UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

LARYSSA THAINÁ MELLO QUEIROZ CUNHA

BIOMARCADORES SALIVARES PARA DIAGNÓSTICO E CORRELAÇÃO DOS SINTOMAS EM PACIENTES COM TRANSTORNO DO ESPECTRO AUTISTA

SALIVARY BIOMARKERS FOR DIAGNOSIS AND SYMPTOM CORRELATIONS IN AUTISM SPECTRUM DISORDER

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Estomatopatologia, na Área de Estomatologia.

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Orientador: Prof. Dr. Jacks Jorge Júnior

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RESUMO

O Transtorno do Espectro Autista (TEA) é um transtorno do neurodesenvolvimento, caracterizado por deficiências na comunicação e interação social, além de padrões restritos e repetitivos de comportamento. O TEA afeta 1 a cada 36 indivíduos e o aumento de sua prevalência pode estar relacionado principalmente a fatores não etiológicos, como a ampliação da sua definição de acordo com o DSM-V, que agora abrange um espectro mais amplo. O diagnóstico de TEA é baseado no julgamento clínico de uma equipe multidisciplinar e realizado entre os 4 e 5 anos nos países desenvolvidos, o que dificulta a intervenção precoce. A identificação de biomarcadores específicos para TEA facilitaria o diagnóstico, a identificação de alvos terapêuticos e a correlação com a sintomatologia e gravidade, possibilitando melhor monitoramento e prognóstico. A saliva, rica em biomarcadores, apresenta vantagens como coleta simples e não invasiva, permitindo repetição em curtos intervalos. Considerando essa coleta minimamente invasiva e a vulnerabilidade ao estresse em indivíduos autistas, a saliva é uma excelente opção para identificação de potenciais biomarcadores do TEA e para validação na prática clínica. Este estudo realiza uma análise sistemática, consolidando informações da literatura sobre biomarcadores salivares associados ao TEA. Além disso, analisamos biomarcadores salivares correlacionados com os sintomas e gravidade do TEA. A revisão seguiu as diretrizes do protocolo PRISMA-2020 e foi devidamente delineada e registrada de acordo com o protocolo PROSPERO. A busca extensiva utilizou as bases de dados MEDLINE/PubMed, EMBASE, Web of Science, Scopus e Lilacs, com pesquisas manuais no Google Scholar, ProQuest, Open Gray e avaliação das referências dos artigos incluídos. A avaliação do risco de viés dos artigos foi realizada utilizando a ferramenta de Avaliação Crítica do Instituto Joanna Briggs. Inicialmente, 361 artigos foram recuperados, dos quais 23 foram utilizados para extração de dados. Os estudos incluídos avaliaram biomarcadores salivares de tipos hormonal, molecular, proteico e metabólico. Treze artigos correlacionaram níveis de biomarcadores salivares com sintomatologia ou escalas comportamentais relacionadas ao TEA. Resultados discrepantes nos níveis de cortisol apontam para a influência de diversos fatores na detecção. Variações nos níveis de ocitocina salivar apresentam associação com TEA em crianças. Hormônios esteroides, especialmente andrógenos, representam potenciais biomarcadores associados a déficits sociais. A expressão de miRNAs salivares, como miRNA-182-5p, miRNA-146b-5p e Staphylococcus, foi identificada como potencial biomarcador diagnóstico para TEA, bem como apresentou alterações após intervenção terapêutica. A análise proteômica salivar identificou desregulação em proteínas associadas à inflamação, resposta

imunológica e distúrbios lipídicos. Níveis mais elevados de nitrito salivar em autistas sugerem um biomarcador potencial, especialmente em crianças do sexo masculino. A diminuição dos níveis salivares de ácido siálico em indivíduos com TEA aponta para uma possível via de investigação diagnóstica. Em conclusão, embora os biomarcadores salivares apresentem potencial para o diagnóstico precoce e prognóstico comportamental do TEA, limitações e divergências nos resultados ressaltam a necessidade de mais pesquisas.
Palavras-chave: Transtorno do Espectro Autista. Biomarcadores. Saliva.

ABSTRACT

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by deficiencies in communication and social interaction, as well as restricted and repetitive patterns of behavior. ASD affects 1 in every 36 individuals, and the increase in its prevalence may be mainly related to non-etiological factors, such as the broadening of its definition according to the DSM-V, which now encompasses a wider spectrum. The diagnosis of ASD is based on the clinical judgment of a multidisciplinary team and is typically made between 4 and 5 years of age in developed countries, which hinders early intervention. The identification of specific biomarkers for ASD would facilitate diagnosis, the identification of therapeutic targets, and correlation with symptomatology and severity, enabling better monitoring and prognosis. Saliva, rich in biomarkers, offers advantages such as simple and non-invasive collection, allowing for repetition at short intervals. Considering this minimally invasive collection and the vulnerability to stress in autistic individuals, saliva is an excellent option for identifying potential ASD biomarkers and for validation in clinical practice. This study comprises a systematic analysis, consolidating information from the literature on salivary biomarkers related to ASD. Additionally, we analyzed salivary biomarkers correlated with ASD symptoms and severity. The review followed the PRISMA-2020 protocol guidelines and was appropriately delineated and registered according to the PROSPERO protocol. The extensive search used the MEDLINE/PubMed, EMBASE, Web of Science, Scopus, and Lilacs databases, with manual searches in Google Scholar, ProQuest, Open Gray, and evaluation of the references of the included articles. The risk of bias assessment of the articles was performed using the Joanna Briggs Institute Critical Appraisal Tool. Initially, 361 articles were retrieved, of which 23 were used for data extraction. The included studies evaluated salivary biomarkers of hormonal, molecular, protein, and metabolic types. Thirteen articles correlated levels of salivary biomarkers with symptomatology or behavioral scales related to ASD. Discrepant results in cortisol levels point to the influence of various factors on detection. Variations in salivary oxytocin levels are associated with ASD in children. Steroid hormones, especially androgens, represent potential biomarkers associated with social deficits. Salivary miRNA expression, such as miRNA-182-5p, miRNA-146b-5p, and Staphylococcus, was identified as a potential diagnostic biomarker for ASD and showed changes after therapeutic intervention. Salivary proteomic analysis identified dysregulation in proteins associated with inflammation, immune response, and lipid disorders. Higher levels of salivary nitrite in autistic individuals suggest a potential biomarker, especially in male children. Decreased salivary levels of sialic

acid in individuals with ASD point to a possible diagnostic investigation pathway. In conclusion, although salivary biomarkers show potential for early diagnosis and behavioral prognosis of ASD, limitations and discrepancies in results emphasize the need for further research.
Keywords: Autism Spectrum Disorder. Biomarkers. Saliva.

SUMÁRIO

1 INTRODUÇÃO

O Transtorno do Espectro Autista (TEA) é caracterizado pela presença concomitante de déficits na comunicação e interação sociais, juntamente com padrões comportamentais restritos e repetitivos (1). Conforme a última edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-V), elaborado pela Associação Americana de Psiquiatria (AAP), e a Classificação Internacional de Doenças, 11ª Revisão (CID-11), o TEA não é categorizado como um transtorno psicótico, mas como um transtorno do neurodesenvolvimento (2).

Em 1911, o psiquiatra Eugen Bleuler foi o pioneiro no uso do termo "autismo" ao descrever sintomas de retração social e pensamento excessivamente interno observados na esquizofrenia (2). A primeira descrição do transtorno autista foi feita em 1943 pelo psiquiatra austríaco Leo Kanner, que descreveu 11 crianças apresentando comportamentos como isolamento extremo desde o início da vida e um interesse obsessivo pela constância (3). No ano seguinte, o pediatra Hans Asperger descreveu crianças com sintomas semelhantes, exceto por habilidade verbal superior e função cognitiva menos comprometida (3,4). Apenas em 1980, o autismo foi oficialmente reconhecido como diagnóstico, com a publicação do DSM-III (5). Na atualização subsequente do DSM, em 1994, os Transtornos Globais do Desenvolvimento foram subdivididos em cinco categorias comportamentais, incluindo Autismo clássico, Transtorno de Asperger, Transtorno Desintegrativo da Infância, Transtorno de Rett e Transtorno Global do Desenvolvimento Sem Outra Especificação (3). No DSM-V, publicado em 2013, os subtipos comportamentais, com exceção do Transtorno de Rett, foram agrupados em um único diagnóstico como TEA (1,5).

O termo “espectro” no TEA refere-se à característica heterogênea das manifestações do transtorno (5). O grau de sintomas nas áreas fundamentais do TEA pode variar amplamente, desde comprometimento cognitivo e habilidades limitadas na comunicação até um funcionamento intelectual e linguístico significativamente acima da média, associado, no entanto, à dificuldades de socialização (5–7). Aproximadamente 70% das pessoas com TEA podem apresentar alguma comorbidade mental, enquanto duas ou mais condições médicas e psiquiátricas podem estar presentes em cerca de 40% dos autista (1,3,6). Estudos relatam que o TEA tem sido frequentemente associado a distúrbios gastrointestinais, restrições alimentares, transtorno de déficit de atenção e hiperatividade (TDAH), irritabilidade aumentada, agressividade, comportamentos autolesivos, ansiedade, depressão, suicídio, transtorno

obsessivo-compulsivo, disforia de gênero, dependência química, catatonia, psicose e transtornos do espectro da esquizofrenia (8,9).

A etiopatogenia do TEA ainda não foi completamente elucidada, sendo associada a diversos fatores, incluindo fatores genéticos, epigenéticos e ambientais (10,11). Por várias décadas, persistiu uma hipótese que relacionava a parentalidade emocionalmente insensível a comportamentos de retração em crianças, as quais buscavam conforto em comportamentos repetitivos. Essa hipótese, conhecida como "mãe geladeira", atribuía a responsabilidade do transtorno à mãe (12). Na década de 1990, observações diversas, como dados neuroanatômicos, neuroimagem, citogenética e associações com síndromes genéticas, apontaram para um desenvolvimento cerebral anormal, substituindo assim a explicação psicogênica que antes predominava (12).

No início dos anos 2000, estudos sobre fatores genéticos associados à etiologia do TEA demonstraram que há uma enorme heterogeneidade genética, com mais de 800 genes reconhecidos, diversas aberrações cromossômicas e dezenas de síndromes identificadas. (7,8,12). A maioria desses genes afeta a regulação da expressão gênica, neurogênese, modificação da cromatina e função sináptica, sobrepondo-se a outros distúrbios do desenvolvimento e psiquiátricos (6,7). O TEA é reconhecido como a condição de neurodesenvolvimento com maior tendência à hereditariedade. (8) Com base em estudos familiares, as taxas de hereditariedade para o TEA variam de 37% até mais de 90%, com gêmeos monozigóticos apresentando índices de concordância cerca de três vezes maiores do que gêmeos dizigóticos (1,8,13). Apesar disso, há indícios de que outras influências genéticas não hereditárias também podem contribuir para a variação de características associadas ao transtorno (14).

Além dos fatores genéticos, diversas exposições ambientais pré-natais, perinatais e infantis representam fatores de risco para o TE (15). Os fatores de risco ambientais incluem uso de medicações, idade da mãe e do pai, exposição fetal a esteroides sexuais ou substâncias tóxicas, infecções e atividade imunológica, tabagismo e uso de álcool, entre outros (16–18).

Considerando a exposição fetal aos esteroides sexuais como um fator de risco para o TEA, Baron-Cohen e colaboradores (2015) sugeriram que a testosterona desempenha um papel importante durante a janela de masculinização pré-natal no desenvolvimento cerebral. Estudos indicam que níveis elevados de testosterona pré-natal podem estar relacionados aos déficits centrais do TEA (20). Além disso, níveis elevados de estrogênio fetal,

importante na sinaptogênese e corticogênese, também foram relacionados a um aumento de risco de autismo (16).

A associação entre alterações imunológicas e o TEA é respaldada por vários estudos (7). O sistema imunológico desempenha um papel significativo no neurodesenvolvimento e na regulação da plasticidade neural (21). Evidências sugerem que uma função imunológica anormal, incluindo inflamação, disfunção de células T, produção de autoanticorpos, aumento no número de células B ativadas e células NK, e desregulação de citocinas pró-inflamatórias, pode contribuir para a etiologia do TEA (7,17,21). Adicionalmente, a ativação das células neurogliais e do sistema imune inato foi observada no tecido cerebral e no líquido cefalorraquidiano (LCR) desses indivíduos (5,7).

Mecanismos epigenéticos, incluindo alterações na metilação do DNA (mDNA) e microRNAs (miRNAs), regulam o estado da cromatina e alteram as expressões genéticas independentemente da sequência do DNA, podendo desempenhar um papel crucial etiologia do TEA (7,17). Os miRNAs influenciam no desenvolvimento e função do sistema nervoso central (SNC), estando associados a distúrbios de cognição, aprendizagem e neurodesenvolvimento (22,23). Perfis de expressão de miRNAs desregulados foram identificados em diferentes matrizes, incluindo saliva, sangue, córtex cerebelar e LCR de indivíduos com TEA (16,24,25).

O aumento no número de casos de TEA nas últimas décadas tem gerado debates questionando se esse fenômeno reflete um aumento real do transtorno e se está diretamente relacionado aos possíveis fatores etiológicos (26,27). Fatores não etiológicos têm sido discutidos como as principais justificativas para os crescentes índices relatados pela Rede de Monitorização do Autismo e das Deficiências do Desenvolvimento (ADDM) (28). Em 2000, a prevalência de TEA era de 1 em cada 150 crianças, aumentando para 1 em 44 em 2018 e para 1 em 36 em 2020 (8,28,29). Entre os indivíduos com TEA, o sexo masculino é mais acometido, sendo que para cada menina, 3,8 meninos são diagnosticados (1,30). Estimativas recentes revelam padrões de prevalência distintos com relação a raça e etnia, com crianças de grupos historicamente menos prevalentes, como crianças negras não-hispânicas e crianças hispânicas, bem como crianças de níveis socioeconômicos mais baixos, demonstrando um aumento na prevalência (31). Esses padrões têm sido associados a melhorias na identificação mais equitativa do TEA, especialmente em grupos que têm menos acesso ou enfrentam maiores barreiras para a obtenção de serviços de saúde (28).

Entre os fatores não etiológicos que podem estar associados ao aumento na prevalência de TEA, destacam-se a ampliação da sua definição, que agora abrange um espectro mais amplo, e as revisões nos critérios diagnósticos do DSM; mudanças nas práticas de notificação; maior conscientização pública; alterações nos padrões de encaminhamento; recomendações para triagem universal (6,26).

O diagnóstico de TEA é realizado através do julgamento clínico dos profissionais e é fundamentado na história detalhada do desenvolvimento, além do uso de instrumentos diagnósticos padronizados. Esse processo envolve uma avaliação abrangente realizada por uma equipe multidisciplinar, que pode incluir pediatras, psicólogos, neurologistas pediátricos, fonoaudiólogos e outros especialistas, como psiquiatras infantis e geneticistas (9). A Academia Americana de Pediatria preconiza a avaliação de todas as crianças através de uma ferramenta de triagem de TEA aos 18 e 24 meses de idade (32,33). Crianças que obtêm resultados positivos devem ser encaminhadas a um especialista para avaliação abrangente e, simultaneamente, para serviços de intervenção precoce (9).

Nesse sentindo, diversos instrumentos de triagem foram desenvolvidos por neurocientistas e psicólogos especializados em TEA, para distinguir os comportamentos autistas de outros transtornos invasivos do desenvolvimento (TID) (34). Exemplos incluem o Quociente do Espectro do Autismo (AQ), o Teste da Síndrome de Asperger na Infância (CAST) e a Lista de Verificação Modificada para o Autismo em Crianças (M-CHAT) (34). Além disso, há instrumentos que podem ser utilizados para diagnóstico formal de TEA e planejamento de tratamento, como o Entrevista Diagnóstica para o Autismo Revisada (ADI-R), Escala de Observação Diagnóstica para o Autismo (ADOS), Escala de Resposta Social (SRS) e Escala de Classificação do Autismo na Infância (CARS) (34).

Dois manuais são internacionalmente utilizados para definir o diagnóstico de TEA: DSM-V e a CID-11, este último sendo o sistema classificatório adotado atualmente no Brasil (1,35). Para atender aos critérios diagnósticos usando o DSM-V, é necessário que todos os três sintomas incluídos no domínio de comunicação e interação social estejam presentes, além de dois dos quatro sintomas associados a comportamentos restritivos e repetitivos (1,26). Esses traços necessitam se manifestar de maneira precoce durante o processo de desenvolvimento e impactar a vida do indivíduo socialmente, profissionalmente ou de outras formas significativas (1).

O DSM-V apresenta cinco especificadores que descrevem a gravidade dos sintomas, levando em consideração o status de deficiência intelectual, deficiência de linguagem, catatonia, comorbidades e fatores etiológicos genéticos ou ambientais conhecidos. (1,26) Com base nesses especificadores e na necessidade de apoio, os indivíduos podem ser classificados em três níveis de gravidade: Nível 1 (“Requer suporte”), Nível 2 (“Requer suporte substancial”) e Nível 3 (“Requer suporte muito substancial”). (1) De maneira similar, o CID-11, publicado em 2022, classifica as subdivisões do TEA de acordo o comprometimento intelectual e/ou da linguagem funcional. (35)

Apesar de o diagnóstico, baseado nos manuais mencionados, poder ser feito antes dos dois anos, a idade média do diagnóstico é entre 4 e 5 anos nos países desenvolvidos, sendo mais elevada nos países em desenvolvimento e ainda mais entre grupos vulneráveis de crianças. (9,36–38) A complexidade do diagnóstico e seu consequente atraso estão associados a vários fatores, como a dificuldade por parte dos prestadores de cuidados primários em identificar sinais do transtorno, encaminhamentos tardios, falta de consciência sobre os marcos do desenvolvimento, incapacidade dos pais de levantar preocupações críticas de desenvolvimento, confusão do TEA com outras condições e sistemas de saúde que não respondem às necessidades das comunidades carentes. (8,9) Além disso, condições socioculturais e econômicas também podem impactar na ocorrência de diagnóstico tardio ou subdiagnóstico. (1)

O prognóstico de TEA está fortemente relacionado ao seu diagnóstico, considerando o início precoce do tratamento. (39,40). Nesse sentido, o diagnóstico do TEA tornou-se um problema significativo de saúde pública. (26,32) As necessidades de cuidados desses indivíduos têm um impacto significativo tanto nas famílias quanto nos sistemas de saúde. Em 2015, os custos diretos e indiretos desses cuidados nos Estados Unidos foram estimados em 268 bilhões de dólares, ultrapassando os custos do AVC e da hipertensão combinados. (26)

Pesquisas recentes têm se concentrado em grandes programas de rastreamento que podem ser eficientes e fáceis de implementar em escala populacional, com o objetivo de auxiliar no diagnóstico precoce de TEA. (37,41) A identificação de biomarcadores específicos ajudaria no diagnóstico, identificação de alvos de tratamento, monitoramento terapêutico e compreensão da etiologia do transtorno. (2,11,42)

Os biomarcadores consistem em características que podem ser mensuradas com precisão e reprodutibilidade, proporcionando medidas objetivas e quantificáveis de processos clinicamente significativos. (43) Embora seu conceito seja relativamente novo na ciência, o

constante surgimento de tecnologias inovadoras tem impulsionado exponencialmente o número de estudos sobre o tema, destacando os biomarcadores como uma abordagem promissora no cuidado de pacientes com diferentes cenários clínicos. (40,44) Nos últimos anos, vários biomarcadores associados ao TEA foram propostos, como tecnologias de rastreamento ocular, eletroencefalografia, imagem por ressonância magnética, carga genética, volume do LCR, assinaturas transcriptômicas e níveis moleculares mensurados em diferentes fluidos biológicos. (25,36,45)

O LCR é considerado padrão-ouro para a detecção de biomarcadores associados a doenças neurodegenerativas ou danos ao tecido cerebral. (46,47) No entanto, a amostragem do LCR é um procedimento invasivo, que apresenta dificuldades para medições repetidas, custos mais elevados e falha na adesão dos pacientes. (46) Recentemente, amostras obtidas a partir de métodos de coleta menos invasivos, como sangue, lágrimas, urina e saliva, ganharam papel fundamental na detecção de biomarcadores. (47)

A saliva demonstrou ser um biofluido rico em biomarcadores, apresentando vantagens como fácil coleta, que pode ser realizada repetidamente durante um período relativamente curto e sem a necessidade de uma equipe treinada. (37,48,49) Além disso, a saliva não exige condições especiais de armazenamento, permite a coleta de uma maior quantidade de amostra, é mais fácil de manusear do que o sangue, não coagula, é estável ao longo do tempo e oferece pouco ou nenhum risco de contaminação cruzada ou exposição a patógenos. (20,47) A saliva é um equivalente funcional do soro em termos de refletir o estado de saúde do corpo humano, e o número de marcadores detectáveis é comparável aos encontrados no sangue, embora se apresentem em menores concentrações. (21,37,49)

As glândulas salivares recebem inervação parassimpática através dos nervos cranianos glossofaríngeo e facial. Essa íntima conexão possibilita que a saliva se torne uma fonte valiosa de biomarcadores que representam diversos processos patológicos no sistema nervoso e no neurodesenvolvimento. (10,21,36) Considerando suas vantagens e o fato de que indivíduos com TEA são mais vulneráveis ao estresse, a saliva é uma excelente opção de fluido para o estudo de biomarcadores nesse grupo. (50)

Este estudo realiza uma análise sistemática, consolidando informações da literatura sobre biomarcadores salivares associados ao TEA. O objetivo é ampliar o entendimento das biomoléculas que têm potencial para serem empregadas no diagnóstico e acompanhamento deste transtorno.

2 ARTIGO

2.1 Title: Salivary biomarkers for diagnosis and symptom correlations in Autism Spectrum Disorder: A Systematic Review.

Artigo submetido para publicação no Frontiers in Neuroscience. (Anexo 1)

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ABSTRACT

Aim: To analyze evidence on salivary biomarkers associated with Autism Spectrum Disorder (ASD), consolidating information from the literature on biomolecules that have the potential to be used in the diagnosis of this disorder. Additionally, we investigated salivary biomarkers correlated with the symptoms and severity of ASD. Design: An electronic search was performed in PubMed, EMBASE, Web of Science, Scopus, and Lilacs databases and a manual search in Google Scholar, ProQuest, and Open Gray and assessing the included articles' reference lists were also performed. Bias assessment for articles was performed utilizing the Joanna Briggs Institute Critical Appraisal tool. Results: 361 articles were initially retrieved, of which 23 were used for data extraction. The included studies assessed salivary hormonal, molecular, protein, and metabolite biomarkers. Thirteen articles correlated salivary biomarker levels with symptoms or behavioral scales related to ASD. Due to the heterogeneity of data regarding individuals and biomarkers across studies, a meta-analysis was not conducted. Discrepant cortisol levels in individuals with ASD suggest the influence of factors in detection, such as collection method and sample volume. Despite reduced levels in some studies, Oxytocin varies in associations with symptoms and severity, necessitating further research. Steroid hormones, particularly androgens, exhibit a correlation between their levels and the social deficits associated with ASD, suggesting their potential as biomarkers. Dysregulated expression of salivary miRNAs, such as miRNA-182-5p, miRNA-146b-5p and Staphylococcus, is associated with ASD. Salivary proteomic analysis identified dysregulation in proteins related to inflammation, immune response, and lipid disorders in individuals with ASD. Proteins like PSP, PIP, transferrin, and alpha-amylase exhibited varied levels, contributing to understanding ASD-associated physiopathology. Higher salivary nitrate levels in ASD suggest a potential biomarker, especially in male children. Decreased salivary sialic acid levels in individuals with ASD indicate a possible avenue for investigation. Conclusion: In summary, although salivary biomarkers hold potential for early diagnosis and behavioral prognosis of ASD, challenges and results inconsistencies highlight the need for further research. Future studies should address the heterogeneity of ASD, improve the acquisition of reliable saliva samples, and establish standardization in collection and analysis methods, aiming for the validation and effective implementation of these biomarkers in clinical practice.

Keywords: Autism Spectrum Disorder. Biomarkers. Saliva.

INTRODUCTION

Autism Spectrum Disorder (ASD) is characterized by the simultaneous presence of deficits in communication and social interaction, along with restricted and repetitive behavioral patterns. (1) The etiopathogenesis of ASD remains partly elucidated and is associated with genetic, epigenetic, and environmental factors. (2,3)

According to data from the Autism and Developmental Disabilities Monitoring Network (ADDM), the prevalence of ASD has seen a significant rise in the past two decades. (4) In 2000, the prevalence was 1 in 150 children, increasing to 1 in 44 in 2018 and 1 in 36 in 2020. (4,5) The escalating number of ASD cases has sparked intense debates, addressing whether it reflects an actual increase in incidence related to etiological factors or is a consequence of non-etiological factors, such as broadening autism definitions to encompass a broader spectrum, changes in reporting practices, increased public awareness, alterations in referral patterns and recommendations for universal ASD screening. (6,7)

The diagnosis of ASD is based on the clinical judgment from professionals, as well as a detailed developmental history and the use of standardized diagnostic instruments. (8) Although diagnosis can occur before the age of two, in developed countries, the diagnosis is generally made between the ages of 4 and 5, in developing countries, registering an even more substantial delay in vulnerable groups of children. (8–11) ASD diagnosis has become a significant public health issue, as early intervention often cannot commence due to diagnostic delays. (7,12) Considering the challenges associated with the diagnosis of ASD, identifying specific biomarkers could assist in the diagnostic process, aiding with therapeutic targets choice, monitoring development, and understanding the disorder's etiology. (3,13,14)

Saliva is gaining a pivotal role among all bodily sampling tissues and has proven to be a biofluid rich in biomarkers. (10,15) Saliva offers various advantages, such as easy and non-invasive collection, repeatability over a relatively short period, and no need for a trained team. (16) The vulnerability of individuals with ASD to stress makes saliva an excellent fluid for studying biomarkers in this group. (14,16) Therefore, this study conducts a systematic analysis, consolidating information from the literature on salivary biomarkers associated with ASD. The aim is to enhance understanding of the biomolecules that have potential for use in the diagnosis and monitoring of this disorder.

MATERIAL AND METHODS

Eligibility Criteria

Following the "PECOS" framework, the review question was formulated as: "What are the potential diagnostic and symptom correlations salivary biomarkers for patients with Autism Spectrum Disorder (ASD)?"

Articles assessing salivary biomarkers for the diagnosis or symptom correlations of individuals diagnosed with ASD were considerably eligible. Observational studies included published cross-sectional, longitudinal, cohort, and case-control studies. Reviews, editorials, letters, personal opinions, book chapters, conference abstracts, experimental in vitro or in vivo studies, and studies published in languages other than English were excluded.

Information Sources and Search Strategy

A literature investigation was performed in the following electronic databases: MEDLINE/PubMed, EMBASE, Scopus, Web of Science, and Lilacs, with an additional gray literature search in Google Scholar and ProQuest. All searches were finalized by December 4, 2023, without time restrictions. Also, a manual search was performed through the reference lists of the included articles to identify other relevant studies that may not have been captured during the screening process. The comprehensive search strategy for each database is available in Supplementary Table 1.

Study Selection and Data Collection Process

First, two reviewers independently screened the articles by reviewing titles and abstracts. Afterward, the full text of the included articles was read to ensure that they met the inclusion criteria, and in instances of discrepancies, a third reviewer was consulted. Rayyan QCRI (Qatar Computing Research Institute) (17) was used for article screening, duplicate exclusion, and recording exclusion reasons.

One reviewer extracted data from each included article, and the second reviewed the extracted data, which incorporated author(s), year of publication, country, age, sex, behavioral characteristics and symptoms, saliva collection process, detection and analysis method for biomarker analysis, and identified markers.

Risk of Bias in Individual Studies

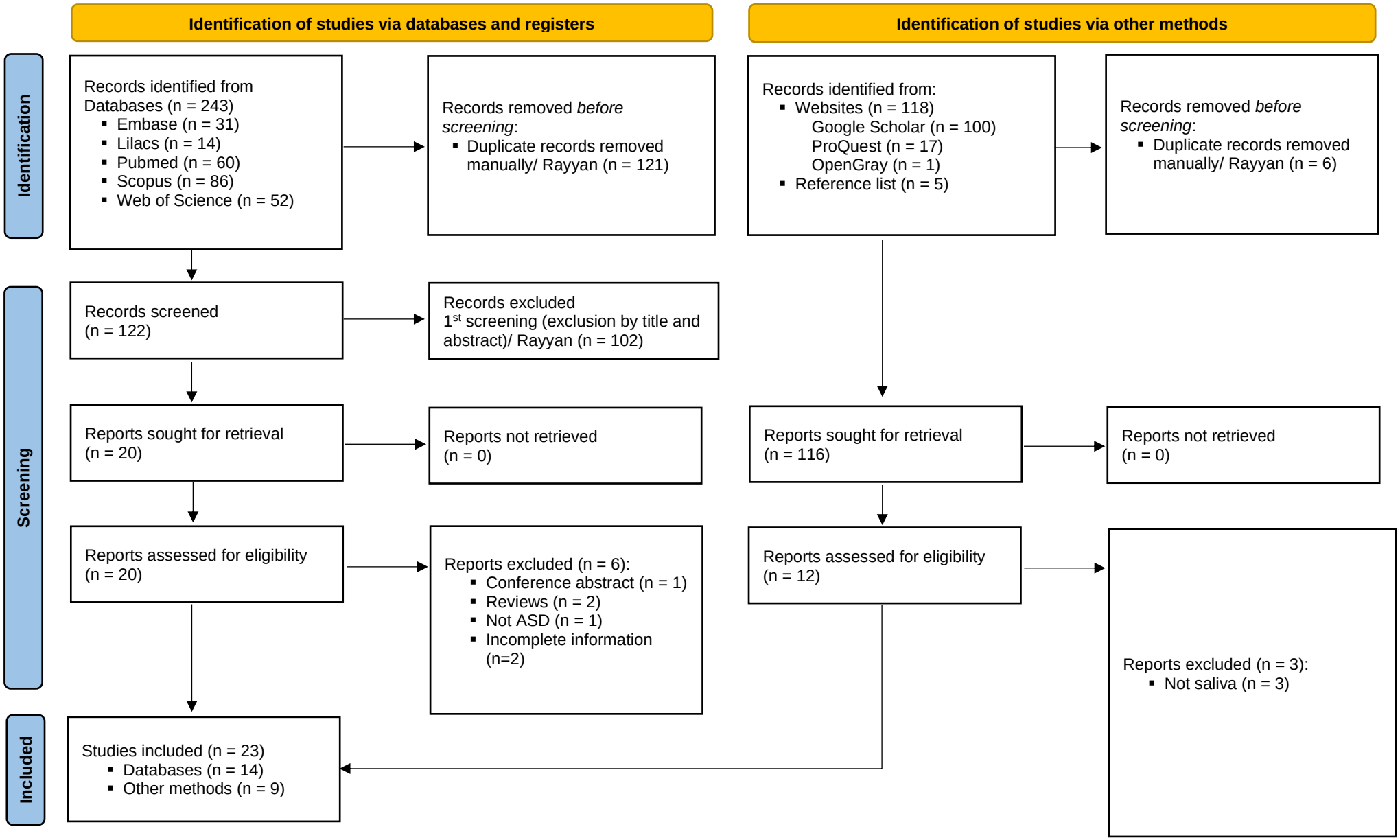
The bias assessment of the articles was carried out independently by two authors, using the Joanna Briggs Institute's Critical Appraisal tool. A calibration process was carried out between the evaluators before carrying out the individual assessments, which consisted of questions answered as “yes”, “not clear”, “no” or “not applicable”. The studies were categorized as high risk of bias if the analysis score was under 49%, moderate between 50 and 69%, and low risk of bias when it was over 70%.

RESULTS

Study selection and characteristics

In the literature screening, 361 records were identified, consisting of 243 studies in the databases and 118 in the grey literature. All of them were managed, and duplicates were removed. Then, the titles and abstracts of 234 studies were read for initial screening. According to the eligibility criteria and resolution of discrepancies through discussion, 32 studies were selected for full-text reading. Finally, after confirming the inclusion criteria, 23 studies were chosen for qualitative analysis. The flowchart describing the selection method of this study is shown in Figure 1.

Figure 1. Process selection flowchart.



Description of the individual studies

Twenty-three articles (2,3,10,13,14,18–35) published between 2008 and 2023 were included in the analysis. Most of these studies originated from the United States of America (n = 9), followed by Italy (n = 3), China (n = 2), and Poland (n = 2). Additionally, Belgium, Bosnia and Herzegovina, Brazil, France, India, the Netherlands, and Turkey contributed to a single study. Table 1 presents comprehensive details on the characteristics of the included studies.

Based on the gathered data, the number of individuals with ASD included in each study ranged from 5 (10) to 238 (18). Regarding gender distribution, out of the total participants with ASD, 1163 were male, and 259 were female. In the control groups, 758 were male, and 368 were female. However, in the study of Castagnola et al. (2017), there was no information about the gender of the patients in the control group.

Likewise, three studies (3,25,26) did not provide or have incomplete data regarding the mean age of participants. Six articles (13,18,23,32,34,35) reported mean ages expressed in months, ranging from 35.47 months (13) to 60.8 months (32) in the ASD group and from 26.23 months (13) to 69.3 months (32) in the control group. Among the other studies that reported age data in years, the mean age varied from 3.7 years (21) to 15 years (30) in the ASD group and from 3.4 years (21) to 15.9 years in the control groups (30). One study (28) provided only the age range of patients (2-10 years).

Considering the type of saliva collection method, Tordjman et al. (2014) (30) and Kidd et al. (2012) (23) employed stimulated saliva collection methods. Dental cotton rolls and cherry-flavored Kool-Aid were used to induce salivation, respectively. Conversely, the remaining 19 articles opted for unstimulated saliva collection for analysis, and two studies did not specify the type of saliva collected (26,32).

Description of salivary biomarkers

The included studies assessed different salivary biomarkers, including hormones, molecular compounds, proteins, and metabolites. Hormonal biomarkers were examined in seven studies (14,19–23,30), with a significant cortisol assessment (14,20–23,30). Molecular compounds were evaluated in seven (2,10,18,32–35) included studies. Additionally, seven articles (3,13,24–27,31) centered on the analysis of proteins as potential biomarkers for ASD. Only two of the records evaluated metabolite levels, with one assessing nitric oxide (NO) levels (28) and another examining salivary sialic acid levels in children with ASD (29). In Table 2, the specific biomarkers identified in individuals with ASD are detailed.

Table 1. Characteristics of the included studies.

AUTHORS	YEAR	COUNTRY	STUDY DESING	TYPE OF SALIVA	TYPE OF SALIVARY BIOMARKERS	METHOD FOR ASSESSING BIOMARKERS	ASD (n)	SEX ASD (n)		AGE ASD	CONTROL (n)	SEX CONTROL		AGE CONTROL
								MALE	FEMALE			MALE	FEMALE	
Evenepoel et al. (19)	2023	Belgium	CC	Unstim	Hormone	Oxytocin ELISA kit and Cortisol ELISA kit and Pyrosequencer	80	64	16	10,5 ±1.3	40	32	8	10,3±1.3
He et al. (14)	2023	China	CC	Unstim	Hormone	ABSS-CIEX QTRAP® 6500+LC/MS/MS	55	55	0	5.08 (4.50, 5.75)	24	24	0	5.17 (4.58, 5.54)
Bakker-Huvenaars et al (30)	2020	The Netherlands	CC	Unstim	Hormone	In-house developed RIA	49	49	0	15±2.1	28	28	0	15,9±1.8
Muscatello; Corbett (20)	2018	USA	CC	Unstim	Hormone	Coat-A-Count® RIA kit	64	57	7	12,02±2.83	49	42	7	11,17±2.94
Majewska et al. (21)	2014	Poland	CC	Unstim	Hormone	GC-MS and RIA - GCMS-QP2010 Plus da Shimadzu	78	(3-4): 23 (7-9): 20	(3-4): 22 (7-9): 13	(3-4) Male: 3,7±0,1 Female: 3,9±0,2 (7-9) Male: 8,2±0,2 Female: 7,7±0,2	70	(3-4): 19 (7-9): 17	(3-4): 16 (7-9): 18	(3-4) Male: 3,5±0,1 Female: 3,4±0,1 (7-9) Male: 8,2±0,2 Female: 8,4±1,4
Tordjman et al. (22)	2014	France	CC	Stim (dental cotton roll)	Hormone	ELISA kit from BIO ADVANCE (Cortisol)	55	36	19	11,3±4,1	32	22	10	11,7±4,9
Kidd et al. (23)	2012	USA	CC	Stim (cherry flavored Kool-Aid)	Hormone	ELISA and Kinetic Reaction Assay (Cortisol and sAA)	26	22	4	45,1 (±8,9; 28–64) *	26	23	3	39,4 (±10,5; 24–61) *
Kalemaj et al. (10)	2022	Italy	CC	Unstim	Molecular	NucleoSpin® miRNA Plasma kit and Agilent 2100 Bioanalyzer RNA assay	5	4	1	5.6	5	3	2	5.4
Levitskiy et al. (34)	2021	USA	CC / Long	Unstim	Molecular	Qiagen miRNeasy MicroKit, Illumina TrueSeq Small RNA Prep protocol and NextSeq500	CC group: 48 Long group: 22	CC group: 37 Long group: 20	CC group: 11 Long group: 2	CC group: 11.5±4.45 (5-23) * Long group: 52,3±13,0 (30-77) *	CC group: 48	CC group: 26	CC group: 22	CC group: 10.4±3.03 (4-19)
Sehovic et al. (32)	2020	Bosnia and Herzegovina	CC	Unstim	Molecular	TaqMan MicroRNA Reverse	39	25	14	60.8 ±15.1 (37-92) *	25	11	14	69.3± 14 (42-95) *

						Transcription Kit and GS1 Thermal Cycler System and SimpliAmp Thermal Cycler								
Hicks et al. (35)	2020	USA	CC	Unstim	Molecular	Illumina TruSeq Small RNA Sample Prep protocol and NextSeq500	TS: 187 Test set: 37	TS: 161 Test set: 29	TS: 26 Test set: 8	TS: 54±15 Test set: 47±14 *	TS: 125 Test set: 8	TS: 76 Test set: 5	TS: 49 Test set: 3	TS: 47±18 Test set: 56±14 *
Ragusa et al. (2)	2020	Italy	CC	Unstim	Molecular	nCounter NanoString technology, TaqMan assays, qPCR and 16S rRNA	76	60	16	6.9±1.5	39	28	11	6.9±1.8
Hicks et al. (18)	2018	USA	CS	Unstim	Molecular	Illumina TruSeq Small RNA Prep and NextSeq500	TS: 188 Test set: 50	TS: 156 Test set: 45	TS: 35 Test set: 5	TS: 54±15 * Test set: 53±15 *	TS: 113 TD / 71 DD Test set: 21 TD / 13 DD	TS: 122 Test set:26	TS: 62 Test set: 8	TS: 49±16 * Test set: 46±16 *
Hicks et al. (33)	2016	USA	CC	Unstim	Molecular	Illumina TruSeq Small RNA Sample Prep	24	16	5	9.1±2.4	21	16	5	9.2±2.5
Wormwood et al. (3)	2023	USA	CC	Unstim	Proteomics	NanoLC-MS/MS - NanoDrop Lite Spectrophotometer	14	13	1	UN	14	13	1	UN
Mota et al. (13)	2022	Brazil	CC	Unstim	Proteomics	Bio-Rad Protein Assay Kit and QExactive Plus Orbitrap mass spectrometer coupled to an EASY-nLC 1000 system	34	27	7	35,47±10,42*	41	21	20	26,23±10,91*
Bhat et al. (24)	2021	India	CC	Unstim	Proteomics	ELISA kit (Human IgG4 Ready-Set-Go Kit, eBiosci-ence)	55	42	13	10.7±4.2 (2.5-18)	Control: 57 PCG-PI: 60	Control: 44 PCG-PI: 40	Control: 13 PCG-PI: 20	Control: 11.2±2.7 (6-18) PCG-PI: 9.2±2.7 (3-14)
Samborska-Mazur et al. (31)	2020	Poland	CC	Unstim	Proteomics	Qiagen Liquichip apparatus with custom-designed 7-plex kits	19	18	1	6.78±2.80	19	15	4	6.84±2.52

Ngounou Wetie et al. (b) (25)	2015	USA	CC	Unstim	Proteomics	2D-PAGE combined with nanoLC-LC-MS/MS and NanoAcquity UPLC coupled to a Q-TOF API MS	6	6	0	UN	6	6	0	9.5
Ngounou Wetie et al. (a) (26)	2015	USA	CC	Unstim	Proteomics	NanoLC-MS/MS using a NanoAcquity UPLC coupled to a QTOF Micro MS	6	6	0	UN	6	6	0	UN
Castagnola et al. (27)	2008	Italy	CC	Unstim	Proteomics	RP-HPLC-ESI Mass Spectrometry	27	20	7	4.65 (1,5-8.5) ‡‡	23	UN	UN	5.00 (1.5-9.0)
Yao et al. (28)	2021	China	CC	Unstim	Metabolomics	Nitric Oxide Analyzer	134	118	16	Range 02-10	135	72	63	Range 02-10
Demirci et al. (29)	2019	Turkey	CC	Unstim	Metabolomics	Sialic acid assay kit	46	36	10	5.50±2.05	30	21	9	5.35±2.15

*Mean age expressed in years; ‡ (One subject is 15 years old and was not included in the mean); **2D-PAGE:** 2-Dimensional Polyacrylamide Gel Electrophoresis; **ASD:** Autism Spectrum Disorder; **CC:** Case-control; **CS:** Cross-sectional; **ELISA:** Enzyme-Linked Immunosorbent; **GC-MS:** Gas Chromatography-Mass Spectrometry; **LC/MS/MS:** Liquid Chromatography-Tandem Mass Spectrometry; **Long:** Longitudinal; **NanoLC-MS/M**S: Nanoliquid Chromatography-Tandem Mass Spectrometry; **PCG-PI:** Positive control group of parasite infection; **qPCR:** Quantitative Polymerase Chain Reaction; **RIA:** Radioimmunoassay; **RP-HPLC-ESI Mass Spectrometry:** Reverse-phase Liquid Chromatography with Electrospray Ionization/Mass Spectrometry; **Stim:** Stimulated; **TS:** Training Set; **UN:** Unclear; **Unstim:** Unstimulated.

Table 2. Biomarkers regulation.

AUTHORS	TIPE OF SALIVARY BIOMARKERS	BIOMARKERS		
		UP-REGULATION	DOWNREGULATED	NO SIGNIFICANT DIFFERENCE
Evenepoel et al. (19)	Hormone	-	Oxytocin (morning)	mDNA OXTR
He et al. (14)	Hormone	DHEA Pregnenolone	-	Cortisol
Bakker-Huvenaars et al. (30)	Hormone	-	Oxytocin	Cortisol Testosterone
Muscatello; Corbett (20)	Hormone	Cortisol (overnight)	-	-
Majewska et al. (21)	Hormone	DHEA Androsterone-C 5-androstene-3β,7α,17β-triol 5-androstene-3β,7β,17β-triol Pregnenolone 20-alpha dihydropregnenolone 20-alpha dihydropregnenolone-C Allopregnanolone Isopregnanolone-C Epipregnanolone-C DHEA-C DHEA-7o Androstenediol Androsterone-C Etiocholanolone Etiocholanolone-C Epiandrosterone Epiandrosterone-C Pregnenolone-C	-	Cortisol -
Tordjman et al. (22)	Hormone	Cortisol	-	-
Kidd et al. (23)	Hormone	-	-	Cortisol Alpha-amylase
Kalemaj et al. (10)	Molecular	miRNA-1246 miRNA-199b-5b miRNA-4516 miRNA-199a-3p miRNA-199b-3p	miRNA-1973 miRNA-203a-5p miRNA-4284 miRNA-660-3p miRNA-545-5p	-
Levitskiy et al. (34)	Molecular	-	-	Correlation between behavior and markers: miRNA-146b-5p, miRNA-29c-3p, miRNA-374a-5p, miRNA-182-5p, miRNA-28-3p, piR-24085, piR-6463, Staphylococcus Biomarkers that have changed over time: let-7e-5p, miRNA-125a-5p, miRNA-125b-5p, miRNA-146b-5p, miRNA-148a-5p, miRNA-182-5p, miRNA-221-3p, Staphylococcus
Sehovic et al. (32)	Molecular	miRNA-7-5p	miRNA-23a-3p	-

		-	miRNA-32-5p miRNA-140-3p miRNA-628-5p	
Hicks et al. (35)	Molecular	miRNA-665 miRNA-4705 miRNA-620 miRNA-1277-5p	miRNA-28–3p miRNA-148a-5p miRNA-151a-3p miRNA-125b-2–3p	-
Ragusa et al. (2)	Molecular	- miRNA-29a-3p miRNA-141-3p miRNA-146a-5p miRNA-200a-3p miRNA-200b-3p miRNA-4454 miRNA-7975 Filifactor Weeksellaceae Ralstonia Actinobacillus parahaemolyticus Pasteurellaceae Haemophilus parainfluenzae Rothia mucilaginosa Aggregatibacter segnis	miRNA-7706 miRNA-16-5p miRNA-205-5p miRNA-451a let-7b-5p Moryella Tannerella TM7-3 -	-
Hicks et al. (18)	Molecular	-	-	Diagnostic panel: miRNA-92a-3p, miRNA-146b, miRNA-146b-5p, miRNA-378a-3p, miRNA-361-5p, miRNA-125-5p, miRNA-106a-5p, miRNA-3916, miRNA-146a, miRNA-10a, miRNA-410, piR-24684, piR-9491, piR-27400, piR-6463, piR-29114, piR-12423, piR-24085, piR-15023, SNORD118, Leadbetterarella byssophia, Alphaproteobacteria, Fusarium, Staphylococcus, Clostridiales, Pasteurella multocida, Corynebacterium uterequi, Lactobacillus fermentum, Oenococcus oeni, Streptococcus gallotycus, Ottowia, Yarrowia lipolytica
Hicks et al. (33)	Molecular	miRNA-628-5p miRNA-27a-3p miRNA-335-3p miRNA-2467-5p miRNA-28-5p	miRNA-127-3p miRNA-30e-5p miRNA-23a-3p miRNA-32-5p -	-

		miRNA-191-5p miRNA-3529-5p miRNA-218-5p miRNA-7-5p miRNA-140-3p		
Wormwood et al. (3)	Protein	Basic Salivary Proline Rich Protein (INF)	PIRG	
		Basic Salivary Proline Rich Protein 2 (INF)	Mucin 5AC	
		LTF	Lipocalin- 1 isoform X1	-
		BPI fold-containing family member 2 precursor	ALB	
		PSP	Common salivary protein 1	
			Mucin-5B precursor	
			Len, Bence-Jones	
		-	Immunoglobulin J chain	
			Immunoglobulin lambda chain	
			Chain A human lysozyme	
			Ig A L	
			Apo-human serum transferrin	
			Chain A human secretory component	
Mota et al. (13)	Protein	Protein disulfide- isomerase A3, EC 5.3.4.1	Protein S100-A2	
		Neutrophil elastase, EC 3.4.21.37	-	-
		Immunoglobulin heavy variable 4-39		
		Cysteine-rich secretory protein 3, CRISP-3		
		Calmodulin-3		
		Plastin-2		
		Protein S100-A7		
Bhat et al. (24)	Protein	IgG4	-	-
Samborska-Mazur et al. (31)	Protein	-	RANTES	Eotaxin
			-	IL-6
				IL-8
				IL-1β
				MCP-1
				TNF-α
Ngounou Wetie et al. (25)	Protein	Proto-oncogene FRAT1	Alpha-amylase	-
		Ig alpha-1 chain C region	CBP	

		Immunoglobulin heavy chain constant region alpha-2 subunit	p532
		V-type proton ATPase subunit C1	Transferrin variant
		Carbonic anhydrase VI nirs variant 3	Protein-l-isoaspartate O-methyltransferase domain-containing protein 1 isoform 3
		KIF14	Chain A of Human Pancreatic Alpha-Amylase In Complex With Myricetin
		Integrin alpha 6 subunit	V-type proton ATPase subunit C 1
		GRTP1 protein	Ig J-chain
		PSP	ZAG
		PIP	Glutamate-rich protein 6B
		Mucin-16	Immunoglobulin heavy chain variable region
		Ca binding protein MRP14	ALB
		Carbonic anhydrase isozyme VI	Sperm activating protein subunit I-Apo A1-SPAP-subunit I
		-	Zymogen granule protein 16 homologue B precursor
			Putative lipocalin 1-like protein 1
			Cystatin D
			Plasminogen
Ngounou Wetie et al. (26)	Protein	PIP	Acidic proline-rich phospho-protein 1/2
		LTF	Submaxillary gland androgen-regulated protein 3B
		Annexin A1	Antileukoproteinase
		Neutrophil-defensin 1	Pleckstrin-homology domain-containing family H member
		Lactoperoxidase	Statherin
		Lipocalin-1	Histatin
		PIRG	-
		Deleted in malignant brain tumors 1 protein	
		IgG gamma-1 chain C region	

-

		IgG kappa chain C region Myeloperoxidase		
Castagnola et al (27)	Protein	-	Phosphorylation level of Statherin Phosphorylation level of Histatin 1 Phosphorylation level of aPRP	-
Yao et al. (28)	Metabolite	Nitrite	-	-
Demirci et al. (29)	Metabolite	Nitric oxide (only in male) -	Sialic Acid	-

ALB: Albumin; **CBP:** CREB-binding protein; **DHEA:** Dehydroepiandrosterone; **DHEA-7o:** Dehydroepiandrosterone 7-oic Acid; **DHEA-C:** Dehydroepiandrosterone-3β-sulfate; **GRTP1:** Growth hormone regulated TBC protein 1; **KIF14:** Kinesin family member 14; **LTF:** Lactotransferrin; **MCP-1:** Monocyte Chemoattractant Protein-1; **mDNA:** DNA methylation; **OXTR:** Oxytocin Receptor Gene; **PIGR:** Polymeric Immunoglobulin Receptor Precursor; **PIP:** Prolactin-Inducible Protein Precursor; **piRNAs:** piwi-interacting RNA; **PSP:** Parotid Secretory Protein; **TNF-α:** tumor necrosis factor-α; **ZAG:** Zn alpha2 glycoprotein.

Relationship between salivary biomarkers and symptoms and behavioral scales in ASD

Thirteen articles (2,14,19–23,29–31,33–35) correlated salivary biomarker levels with symptoms or behavioral scales related to ASD (Table 3).

Risk of bias

Nine studies (39.13%) were categorized with a moderate risk of bias, while eight studies (34.78%) demonstrated a low risk, and six (26.08%) exhibited a high risk of bias. The first measure for bias risk in the studies was the methodology applied to saliva collection, which several studies collected at home, by parents or guardians, and not by a trained and standardized team. Other factors influencing the bias assessment were the absence of a sample matched for sex and age, unclear data on sample sources, and the lack of identification of confounding factors and strategies to address these issues. Further information is summarized in Figure 2 and described in detail in supplementary file 2. Due to the heterogeneity of data regarding individuals and biomarkers across studies, a meta-analysis was not conducted. Consequently, only descriptive assessments of the data are provided.

Figure 2. Risk of bias: reviewers' judgments about each checklist item across the studies. **(A)** Case-control studies. **(B)** Cross-sectional studies.

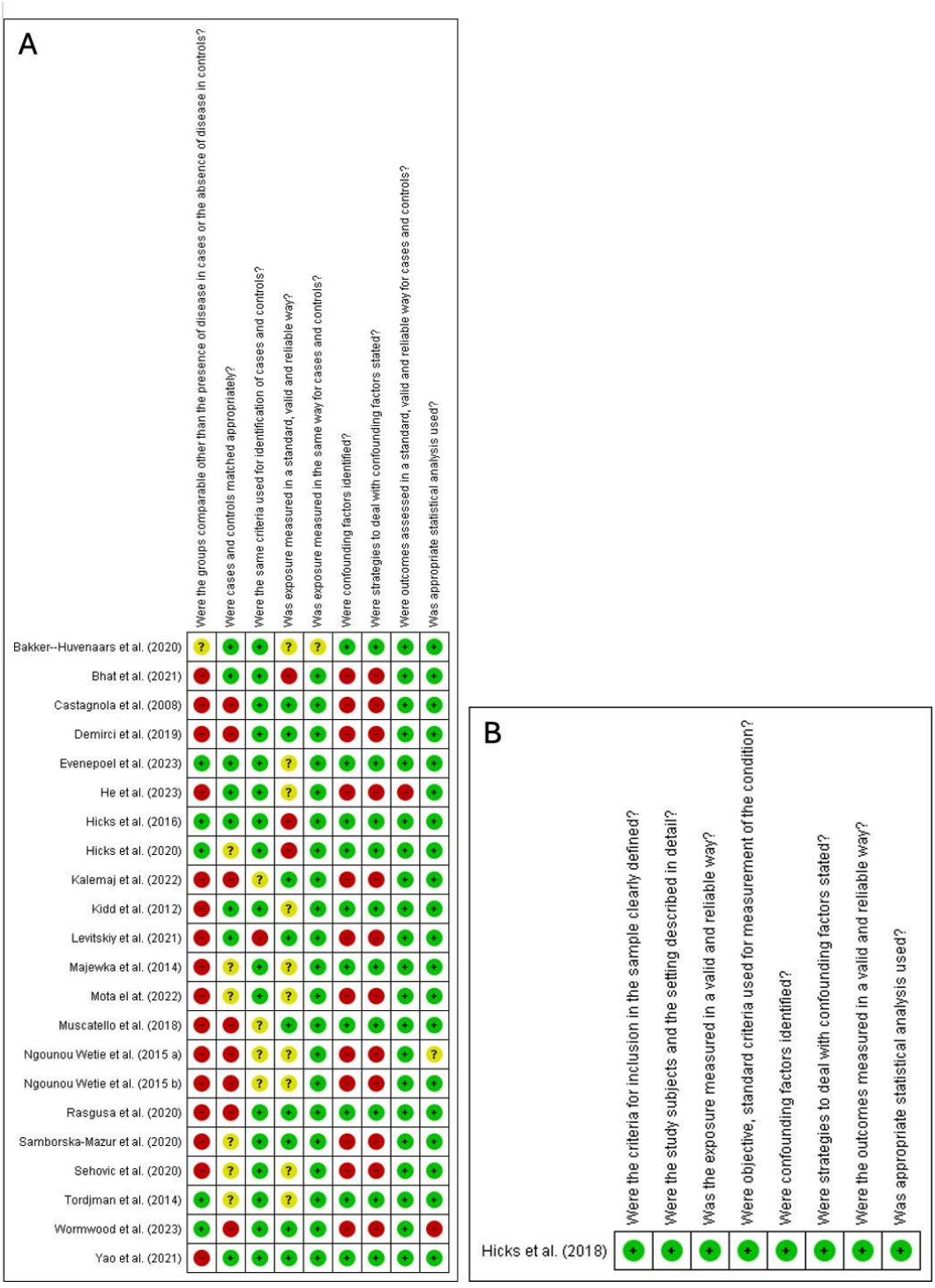


Table 3. Correlation between biomarkers and symptoms/behaviors.

AUTHORS	CORRELATIONS
Evenepoel et al. (19)	Oxytocin levels in the control group and CBCL
He et al. (14)	Pregnenolone and ABC score
Bakker-Huvenaars et al. (30)	Cortisol and testosterone levels and self-assessed empathy deficits
Muscatello; Corbett (30)(20)	Elevated cortisol levels and more symptoms of withdrawal and depression (CBCL- Withdrawn)
Majewska et al. (21)	Autistic girls (7 to 9 years old): higher concentrations of most steroids positively correlated with CARS scores Autistic boys (7-9 years old): levels of allopregnanolone and isopregnanolone positively correlated, and levels of DHEA, DHEA-C, and epiandrosterone negatively correlated with CARS scores
Tordjman et al. (22)	Cortisol levels and severity of deficits in social interaction and verbal language
Kidd et al. (23)	sAA levels 1.5 times higher in individuals with LFA Higher cortisol and sAA levels associated with lower IQ
Levitskiy et al. (34)	15 significant associations between RNAs and VABS-II, AQ, and BASC scores Most significant association: between AQ Total Score and piR-24085 levels Staphylococci: correlated with the highest number of behavioral scores (BASC Parent Behavioral Symptoms Index; VABS-Composite; VABS-Daily Living Skills; VABS-Social) miRNA-4700-3p and miRNA-4485-3 and the presence of GI disturbance.
Hicks et al. (35)	miRNA-152–3p, miRNA-379–5p, miRNA-4781–3p, miRNA-26a-5p, and miRNA-221–3p and VABS-Social miRNA-223–3p, miRNA-142–3pa, miRNA-182–5pa, miRNA-142–5p, miRNA-125b-2–3p, miRNA-181c-5p, miRNA-148b-3p, miRNA-143–3p and ADOS-B miRNA-136–3p, miRNA-8485, miRNA-106a-5p, miRNA-3679–5p, miRNA-573, miRNA-6733–5p, miRNA-8061, miRNA-130a-3p, miRNA-766–5p, miRNA-431–5p and ADOS-D miRNA-223–3p, miRNA-142–3p, miRNA-142–5p, miRNA-182–5p, miRNA-148b-3p, miRNA-151a-3pa, and total score on ADOS-II
Ragusa et al. (2)	Negative association between let-7b-5p, miRNA-451a, and ADI-A, ADI-B, ADOS-A, and ADOS-D. Negative correlation between miRNA-451a and ADI-D and ADOS-B. miRNA-16-5p was negatively related to ADOS-C and ADOS-D. Positive correlation between miRNA-29a-3p and ADI-B and ADOS-A. Positive correlation between Moryella and VIQ. Negative relationship between Moryella and ADI-D. Negative relationship of TM7-3 with ADI-B and ADI-C. Positive correlation between Ralstonia and ADI-A.
Hicks et al. (33)	The abundance of seven microbial species was significantly related to cognitive impairments. Significant relationship between VIQ, PIQ, and TIQ and the abundances of Weeksellaceae and Ralstonia. 13 miRNAs (miRNA-628-5p; miRNA-127-3p; miRNA-27a-3p; miRNA-335-3p; miRNA-2467-5p; miRNA-30e-5p; miRNA-28-5p; miRNA-191-5p; miRNA-23-3p; miRNA-3529-5p; miRNA-218-5p; miRNA-7-5p; miRNA-32-5p; miRNA-140-3p) and VABS RANTES level and aggression and gait disturbances
Samborska-Mazur et al. (31)	IL-8 level associated with fixations/stimulations IL-1 β level associated with no active speech
Demirci et al. (29)	Negative correlation between AuBC total score, AuBC - Body and object use behaviors, ABC - Stereotypic behavior factor, and ABC - Hyperactivity/noncompliance factor, and Sia levels

ABC: Aberrant Behavior Checklist; **AuBC:** Autism Behavior Checklist; **AQ:** Autism Spectrum Quotient; **ADOS-II:** Autism Diagnostic Observation Schedule; **ADOS-A:** Communication; **ADOS-B:** Social Interaction; **ADOS-C:** Imagination; **ADOS-D:** Repetitive and Restricted Behavior; **ADI-A:** Qualitative anomalies in social interaction; **ADI-B:** Qualitative anomalies in communication; **ADI-C:** Repetitive and restricted behavior; **ADI-D:** Anomalies in neurodevelopment arisen before 36 months old; **CARS:** Childhood Autism Rating Scale; **CBCL:** Child Behavior Checklist; **GI:** Gastrointestinal; **LFA:** low functioning autism; **PIQ:** Performance Intelligence Quotient; **TIQ:** Total Intelligence Quotient; **VABS-II:** Vineland Adaptive Behavioral Scales 2nd Edition; **VIQ:** Verbal Intelligence Quotient; **sAA:** salivary alpha-amylase; **Sia:** Sialic acid.

DISCUSSION

The clinical heterogeneity of ASD has been an obstacle in understanding the pathophysiological mechanisms involved and identifying associated biomarkers. (36,37) The absence of specific biomarkers complicates the early diagnosis, treatment, and prognosis of the disorder. In this systematic review, we explored saliva as a biofluid for identifying biomarkers that could differentiate individuals with ASD from those with typical development, aiding in the diagnostic process. Additionally, we analyzed salivary biomarkers correlated with ASD symptoms and severity, providing an overview of the patient's developmental trajectory and assisting in the selection of therapeutic goals and treatment efficacy monitoring.

This systematic review reinforces the notion of most of the research on ASD is conducted in high-income Western countries. This discrepancy may reflect inequalities in countries' access to financial resources and research infrastructure.

In the included studies, the gender ratio of patients with ASD was 4.49 males to every female. This data may be reflective of the higher prevalence of ASD in males, as boys are diagnosed four times more frequently than girls. (1) However, numerous studies have investigated whether this higher male prevalence is associated with a significant gender diagnostic bias. (8) Evidence reveals that higher levels of camouflaging in autistic women compared to men result in their under-recognition and/or incorrect diagnosis. (38,39) The term "camouflaging" refers to an attempt to conceal socially salient autistic behaviors and compensate for socio-communicative deficits, consciously or unconsciously, to fit into the social environment. (39)

The population addressed in all studies of this review consists predominantly of children and adolescents. This choice is closely related to the goal of identifying tools that enable early diagnosis, even during childhood. Additionally, by restricting the age range of participants, researchers seek to mitigate potential influences of patient development and puberty stage on the levels of specific biomarkers, such as steroid hormones. (40) Another justification for including an initial sample of children is the need for a longitudinal study design to track development over time and understand the trajectories of ASD. (37) However, it is essential to recognize the limited knowledge regarding the elderly phase of ASD, and there is an increasing recognition of the necessity for research in adults. (1)

Several factors, such as the collection of total saliva or from a specific gland, the collection time, and the presence of stimuli, can influence the results of salivary analyses, making the collection process challenging. (16,41) Only two included studies used stimulated

saliva for analysis (22,23). Stimulated saliva offers several advantages, such as time efficiency, suitability for low flow rates, and minimal patient adherence requirements. (41,42) However, it is essential to note that different stimulating agents can alter saliva content by modifying the concentration of specific biomarkers. (16,42,43) A crucial aspect to consider when selecting the type of saliva for research in individuals with ASD is the acceptability of collection methods, given the specific sensory sensitivities in these individuals. (16,44)

Among the included studies that investigated hormonal biomarkers, cortisol was examined in six (14,20–23,30). Cortisol is the primary product of the hypothalamic-pituitary-adrenal (HPA) axis, an essential system in the stress reaction. (22,45) Studies conducted in animals have indicated a relationship between the HPA axis and characteristic behaviors of ASD, suggesting that these behaviors can be alleviated through regulating the HPA axis. (45,46) Additionally, chronic stress and the resulting excessive release of cortisol tend to activate microglia, associated with synaptic pruning and imbalance of excitation/inhibition in the central nervous system (CNS). (45) When assessing the amniotic fluid of pregnant women who had male children with ASD, Baron-Cohen et al. (2015)(47) identified an elevated level of cortisol, suggesting that this hormone may be involved in the neurodevelopmental process. Therefore, peripheral cortisol may be involved in the etiology of ASD.

Studies by Bakker-Huvenaars et al. (2020) (30), He et al. (2023) (14), Kidd et al. (2012) (23), and Majewska et al. (2014) (21) did not reveal significant differences in cortisol levels between individuals with ASD and those with typical development (TD). On the other hand, Tordjman et al. (2014) (22) and Muscatello and Corbett (2018) (20) identified significantly higher levels of salivary cortisol in individuals with ASD. This discrepancy in results may be attributed to various factors interfering with cortisol detection in saliva, such as substantial cortisol retention in the absorbent material of specific collection devices, sample volume, type of saliva collected, collection time, and food or beverage consumption. (42–44)

Tordjman et al. (2014) (22) and Muscatello and Corbett (2018) (20) found that compared to individuals with TD, the ASD group exhibited flatter diurnal and nocturnal cortisol slopes. This persistence of elevated cortisol levels during the night in this group suggests cumulative effects of stress throughout the day or difficulty adapting to stressors. Other studies support the findings in this review. The study by Gao et al. (2022) (45) identified significantly elevated peripheral (serum and plasma) cortisol levels in ASD patients compared to controls. In a meta-analysis conducted to investigate potential differences in cortisol awakening response

(CAR) between young people with ASD and TD, the authors identified that individuals with ASD exhibit diminished CAR. However, there was no statistically significant difference compared to the TD group. (46)

Regarding behavioral and severity associations, cortisol levels were significantly correlated with deficits in social interaction and verbal language (22), symptoms of withdrawal and depression (CBCL-Withdrawn) (20), self-reported traits of lack of empathy (30), and lower IQ (23). A study evaluated cortisol levels in hair and saliva samples of beings with ASD with and without a stressful stimulus and identified a positive correlation between cortisol elevation and more severe ASD symptoms. (48) In a study that assessed morning levels of salivary cortisol in children with TD, the results suggested that stress, and consequently, elevated cortisol levels, act as modulators on the HPA axis and influence cognitive performance. (49)

Oxytocin, another hormone evaluated in this study, is linked to pro-social behaviors and may play a role in the etiology of several socioemotional dysfunctions, including ASD. (50–52) In this review, two studies assessed salivary oxytocin and found reduced levels in participants with ASD compared to individuals with TD. (19,30) These findings align with recent research proposing that individuals with autistic traits had lower serum levels of oxytocin and elevated levels of testosterone and androstenedione. (53) Additionally, two recent meta-analyses identified lower endogenous levels of oxytocin in populations with ASD compared to individuals with TD. (52,54) However, when performing a subgroup analysis by age, this effect disappears, especially in adolescents and adults. Albantakis et al.'s study (2021) (50) also demonstrated no significant differences in baseline levels of salivary and serum oxytocin in adult individuals with ASD.

In addition to assessments of circulating oxytocin, Evenepoel et al. (2023) (19) examined variations in the epigenetic level of the oxytocin receptor gene (OXTR). They observed no statistically significant distinctions in OXTR DNA methylation (mDNA) between the ASD and control cohorts. mDNA is an epigenetic mechanism involved in regulating gene transcription, and an increase in its frequency is associated with a decrease in transcription, resulting in reduced expression and availability of receptors. (55) A systematic review was conducted to identify evidence related to single nucleotide polymorphisms (SNPs) in OXTR, particularly in the context of psychopathologies and socioemotional and behavioral disorders among youth. The results highlighted a direct association between ASD and various SNPs in the OXTR gene, correlating with the diagnosis of ASD, symptom severity, phenotypes, brain

function, and pro-social skills. (56) A recent review aimed at analyzing the correlation between OXTR mDNA and behavioral variations in individuals with ASD identified hypomethylation in children, while adults exhibited hypermethylation. (57)

Evenepoel et al. (2023) (19) did not specify an association between morning or evening oxytocin levels and the degree of severity of ASD symptoms. However, a significant correlation was identified between CBCL competencies and afternoon oxytocin levels in the TD group. This finding indicates a relationship between these levels and more substantial performance. Bakker-Huvenaars et al. (2020) (30), analyzing the association between lower oxytocin levels and the severity and subtype of aggressive behavior and traits of lack of empathy, also did not identify any association. These results contradict recent evidence suggesting that dysfunctions in oxytocin in the CNS may be associated with critical symptoms of social deficits in ASD. (52) A randomized clinical trial demonstrated elevated levels of oxytocin after four weeks of treatment with intranasal oxytocin, associated with reduced OXTR mtDNA and improvement in the sensation of secure attachment. (58)

Another hormone category included in this review was steroid hormones, which play an essential role in neurodevelopment, encompassing aspects such as neural cell proliferation, transmission of nerve signals, management of oxidative stress, and regulation of the immune system. (59,60) Steroid hormones play an additional role in fetal epigenetic elements, influencing the early stages of brain development. (59) Therefore, prenatal and perinatal alterations in steroid pathways can significantly affect neurodevelopment, resulting in conditions such as ASD. (47,61) Considering the pronounced gender bias associated with ASD, several studies suggest the involvement of steroid hormones, especially androgens, in the pathogenesis of the disorder. (14,21,59) There is evidence establishing a connection between increased exposure to androgens during the period of fetal development and elevated risk of ASD, along with persistent implications of these hormones on the human brain and cognitive functions. (14)

Studies indicate that the higher prevalence of ASD is related to increased exposure to testosterone during the prenatal period. (62) However, Bakker-Huvenaars et al. (2020) (30) found no statistically significant differences in testosterone levels between individuals with ASD and those with TD. Majewska et al. (2014) (21) identified a substantial increase in androgen levels in autistic people, signaling the presence of precocious adrenarche leading to precocious puberty. These findings are consistent with the study by Gasser et al. (2019) (63),

which identified higher levels of androgens in the urine of boys with ASD compared to compared to individuals in the control group. Some steroids identified by Majewska et al. (2014) (21) as elevated in the ASD group influence neurodevelopment and brain function, potentially contributing to the pathobiology of ASD. DHEA-C and androsterone-C levels were elevated in all cohorts of children with autism, indicating the potential utility of these steroids as biomarkers for ASD.

Similarly, He et al. (2023) (14) found significantly higher levels of DHEA and pregnenolone in ASD. In contrast to these results, when comparing plasma samples from pre-pubertal boys with ASD and controls, Janšáková et al. (2020) (60) identified reduced levels of pregnenolone sulfate, androsterone, epiandrosterone, and etiocholanolone in the ASD group. Through a logistic regression analysis, He et al. (2023) (14) suggested salivary pregnenolone as a predictor of ASD, indicating that this hormone exhibits good sensitivity in discriminating between individuals with ASD and those with typical development (TD). Therefore, pregnenolone play may be a promising diagnostic biomarker or therapeutic target in individuals with ASD. Pregnenolone acts as an androgen precursor, undergoing conversion to DHEA and, subsequently, to androstenediol, also serving as a precursor to glucocorticoids. (14,60) Recognized as a neurosteroid, pregnenolone plays a vital role in brain development and neural adaptability. Additionally, this hormone exhibits anxiolytic effects and contributes to improving depression symptoms, enhancing social functioning, and attenuating sensory anomalies and cognitive deficits. (60)

Studies in humans and animals have shown a correlation between androgens and a decrease in social interaction. (14,60) Through Spearman correlation analysis, He et al. (2023) (14) identified that pregnenolone was associated with behavioral deficits assessed from the ABC scale. Majewska et al. (2014) (21) found a positive correlation between higher concentrations of most steroids and scores on the CARS scale in autistic girls aged 7 to 9 years. Among autistic boys of the same age group, levels of allopregnanolone and allopregnanolone positively correlated with CARS, while DHEA, DHEA-C, and epiandrosterone levels were negatively correlated. Bakker-Huvenaars et al. (2020) (30) identified that higher levels of testosterone and cortisol were related to characteristics of lack of empathy among individuals with autism.

As mentioned earlier, epigenetic factors may be associated with the pathogenesis of ASD. (35) In this context, a growing category of epigenetic markers encompasses non-

coding ribonucleic acids, such as micro-ribonucleic acids (miRNAs). (10,64) MiRNAs can bridge genetic and environmental contributions by influencing gene expression and regulating messenger RNA (mRNA) translation into functional proteins. (64,65) Studies have linked the activity of miRNAs to neurodevelopment, associating it with cognitive, learning, and neurological developmental disorders. (35,64)

Patterns of dysregulated miRNA expression have been identified in various biomaterials from individuals with ASD, including post-mortem cerebellar cortex, peripheral blood, total blood, olfactory mucosa stem cells, and lymphoblast cell lines. (65,66) Several miRNAs associated with ASD have been identified, most of which are related to cellular respiration and the functioning of the nervous system. (10,32) Among the studied miRNAs in ASD are microbial miRNAs. The high prevalence of immunological and gastrointestinal disorders among autistic people has stimulated research into the intestinal microbiome in this group. (2) Furthermore, environmental risk factors associated with microbial dysbiosis have been related to the etiology of ASD. (18)

Among the salivary miRNAs identified in this review, eight were identified in more than one study. MiRNA-7-5p levels were elevated in the ASD group, as specified in both the Hicks et al. (2016) (33) study and the Sehovic et al. (2020) (32) study. Both studies identified reduced levels of miRNA-23a-3p and miRNA-32-5p in this group. There was a discrepancy regarding miRNA-140-3p levels, which were increased in the Hicks et al. (2016) study (33) and decreased in the Sehovic et al. (2020) study (32). This was also identified for miRNA-628-5p. MiRNA-148a-5p was identified as down-regulated in the Hicks et al. (2020) research (33) and showed a change over time when longitudinally assessed in the Levitskiy et al. (2021) study (34). This discrepancy in results may be justified by the heterogeneity of ASD, differences in diagnostic criteria, collection techniques, sensitivity and specificity of miRNANA detection methods, inadequate control of confounding factors, and differences in statistical methods.

The Levitskiy et al. (2021) (34) study identified seven RNAs associated with scores on VABS-II, AQ, and BASC, as observed in Table 3. Within the longitudinal cohort, 9 RNAs exhibited notable alterations throughout the study. Three of these nine RNAs were also associated with behavioral scores (miRNA-182-5p, miRNA-146b-5p, Staphylococcus). In the Hicks et al. (2020) study (35), miRNA-182–5p levels were also associated with behavioral scores, especially social scores and total scores on ADOS-2. MiRNA-146b-5p, identified by Levitskiy et al. (2021), related to behavioral scores and changing over time after the therapeutic

intervention, was suggested by Hicks et al. (2018) (18) as a possible salivary biomarker to distinguish ASD status in a training set accurately. The microbial miRNANA Staphylococcus, identified in the Hicks et al. (2018) (18) study as a potential diagnostic biomarker for ASD, correlated with the highest number of behavioral scores (BASC Parent Behavioral Symptoms Index; VABS-Composite; VABS-Daily Living Skills; VABS-Socialization) in the Levitskiy et al. (2021) study (34). These results suggest that miRNA-182-5p, miRNA-146b-5p, and Staphylococcus may be used as salivary biomarkers to differentiate between individuals with ASD and neurotypicals for therapeutic monitoring and to provide predictive feedback.

Proteomic analysis comprises a set of analytical approaches used to assess a group of proteins qualitatively and quantitatively. (67) This methodology enables the investigation of isoform diversity, interactions, locations, and modifications, with the potential to aid researchers in identifying biological markers for medical diagnosis, therapeutic management, and understanding the pathophysiological mechanisms of ASD. (67,68) The significant proteins identified as dysregulated in ASD include those involved in inflammation, immune response, and lipid disorders. (3) The immune and nervous systems interact reciprocally. Several studies describe the presence of immunological disorders in individuals with ASD, such as alterations in the function and number of immune cells, as well as variations in the levels of immunoglobulins and cytokines. (24,31,69)

In this study, nine proteins were identified in more than one study, namely Parotid Secretory Protein (PSP), Polymeric immunoglobulin receptor precursor (PIGR), Prolactin-inducible protein precursor (PIP), Transferrin, Lactotransferrin (LTF), Albumin (ALB), Statherin, Histatin, and alpha-amylase. Wormwood et al. (2023) (3) and Ngounou Wetie et al. (2015b) (25) identified elevated levels of Parotid Secretory Protein (PSP). PSP has an affinity for the Bactericidal/Permeability-Increasing protein (BPI), performing antibacterial and anti-inflammatory functions. (25) Additionally, PSP is associated with HDL, suggesting a possible association with cholesterol metabolism, which has been identified as dysregulated in ASD. (70)

PIGR was identified as down-regulated in the Wormwood et al. (2023) study, while it was up-regulated in the Ngounou Wetie et al. (2015a) (26) study. PIGR is involved in transporting immunoglobulins (antibodies) across mucous membranes, playing a crucial role in mucosal immune defense. (71)

PIP was identified as upregulated in the Ngounou Wetie et al. (2015a) (26) study and subsequent research by the same researchers (25). This protein regulates the immune system, antimicrobial functions, apoptosis, and tumor progression. Prolactin and androgens positively influence PIP expression, while estrogens exert a negative regulatory impact. (25) The association of this protein with androgens may explain the high levels found in the saliva of patients with ASD. Although PIP has been positive in ASD, Ngounou Wetie et al. (2015b) (25) observed negative regulation in specific isoforms of this protein. Therefore, even with the global increase in PIP, the presence of isoforms or post-translational variations must also be considered.

Transferrin, a crucial antioxidant protein that binds to iron and acts as a growth factor, was found at reduced levels in Wormwood et al. (2023) (3) and Ngounou Wetie et al. (2015b) (25) studies. Decreased levels of transferrin were also detected in the serum of individuals with ASD in a prior investigation. (72) Interestingly, elevated levels of lactotransferrin were determined by Wormwood et al. (2023) (3) and in the pilot study by Ngounou Wetie et al. (2015a) (26). Lactotransferrin is also associated with iron transport, and increased protein levels may compensate for the deficits observed in transferrin. (25)

Reduced albumin levels were identified in the studies by Wormwood et al. (2023) (3) and Ngounou Wetie et al. (2015b) (25). Albumin plays a crucial role in cerebrospinal fluid (CSF) composition and exhibits various antioxidant and anti-inflammatory properties. (73,74) Additionally, its association with various neurological diseases is attributed to its relationship with the hemodynamic properties of the brain and its neuroprotective effects. (73,75)

The decreased levels of albumin identified in the studies included in this review are consistent with the findings of Morimoto et al. (2023) (74), who identified lower serum albumin levels in children with ASD than in children with typical development (TD). However, these results diverge from those of Ceylan et al. (2021) (76), where no statistically significant differences in albumin levels were observed between the groups of individuals with ASD and the TD control. Justifications for these differences in results include factors associated with sample size and, primarily, the influence of secondary disorders, such as depression and anxiety, common among individuals with autism and subject to the impact of oxidative stress.

Ngounou Wetie et al. (2015a) (26) observed reduced levels of histatin and statherin, while Castagnola et al. (2008) (27) identified hypophosphorylation of these proteins in ASD

patients. As explained by Castagnola et al. (2008) (27), the observed hypophosphorylation in salivary peptides of ASD patients may indicate incomplete phosphorylation of some relevant secretory phosphopeptides still unknown in the CNS. Delayed phosphorylation of the peptide during childhood may result in asynchrony or temporal dysregulation in neuronal maturation, development, or differentiation events, leading to characteristic features of ASD. This phenomenon appears to be more associated with behavioral aspects rather than the overall developmental trajectory of children with ASD. (27)

The alpha-amylase enzyme is involved in starch digestion in the oral cavity. Its production decreases immediately upon waking and gradually increases throughout the day. Additionally, its production can be heightened in response to stress. (23,25) Salivary amylase behavior can be considered an indirect indicator of the autonomic nervous system activity, as its release occurs with autonomic activation. (23) The studies by Ngounou Wetie et al. [OBJ] and Kidd et al. (2012) [OBJ] identified reduced alpha-amylase levels in the saliva of patients with ASD. These results corroborate a previous study that identified decreased concentrations of salivary alpha-amylase and an altered daily pattern in autistic children compared to TD individuals. (77)

The only study that established a correlation between salivary proteome and behavioral characteristics related to ASD was conducted by Samborska-Mazur et al. (2020) (31). This study identified significant associations, including the relationship between salivary levels of RANTES and aggressive behavior, between salivary levels of IL-8 and fixations/stimulations, between gait disturbances and salivary levels of RANTES, and between the absence of active speech and IL-1β. RANTES is essential for recruiting specific types of leukocytes and regulates the Th1 and Th2 immune response mechanisms. (31) Differing from the findings by Samborska-Mazur et al. (2020), Masi et al. (2017) (78) identified increased levels of RANTES in plasma, accompanied by lower proportions of Th1 cells and higher proportions of Th2 cells in children with ASD. These results suggest a cytokine imbalance associated with Th1 and Th2 in ASD. (78) The association between IL-1β and IL-4 and ASD severity was identified in studies of neonatal blood samples. (69) High concentrations of IL-6 and IL-8 have previously been correlated with an ASD phenotype, with elevated IL-8 levels suggesting an association with the presence of intellectual disability. (79)

Another factor associated with the pathogenesis of ASD is oxidative stress. This type of stress results from a disharmony between antioxidants and pro-oxidants, producing free radicals and peroxides. (69) An excess of nitric oxide (NO), a pro-oxidant, can cause neuronal

damage and cell death, subsequently leading to the development of neurological diseases. (80) On the other hand, maintaining adequate NO levels is essential for regulating normal neurological functions. (81) Increased levels of NO result in the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to mitochondrial dysfunction, neuroinflammation, and oxidative stress, which are related to the pathophysiology and severity of ASD symptoms. (81,82) Brain concentrations of nitrite, an endogenous reservoir of NO, influence the occurrence of neurodevelopmental disorders. (28,81) Alongside its metabolites, NO has been identified at significantly higher levels in the blood or CSF of individuals with ASD. (28) Yao et al. (2021) (28) quantified salivary nitrite and explored its relationship with serum NO. The results indicated the presence of salivary nitrite in statistically higher concentrations in participants with ASD than in neurotypical individuals.

Additionally, males with ASD exhibited significantly higher concentrations of NO compared to boys with TD, with no significant difference observed between girls with ASD or TD. Evidence indicates a relationship between NO and estrogen, including estrogen control of endothelial/inducible nitric oxide synthase. These enzymes are essential for younger women's immune and cardiovascular protection, promoting NO production instead of superoxide ($O2^-$). Thus, estrogen seems to protect female children with ASD from dysregulated oxidative stress. (28) These data suggest the possibility of using salivary nitrite as a biomarker to distinguish male children with ASD.

Sialic acid (Sia) was another metabolite analyzed in this review and is an essential nutrient for neurodevelopment and cognitive functions. Sia can be synthesized into polysialic acid (polySia). This glycan is associated with synaptic plasticity and, consequently, brain functions. (83,84) Studies in autistic rodent models indicated that variants of the ST8SIA2 gene, which plays a role in polySia synthesis, were correlated with behaviors resembling those observed in ASD, including decreased social interaction, aggression, and hyperactivity. (29,83)

The study by Demirci et al. (2019) (29) investigated a possible link between salivary Sia levels and ASD. The results pointed to significantly lower Sia levels in participants with ASD compared to TD controls. These findings align with the study by Yang et al. (2018) (84), where plasma Sia levels were considerably higher in the control group. However, a recent study evaluating plasma Sia levels in children with ASD identified that Sia levels in these individuals were significantly higher than in healthy controls. (83) According to Ashaat et al. (2023) (83), discrepancies in results may be related to how Sia fractions were measured, as in their study,

measurements were performed in free and conjugated forms. However, this information needed to be specified in previous studies.

This systematic review has some limitations, specifically a significant heterogeneity in participant characteristics and the methodologies used to collect and analyze biomarkers in the included studies. The absence of a standardized method introduces a potential source of variability in the results, impacting interpretation and future implications, which makes a meta-analysis impossible. Despite the existence of validated questionnaires for behavioral assessment in ASD, the lack of uniformity in the instruments used was also a factor in not carrying out a meta-analysis. Nine studies were classified with a moderate risk of bias, and six presented a high risk, potentially influencing the reliability of the results and diminishing the certainty of the conclusions of this study. Also, the predominantly male sample from the studies may limit the findings to the overall ASD population, given the scarcity of female participants, especially considering gender-specific variations in specific biomarkers, such as hormones.

Moreover, despite the search in multiple databases, the risk of publication bias persists, potentially excluding studies incongruent with the search strategy or those reporting non-significant or negative results, which may have yet to be published. Considering this, the recommendation is for well-designed primary studies following rigorous protocols with clear eligibility criteria, standardized methods for data collection, and statistical analyses. Establishing a methodological standard for management (collection, processing, and storage) and analyzing samples, combined with evaluating behavioral correlations, would significantly enhance the groundwork for future meta-analyses. The comprehensive sharing of demographic and phenotypic data for each sample, encompassing details such as age, gender, medications, and comorbidities, would facilitate the incorporation of covariates and aid in identifying more homogeneous subgroups.

CONCLUSION

Due to the heterogeneity of ASD and considering the results of this review, it is unlikely that the disorder is exclusively linked to a single type of biomarker. The association between different biomarkers may be the best strategy for the diagnosis and monitoring of patients.

The results of this study indicate saliva is a promising tool for identifying biomarkers related to ASD. Discrepant cortisol levels in individuals with ASD suggest the influence of factors in detection, such as collection method and sample volume. Variations in oxytocin levels are associated with ASD, particularly in children. Steroid hormones, especially androgens, represent potential biomarkers related to social deficits. The dysregulated expression of salivary miRNAs, such as miRNA-182-5p, miRNA-146b-3p, and Staphylococcus, is associated with diagnosis and prognosis of ASD. Salivary proteomic analysis identified protein dysregulation related to inflammation, immune response, and lipid disorders. Higher salivary nitrate levels in ASD suggest a potential biomarker, especially in male children. Decreased levels of salivary sialic acid in individuals with ASD suggest a potential area for further investigation.

Although salivary biomarkers have potential for early diagnosis of ASD and behavioral prognosis, challenges and inconsistencies in results highlight the need for further research. Except for miRNAs, all identified biomarkers are nonspecific and may be associated with other disorders. Future studies should consider the heterogeneity of ASD, improve the reliable saliva sample collection and standardize collection and analysis methods, in addition to considering confounding factors, such as the effects of drugs used by these individuals, aiming at the validation and effective implementation of these biomarkers in clinical practice.

Protocol and Registration

This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) checklist. (85) A systematic review protocol was registered on the International Prospective Register of Systematic Reviews (PROSPERO) (86) under the registration number CRD42024504339, according to PRISMA-P. (87)

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Ethics statement

This article contains no studies with human participants or animals performed by authors.

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Supplementary Table 1. Full search strategies

Database	**Query (**Search date: December 4th, 2023)	**Results**
PubMed	(("Autism Spectrum Disorder" [MeSH Terms] OR "Autism Spectrum Disorders" OR "Autistic Spectrum Disorder" OR "Autistic Spectrum Disorders" OR "Autistic Disorder" [MeSH Terms] OR "Disorders, Autistic" OR "Kanner's Syndrome" OR "Kanner Syndrome" OR "Infantile Autism" OR Autism OR "Early Infantile Autism" OR "Asperger Syndrome" [MeSH Terms] OR "Asperger's Disease" OR "Asperger Disease" OR"Asperger Disorder" OR "Asperger Disorders" OR "Asperger's Disorder" OR "Aspergers Disorder" OR "Asperger's Syndrome" OR "Aspergers Syndrome") AND (Biomarkers [MeSH Terms] OR "Biological Marker" OR "Biologic Marker" OR "Biological Markers" OR "Biologic Markers" OR Biomarker OR "Immune Markers" OR "Immunologic Markers" OR "Immune Marker" OR "Immunologic Marker" OR "Serum Markers" OR "Serum Marker" OR "Surrogate Endpoints" OR "Surrogate End Point" OR "Surrogate End Points" OR "Surrogate Endpoint" OR "Clinical Markers" OR "Clinical Marker" OR "Viral Markers" OR "Viral Marker" OR "Biochemical Marker" OR "Biochemical Markers" OR "Laboratory Markers" OR "Laboratory Marker" OR "Surrogate Markers" OR "Surrogate Marker")) AND (Saliva [MeSH Terms] OR Salivas)	60
Embase	("Autism Spectrum Disorder" OR "Autism Spectrum Disorders" OR "Autistic Spectrum Disorder" OR "Autistic Spectrum Disorders" OR "Autistic Disorder" OR "Disorders, Autistic" OR "Kanner Syndrome" OR "Infantile Autism" OR Autism OR "Early Infantile Autism" OR "Asperger Syndrome" OR "Asperger Disease" OR "Asperger Disorder" OR "Asperger Disorders" OR "Aspergers Disorder" OR "Aspergers Syndrome") AND (Biomarkers OR "Biological Marker" OR "Biologic Marker" OR "Biological Markers" OR "Biologic Markers" OR Biomarker OR "Immune Markers" OR "Immunologic Markers" OR "Immune Marker" OR "Immunologic Marker" OR "Serum Markers" OR "Serum Marker" OR "Surrogate Endpoints" OR "Surrogate End Point" OR "Surrogate End Points" OR "Surrogate Endpoint" OR "Clinical Markers" OR "Clinical Marker" OR "Viral Markers" OR "Viral Marker" OR "Biochemical Marker" OR "Biochemical Markers" OR "Laboratory Markers" OR "Laboratory Marker" OR "Surrogate Markers" OR "Surrogate Marker") AND (Saliva OR Salivas)	31

Scopus	TITLE-ABS-KEY("Autism Spectrum Disorder" OR "Autism Spectrum Disorders" OR "Autistic Spectrum Disorder" OR "Autistic Spectrum Disorders" OR "Autistic Disorder" OR "Disorders, Autistic" OR "Kanner's Syndrome" OR "Kanner Syndrome" OR "Infantile Autism" OR Autism OR "Early Infantile Autism" OR "Asperger Syndrome" OR "Asperger's Disease" OR "Asperger Disease" OR"Asperger Disorder" OR "Asperger Disorders" OR "Asperger's Disorder" OR "Aspergers Disorder" OR "Asperger's Syndrome" OR "Aspergers Syndrome") AND TITLE-ABS-KEY(Biomarkers OR "Biological Marker" OR "Biologic Marker" OR "Biological Markers" OR "Biologic Markers" OR Biomarker OR "Immune Markers" OR "Immunologic Markers" OR "Immune Marker" OR "Immunologic Marker" OR "Serum Markers" OR "Serum Marker" OR "Surrogate Endpoints" OR "Surrogate End Point" OR "Surrogate End Points" OR "Surrogate Endpoint" OR "Clinical Markers" OR "Clinical Marker" OR "Viral Markers" OR "Viral Marker" OR "Biochemical Marker" OR "Biochemical Markers" OR "Laboratory Markers" OR "Laboratory Marker" OR "Surrogate Markers" OR "Surrogate Marker") AND TITLE-ABS-KEY(Saliva OR Salivas)	86
Lilacs	(“Transtorno do Espectro Autista” OR “Autism Spectrum Disorder” OR “Trastorno del Espectro Autista”) AND (Biomarcadores OR Biomarcador OR Desfecho Substituto OR “Desfechos Substitutos” OR “Endpoints Substitutos” OR “Marcadores Biológicos” OR “Marcadores Bioquímicos” OR “Marcadores Clínicos” OR “Marcadores de Laboratório” OR “Marcadores de Soro” OR “Marcadores Imunológicos” OR “Marcadores Séricos” OR “Marcadores Substitutos” OR “Marcadores Virais” OR “Resultado Substituto” “Resultados Substitutos” OR Biomarkers) AND (Saliva OR Salivas)	14
Web of Science	((TS=("Autism Spectrum Disorder" OR "Autism Spectrum Disorders" OR "Autistic Spectrum Disorder" OR "Autistic Spectrum Disorders" OR "Autistic Disorder" OR "Disorders, Autistic" OR "Kanner's Syndrome" OR "Kanner Syndrome" OR "Infantile Autism" OR Autism OR "Early Infantile Autism" OR "Asperger Syndrome" OR "Asperger's Disease" OR "Asperger Disease" OR"Asperger Disorder" OR "Asperger Disorders" OR "Asperger's Disorder" OR "Aspergers Disorder" OR "Asperger's Syndrome" OR "Aspergers Syndrome")) AND TS=(Biomarkers OR	52

	"Biological Marker" OR "Biologic Marker" OR "Biological Markers" OR "Biologic Markers" OR Biomarker OR "Immune Markers" OR "Immunologic Markers" OR "Immune Marker" OR "Immunologic Marker" OR "Serum Markers" OR "Serum Marker" OR "Surrogate Endpoints" OR "Surrogate End Point" OR "Surrogate End Points" OR "Surrogate Endpoint" OR "Clinical Markers" OR "Clinical Marker" OR "Viral Markers" OR "Viral Marker" OR "Biochemical Marker" OR "Biochemical Markers" OR "Laboratory Markers" OR "Laboratory Marker" OR "Surrogate Markers" OR "Surrogate Marker")) AND TS=(Saliva OR Salivas)	
Google Scholar	“Autism” AND “Biomarker”	100
ProQuest	Summary("Autism Spectrum Disorder" Autism) AND summary(Biomarkers OR "Biological Marker" OR "Biological Markers" OR Biomarker) AND summary(Saliva)	17
OpenGray	("Autism Spectrum Disorder" Autism) AND (Biomarkers OR "Biological Marker" OR "Biological Markers" OR Biomarker) AND (Saliva)	1

Supplementary Table 2. Critical appraisal of case-control studies

Authors	Q.1	Q.2	Q.3	Q.4	Q.5	Q.6	Q.7	Q.8	Q.9	Q.10	% yes/ risk
Evenepoel et al. (2023)	Y	Y	Y	UN	Y	Y	Y	Y	NA	Y	80%/Low
He et al. (2023)	N	Y	Y	UN	Y	N	N	N	NA	Y	40%/High
Bakker-Huvenaars et al. (2020)	UN	Y	Y	UN	UN	Y	Y	Y	NA	Y	60%/Moderate
Muscatello; Corbett (2018)	N	N	Y	UN	Y	Y	Y	Y	NA	Y	60%/Moderate
Majewska et al. (2014)	N	UN	Y	UN	Y	Y	Y	Y	NA	Y	60%/Moderate
Tordjman et al. (2014)	Y	UN	Y	UN	Y	Y	Y	Y	NA	Y	70%/Low
Kidd et al. (2012)	N	Y	Y	UN	Y	Y	Y	Y	NA	Y	70%/Low
Kalemaj et al. (2022)	N	N	UN	Y	Y	N	N	Y	NA	Y	40%/High
Levitskiy et al. (2021)	N	Y	N	Y	Y	N	N	Y	NA	Y	50%/Moderate
Ragusa et al. (2020)	N	N	Y	Y	Y	Y	Y	Y	NA	Y	70%/Low
Sehovic et al. (2020)	N	UN	Y	UN	Y	N	N	Y	NA	Y	40%/High
Hicks et al. (2020)	Y	UN	Y	N	Y	Y	Y	Y	NA	Y	70%/Low
Hicks et al. (2016)	Y	Y	Y	N	Y	Y	Y	Y	NA	Y	80%/Low
Wormwood et al. (2023)	Y	N	Y	Y	Y	N	N	Y	NA	N	50%/Moderate
Mota et al. (2022)	N	UN	Y	UN	Y	N	N	Y	NA	Y	40%/High
Bhat et al. (2021)	N	Y	Y	N	Y	N	N	Y	NA	Y	50%/Moderate
Samborska-Mazur et al. (2020)	N	UN	Y	Y	Y	N	N	Y	NA	Y	50%/Moderate
Ngounou Wetie et al (2015a)	N	N	UN	UN	Y	N	N	Y	NA	UN	20%/High
Ngounou Wetie et al. (2015b)	N	N	UN	UN	Y	N	N	Y	NA	Y	30%/High
Castagnola et al. (2008)	N	N	Y	Y	Y	N	N	Y	NA	Y	50%/Moderate
Yao et al. (2021)	N	Y	Y	Y	Y	Y	Y	Y	NA	Y	80%/Low
Demirci et al. (2019)	N	N	Y	Y	Y	N	N	Y	NA	Y	50%/Moderate

Supplementary Table 3. Critical appraisal of cross-sectional studies.

Authors	Q.1	Q.2	Q.3	Q.4	Q.5	Q.6	Q.7	Q.8	% yes/ risk
Hicks et al. (2018)	Y	Y	Y	**Y**	Y	Y	Y	Y	100%/Low

3 CONCLUSÕES

Devido a heterogeneidade do TEA e considerando os resultados desta revisão, é improvável que este transtorno esteja associado exclusivamente a um único tipo de biomarcador. A associação entre diferentes biomarcadores pode ser a melhor estratégia para a realização do diagnóstico e monitoramento dos pacientes. Os resultados deste estudo indicam a saliva como uma ferramenta promissora para identificar biomarcadores relacionados ao TEA.

Resultados discrepantes nos níveis de cortisol apontam para a influência de diversos fatores na detecção, como método de coleta e volume da amostra. Variações nos níveis de ocitocina apresentam associação com o TEA, principalmente em crianças. Hormônios esteroides, especialmente andrógenos, representam potenciais biomarcadores associados ao diagnóstico e a déficits sociais. A expressão desregulada de miRNAs salivares, como miRNA-182-5p, miRNA-146b-3p, e Staphylococcus, está associada ao TEA. A análise proteômica salivar identificou desregulação em proteínas associadas à inflamação, resposta imunológica e distúrbios lipídicos. Níveis mais elevados de nitrato salivar em autistas sugerem um biomarcador potencial, especialmente em crianças do sexo masculino. A diminuição dos níveis salivares de ácido siálico em indivíduos com TEA aponta para uma possível via de investigação diagnóstica.

Embora os biomarcadores salivares apresentem potencial para o diagnóstico precoce e prognóstico comportamental do TEA, desafios e inconsistências nos resultados ressaltam a necessidade de mais pesquisas. Exceto os miRNAs, todos os biomarcadores identificados são inespecíficos e podem estar associados a outros distúrbios. Estudos futuros devem considerar a heterogeneidade do TEA, tonar a coleta de amostras de saliva mais confiável e padronizar os métodos de coleta e análise. Além disso, é importante considerar fatores de confusão, como os efeitos dos medicamentos utilizados por esses indivíduos, visando a validação e implementação eficaz desses biomarcadores na prática clínica.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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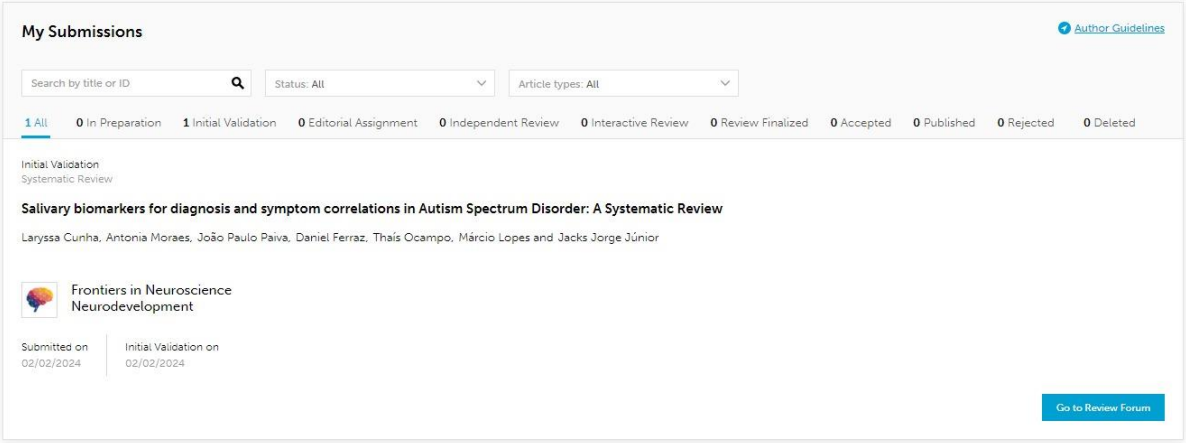
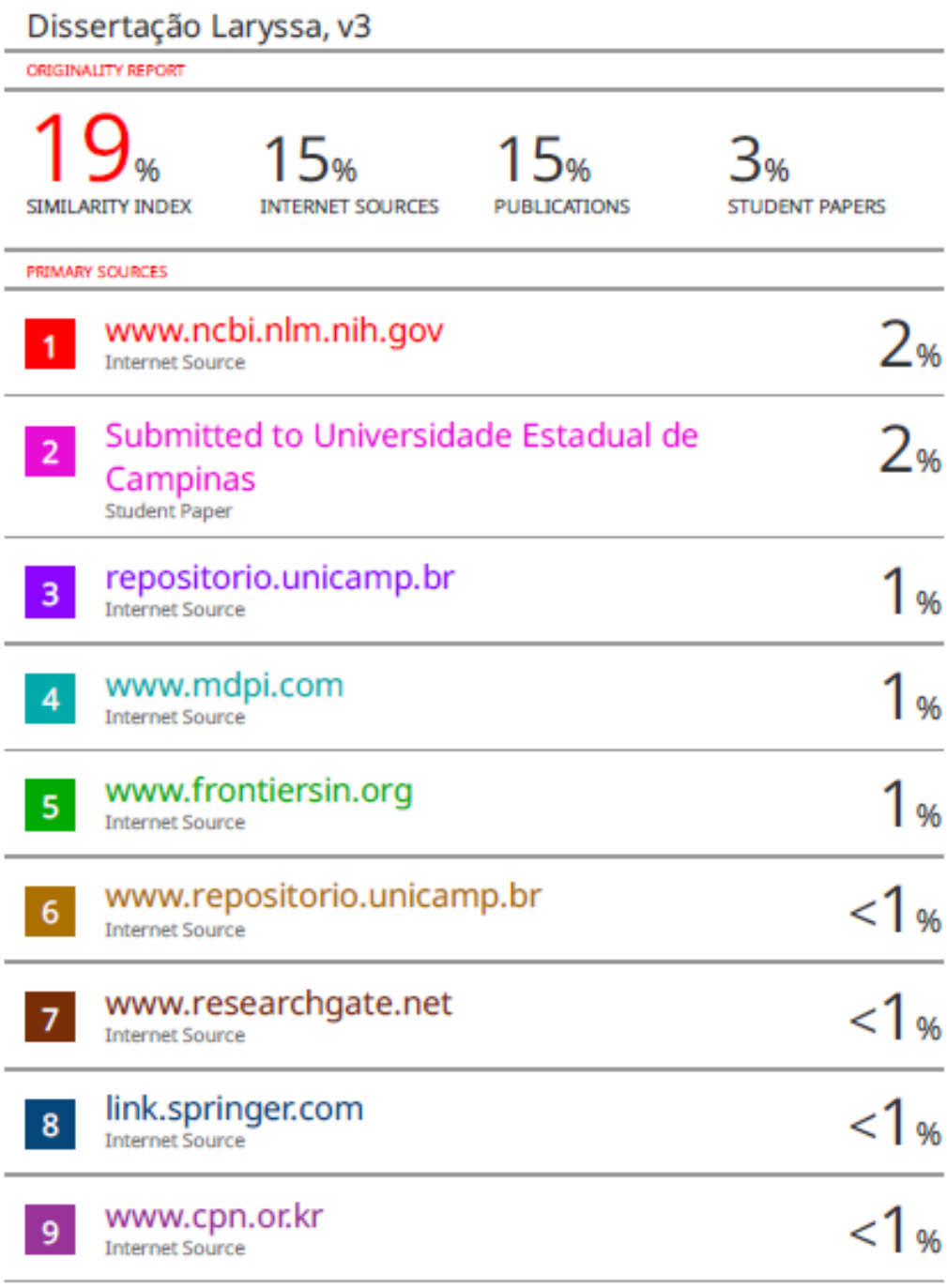
ANEXOS

Anexo 1 - Documento de submissão do artigo (print do sistema online de submissão)



Anexo 2 - Relatório de similaridade da Plataforma Turnitin



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