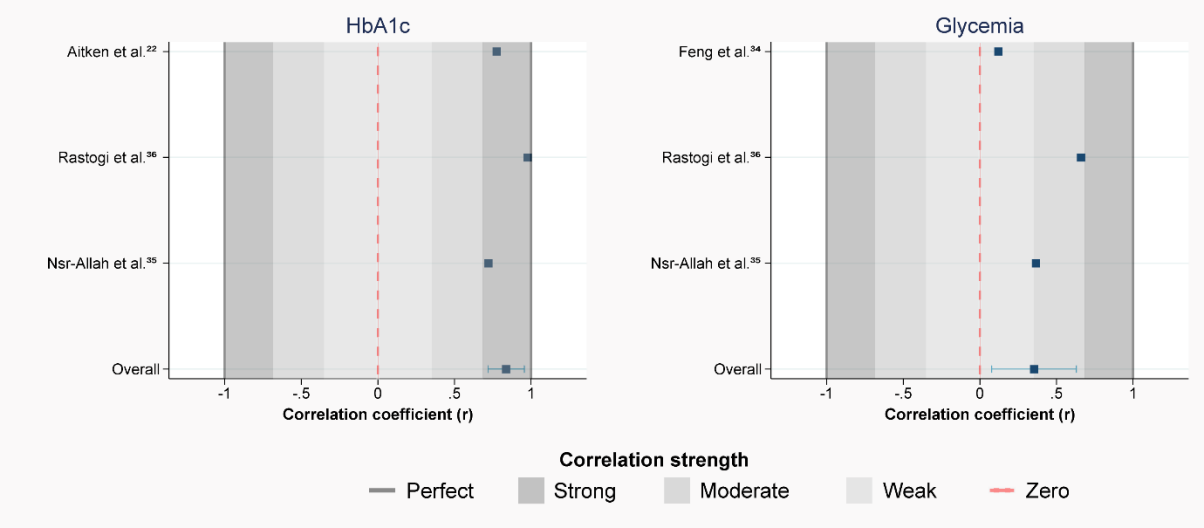

Figure 2. Correlations of salivary alpha 2-macroglobulin (A2MG) with hemoglobin-A1c (HbA1c) and glycemia.

Table 1. Strategies for database search

Database	Search strategy (November 2020)
PubMed (Best Match) http://www.ncbi.nlm.nih.gov/pubmed	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))
SCOPUS http://www.scopus.com/	("Diabetes Mellitus Type 2" OR "Diabetes Mellitus, Noninsulin-Dependent" OR "Diabetes Mellitus, Non-Insulin-Dependent" OR "Diabetes Mellitus, Type II" OR "NIDDM" OR "Type 2 Diabetes" OR "DM2" OR "T2DM") AND ("A2M protein, human" OR "α2-macroglobulin" OR "salivary α2-macroglobulin" OR "α2-MG" OR "alpha 2-macroglobulin" OR "A2MG")
LILACS http://lilacs.bvsalud.org/	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))
Web of Science http://apps.webofknowledge.com/	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))
EMBASE https://www.embase.com	('diabetes mellitus type 2'/exp OR 'diabetes mellitus type 2' OR 'diabetes mellitus, noninsulin-dependent' OR 'diabetes mellitus, non-insulin-dependent'/exp OR 'diabetes mellitus, non-insulin-dependent' OR 'diabetes mellitus, type ii'/exp OR 'diabetes mellitus, type ii' OR 'niddm'/exp OR 'niddm' OR 'type 2 diabetes'/exp OR 'type 2 diabetes' OR 'dm2' OR 't2dm'/exp OR 't2dm') AND ('a2m protein, human' OR 'α2-macroglobulin' OR 'salivary α2-macroglobulin' OR 'α2-mg' OR 'alpha 2-macroglobulin'/exp OR 'alpha 2-macroglobulin' OR 'a2mg')
SciELO https://www.scielo.org/	((“diabetes mellitus type 2” OR “diabetes mellitus, noninsulin-dependent” OR “diabetes mellitus, non-insulin-dependent” OR “diabetes mellitus, type ii” OR “niddm” OR “type 2 diabetes” OR “dm2” OR “t2dm”) AND (“a2m protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-mg” OR “alpha 2-macroglobulin” OR “a2mg”))
OpenGrey http://www.opengrey.eu/	“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM” AND “A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”
OpenThesis http://www.openthesis.org/	((“Diabetes Mellitus Type 2” OR “Diabetes Mellitus, Noninsulin-Dependent” OR “Diabetes Mellitus, Non-Insulin-Dependent” OR “Diabetes Mellitus, Type II” OR “NIDDM” OR “Type 2 Diabetes” OR “DM2” OR “T2DM”) AND (“A2M protein, human” OR “α2-macroglobulin” OR “salivary α2-macroglobulin” OR “α2-MG” OR “alpha 2-macroglobulin” OR “A2MG”))

Table 2. Characteristics of the populations of the eligible studies included

Author/Year	Country	Type of DM2 population	Control population	Average age (years)	Average age range (years)	Sex ratios	Diagnosis	Data collection period
Aitken et al.[22]/2015	Chile	120 patients (75 patients with uncontrolled glycemia and 45 patients with well-controlled glycemia)	NA	61.6 ± 10.1	31-79	32.5% ♂, 67.5% ♀	Patients with HbA1c levels < 7% were classified as having adequate glycemic control and those with levels > 7% were classified as having inadequate glycemic control	July 2013 to December 2013
Feng et al.[34]/2015	China	116 patients with DM2 and 60 patients with IFG (impaired fasting glucose)	60 healthy volunteers	DM2 (57 ± 12.3); IFG (55 ± 14.3); Control (51 ± 11.3)	Not reported	DM2 (54♂/62♀); IFG (27♂/33♀); Control (22♂/38♀)	American Diabetes Association in 2010 for DM2; IFG ≥ 7.0 mM (pre-diabetic); fasting blood glucose ranged from 5.6-6.9 mM (control)	February 2011 to March 2012
Nsr-Allah et al.[35]/2019	Egypt	40 patients: 20 patients with uncontrolled glycemia (group 1) and 20 patients with well-controlled glycemia (group 2)	20 healthy volunteers (group 3)	Group 1 (49.75 ± 10.74); Group 2 (50.90 ± 10.54); Group 3 (48.9 ± 11.47)	23-65	Group 1 (7♂/13♀); Group 2 (9♂/11♀); Group 3 (13♂/7♀)	Patients with HbA1c levels < 7% were classified as having adequate glycemic control and those with levels ≥ 7% were classified as having inadequate glycemic control. Group 3 included with fasting plasma glucose less than 100 mg/dl and HbA1c less than 5.7%.	April 2016 and June 2017
Rastogi et al.[36]/2019	India	87 patients: 53 patients with	NA	52.4 ± 8.1	35-65	43♂, 44♀	Not reported	August 2018 to October 2018

	uncontrolled glycemia and 34 patients with well-controlled glycemia	

NA = not applicable; ♀ = women; ♂ = men; DM2 = type 2 diabetes mellitus; HbA1c = hemoglobin-A1c.

Table 3. Risk of bias assessed using the Joanna Briggs Institute Critical Appraisal Tools for use in JBI Critical Appraisal Checklist for Analytical Cross-Sectional Studies[28]

Authors	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	% Yes	Risk
Aitken et al.[22]	√	√	√	√	√	√	√	√	100	Low
Feng et al.[34]	√	√	√	√	√	√	√	√	100	Low
Nsr-Allah et al.[35]	√	√	√	√	√	√	√	√	100	Low
Rastogi et al.[36]	√	U	√	U	√	√	√	√	75	Low

Q1. Were the criteria for inclusion in the sample clearly defined?; Q2. Were the study subjects and the setting described in detail?; Q3. Was the exposure measured in a valid and reliable way?; Q4. Were objective, standard criteria used for measurement of the condition?; Q5. Were confounding factors identified?; Q6. Were strategies to deal with confounding factors stated?; Q7. Were the outcomes measured in a valid and reliable way? Q8. Was appropriate statistical analysis used? √ = yes; -- = no; NA = not applicable; U = unclear.

Table 4. Summary of the main results from the studies included in the quantitative analysis.

Study	Mean glycemia	Mean HbA1c	Mean A2MG	Correlation of salivary A2MG with glycemia	Correlation of salivary A2MG with HbA1c
Aitken et al.[22]	NA	HbA1c > 7% (62.5%); HbA1c < 7% (37.5%)	Not reported	NA	r = 0.7748; P < 0.0001
Feng et al.[34]	DM2 (10.08 ± 2.44 mM); IFG (6.58 ± 0.24 mM); Control (5.01 ± 0.41 mM)	DM2 (8.7 ± 1.7%); IFG (5.8 ± 1.1%); Control (5.7 ± 0.7%)	Salivary A2MG (ng/ml): DM2 (192.6 ± 65.3); IFG (158.1 ± 60.1); Control (134.8 ± 63.2). Plasmatic A2MG (g/l): DM2 (1.70 ± 0.55); IFG (1.57 ± 0.36); Control (1.54 ± 0.38)	DM2 (r = 0.12, P = 0.199)	NA
Nsr-Allah et al.[35]	Group 1 (172.20 ± 26.52 mg/dl); Group 2 (100.65 ± 21.30 mg/dl); Group 3 (90.95 ± 8.66 mg/dl)	Group 1 (9.02 ± 1.38%); Group 2 (6.20 ± 0.61%); Group 3 (5.35 ± 0.44%)	Salivary A2MG (ng/ml): Group 1 (820.65 ± 190.17); Group 2 (331 ± 98.01); Group 3 (146.90 ± 42.01)	Group 1 (r = 0.586, P < 0.05); Group 2 (r = 0.146, P = 0.539); Group 3 (r = 0.650 ,P < 0.05); All subjects (r = 0.788, P < 0.001)	Group 1 (r = 0.778, P < 0.001); Group 2 (r = 0.666, P < 0.05); Group 3 (r = 0.474, P < 0.05); All subjects (r = 0.927, P < 0.001)
Rastogi, et al.[36]	Uncontrolled glycemia (290.58 ± 96.126 mg/dl); Well-controlled glycemia (172.83 ± 39.955 mg/dl)	HbA1c > 7% (60.9%); HbA1c < 7% (39%)	Salivary A2MG (ng/mL): Uncontrolled glycemia (2017.42 ± 575.133); Well-controlled glycemia (772.54 ± 118.324)	r = 0.660, P < 0.001	r = 0.977, P < 0.001

NA = not applicable; DM2 = type 2 diabetes mellitus; HbA1c = hemoglobin-A1c. A2MG = alpha 2-macroglobulin; IFG = impaired fasting glucose.

Table 5. Grading of Recommendations Assessment, Development, and Evaluation (GRADE) summary of findings table for the outcomes of the systematic review and meta-analysis

Quality assessment							Summary of results		
Number of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Publication biases**	**Number of participants**	**Effect** r (95% CI)	**General quality**
Outcome 1: Correlation between A2MG and HbA1c									
3	Cross-sectional studies	Not serious	Serious[1]	Not serious	Serious[2]	Not serious	247	0.838 (0.719-0.956)	⊕ VERY LOW
Outcome 2: Reduction of salivary creatinine after dialysis									
3	Cross-sectional studies	Not serious	Not serious	Not serious	Serious[2]	Not serious	243	0.354 (0.077-0.630)	⊕ VERY LOW

CI = confidence interval, A2MG = alpha-2-macroglobulin; HbA1c = hemoglobin-A1c.

GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect
[1]The heterogeneity (I^2) among the studies was high (> 75%); [2]The number of participants included in the meta-analysis was too low.