Plasmatic and salivary biomarkers for early diagnosis of Autism Spectrum Disorder: a systematic review

Biomarcadores plasmáticos e salivares para diagnóstico precoce de Transtorno do Espectro Autista: revisão sistemática

Biomarcadores plasmáticos y salivales para el diagnóstico temprano del Trastorno del Espectro Autista: revisión sistemática

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Abstract
Autistic Spectrum Disorder (ASD) is considered a neurological development disorder characterized by different degrees of deficit in communication, social interaction, and learning, accompanied by repetitive and stereotyped patterns of behavior. ASD diagnosis is extremely complex due to the still unknown etiopathology, the diversity of symptoms presented by the individuals, and it is carried out solely from clinical observations of the individual's behavior. This study aims to review the main plasma and salivary biomarkers currently studied for the early diagnosis of ASD. For this systematic literature review, we used the online data directory and database "Google Scholar" and "Publish Medliner" (PubMed), respectively, with the descriptors: "Autism", "Biomarker", "Diagnostic", "Saliva", and "Plasma". We selected 564 studies in PubMed and 185 in Google Scholar, by screening the titles. After reading the abstracts, we excluded 647 studies, either due to irrelevance or because they were review articles, genetics studies or did not use plasma or saliva samples. The remaining 102 original studies were evaluated in full, and 83 were excluded. Thus, nineteen complete articles that met the inclusion criteria were included in the qualitative analysis. Results identified Cortisol, glutamate/GABA, glutathione, Lipid peroxidation, markers of oxidative stress, mitochondrial dysfunction, and pro-inflammatory cytokines, especially IL-6, as the main plasma and salivary biomarkers currently studied for the early diagnosis of ASD. However, considering that several results were controversial and inconclusive, further studies are needed to validate specific biomarkers as diagnostic tools. The current findings encourage studies that are controlled, multicentric, prospective, and of greater diagnostic precision.
Keywords: Biomarkers; Autism spectrum disorder; Diagnosis; Plasma; Saliva.

Resumo
Transtorno do Espectro Autista (TEA) é uma desordem de desenvolvimento neurológico caracterizada por diferentes graus de déficit na comunicação, interação social, aprendizagem, acompanhado por padrões repetitivos e estereotipados de comportamento. O diagnóstico de TEA é extremamente complexo devido à etiopatologia ainda desconhecida e à diversidade de sintomas apresentados pelos indivíduos, sendo realizado unicamente a partir de observações clínicas do comportamento do indivíduo. Este estudo tem como objetivo revisar os principais biomarcadores plasmáticos e salivares atualmente estudados para o diagnóstico precoce de TEA. Para esta revisão sistemática de literatura, foram utilizados o diretório e a base de dados online "Google Scholar" e "Publish Medliner" (PubMed), respectivamente, com os descritores: "Autism", "Biomarker", "Diagnostic", "Saliva" e "Plasma". Foram selecionados 564 estudos no PubMed e 185 no Google Scholar, por meio da triagem dos títulos. Após a leitura dos resumos, foram excluídos 647 estudos,

seja por irrelevância ou por serem artigos de revisão, estudos genéticos ou não utilizarem amostras de plasma ou saliva. Os 102 estudos originais restantes foram avaliados na íntegra e 83 foram excluídos. Assim, dezenove artigos completos que atenderam aos critérios de inclusão foram utilizados na análise qualitativa. Os resultados identificaram cortisol, glutamato/GABA, glutationa, peroxidação lipídica, marcadores de estresse oxidativo, disfunção mitocondrial e citocinas pró-inflamatórias, especialmente IL-6, como os principais biomarcadores plasmáticos e salivares atualmente estudados para o diagnóstico precoce de TEA. No entanto, considerando que vários resultados foram controversos e inconclusivos, mais estudos são necessários para validar biomarcadores específicos como ferramentas diagnósticas. Os achados atuais incentivam estudos de maior precisão diagnóstica, controlados, multicêntricos e prospectivos.
Palavras-chave: Biomarcadores; Transtorno do espectro autista; Diagnóstico, Plasma; Saliva.

Resumen
El Trastorno del Espectro Autista (TEA) es un trastorno del desarrollo neurológico que se caracteriza por diferentes grados de déficit en la comunicación, la interacción social, el aprendizaje, acompañado de patrones de comportamiento repetitivos y estereotipados. El diagnóstico de TEA es sumamente complejo debido a la etiopatología aún desconocida y la diversidad de síntomas que presentan los individuos, siendo realizado únicamente a partir de observaciones clínicas del comportamiento del individuo. Este estudio tiene como objetivo revisar los principales biomarcadores plasmáticos y salivales actualmente estudiados para el diagnóstico precoz de los TEA. Para esta revisión sistemática de la literatura se utilizó el directorio y la base de datos en línea "Google Scholar" y "Publish Medliner" (PubMed), respectivamente, con los descriptores: "Autismo", "Biomarcador", "Diagnóstico", "Saliva" y "Plasma". Se seleccionaron 564 estudios en PubMed y 185 en Google Scholar, mediante la selección de los títulos. Después de leer los resúmenes, 647 estudios fueron excluidos, ya sea por irrelevancia o porque eran artículos de revisión, estudios genéticos o no utilizaron muestras de plasma o saliva. Los 102 estudios originales restantes se evaluaron en su totalidad y se excluyeron 83. Así, se utilizaron en el análisis cualitativo diecinueve artículos completos que cumplieron con los criterios de inclusión. Los resultados identificaron cortisol, glutamato/GABA, glutatión, peroxidación lipídica, marcadores de estrés oxidativo, disfunción mitocondrial y citocinas proinflamatorias, especialmente IL-6, como los principales biomarcadores plasmáticos y salivales actualmente estudiados para el diagnóstico precoz de TEA. Sin embargo, considerando que varios resultados fueron controvertidos y no concluyentes, se necesitan más estudios para validar biomarcadores específicos como herramientas de diagnóstico. Los hallazgos actuales fomentan estudios de mayor precisión diagnóstica, controlados, multicéntricos y prospectivos.
Palabras clave: Biomarcadores; Trastorno del espectro autista; Diagnóstico, Plasma; Saliva.

1. Introduction

Autistic Spectrum Disorder (ASD) is considered a neurological development disorder characterized by deficit in verbal and non-verbal communication, learning, and social interaction, with persistent repetitive and stereotyped patterns of behavior, such as continuous movements, unusual and limited interests, and hypo or hyper-reactivity to sensory stimuli (American Psychiatric Association, 2014). These characteristics vary both in the way and levels of intensity, but they are present in a very particular and heterogeneous way in more than one person. These characteristics influence how each individual relates, expresses him/herself, and behaves (Guedes & Tada, 2015).

According to the World Health Organization (2019), the estimated prevalence of ASD was 1 in 160 children, affecting four times more boys than girls, with a prevalence in caucasians, compared to afro-descendant and Hispanic children (Jarquin et al., 2011; WHO, 2019).

ASD diagnosis has increased significantly in recent decades, emerging as one of the most frequent disorders of neurodevelopment and a major public health problem (Brentani et al., 2013).

The diagnosis of ASD is puzzling and complex, and it is carried out by investigation and clinical observation of the individual's behavior by a multidisciplinary team, with being rarely diagnosed in children younger than 2 years of age (Buemo et al., 2019; Frustaci et al., 2012). The use of previously established criteria is recommended, according to the International Statistical Classification of Diseases and Related Health Problems (ICD-11) and the Manual Statistics and Diagnosis of the

American Psychiatric Association (DSM-V). Neuroimaging and neurofetology exams, as well as genetic studies, also contribute to better understand the neurobiology of autism (Gadia, Tuchman, & Rotta, 2004).

However, there are currently no biological tests or specific markers to confirm this diagnosis (Assumpção & Kuczynski, 2011). This hinders the early identification of ASD and, consequently, delays the start of specific therapeutic and pharmacological interventions, which contribute to the biopsychosocial development and enable a better prognosis, by providing greater independence for these individuals, decreasing the challenges experienced by the family and supplying data to implemente public policies (Magalhães et al., 2021). The lack of knowledge about the etiology is another factor that makes early diagnosis difficult. Several studies suggest that genetic, environmental, inflammatory, immunological, and metabolic factors play a prominent role in ASD (Marchezan et al., 2019).

According to Magalhães et al. (2021), other aspects associated with ASD may justify the difficulty of correct diagnosis, including a high prevalence of mental disorders (70%) or the sum of two or more situations of associated mental disorders (40%). Among the most common comorbidities are intellectual development disorder and/or communication and attention disorder, hyperactivity, anxiety, depression and epilepsy (Hyman, Levy, & Myers, 2020).

Several research studies seek to establish an easy, fast, and economical diagnosis protocol for ASD. Biomarkers have emerged as biological indicators that can distinguish different groups of diseases or products associated with a given condition. These biomarkers must be able to perform an early diagnosis of ASD. In addition, a possible definition of subgroups of these individuals would assist in the prognosis and also predict a more favorable response to a particular type of treatment (Anderson, 2015).

Numerous biochemical biomarkers have been studied using blood, urine, hair, and saliva samples, including neurotransmitters, hormones, and immune and inflammatory markers (Bjørklund et al., 2018). However, scientific, ethical, clinical, and practical issues still represent a major challenge for the use of these biomarkers in the clinical area (Walsh et al., 2011). One of these challenges is due to the fact that ASD is a highly idiopathic and heterogeneous disorder, requiring a diversity of different markers for the ASD diagnosis and making one-dimensional studies extremely challenging in terms of reproducibility (Hewitson et al., 2021).

Saliva is a biological fluid that has several functions, such as protecting and cleaning the oral mucosa, as well as antibacterial action and initiation of the digestive process in the oral cavity. The progress of diagnostic techniques demonstrated that saliva presents biological markers capable of detecting numerous oral and systemic diseases, beyond monitoring the evolution of these pathologies and the dosage of some drugs (Zhang et al., 2016).

On the other hand, plasma is the most used material for analysis of biomarkers due to the high concentration of markers present in this material. As an alternative to blood testing, saliva contains most of the blood components and both are functionally equivalent. Therefore, its use for the diagnosis of various disorders, such as ASD, is extremely relevant, since it is a fast, easy, safe, low-cost, and highly sensitive method. In addition, saliva collection does not use invasive or sharp drill procedures, responsible for skin lesions and transmission of infectious diseases (Pandey et al., 2014; Samaranayake, 2007). Furthermore, the procedure to collect the saliva triggers less anxiety in people, a very important factor when ASD is suspected (Putnam et al., 2012).

Considering the importance of diagnosing ASD to institute intervention actions, minimizing the losses in the development of these individuals, this study aims to review the main plasma and salivary biomarkers currently studied for the early diagnosis of ASD.

2. Methodology

For this systematic literature review addressing the theme "Plasma and salivary biomarkers for the diagnosis of Autistic Spectrum Disorder (ASD)" we used the online data directory and database "Google Scholar" and "Publish Medliner" (PubMed), respectively. The following descriptors were used in a combined manner: "Autism", "Biomarker", "Diagnostic", "Saliva", and "Plasma". Considering the complexity of the subject, no deadline was set for the study to be eligible for analysis. Duplicate titles were removed after initial search and all the reference lists of the identified studies were reviewed by hand. The Impact Factor (IF) survey of the journals where the articles were published served as a qualitative analysis of the selected studies.

The eligibility criteria for study selection focused on original clinical research conducted with plasma and saliva samples with the objective of mapping possible biochemical biomarkers for ASD. Only biomarkers that were the object of at least 2 independent studies were included. Studies that did not use plasma and saliva, genetic studies and those that did not aim at diagnosing ASD were excluded.

Studies were selected for further data extraction after screening the titles and the abstracts based on the eligibility criteria listed above. After a full analysis, studies with promising findings of diagnosis biomarkers for clinical applicability and with validated techniques to measure biomarkers and to diagnose ASD were included in this review. Data on the study sample, researched biomarkers and the outcome measures were extracted and organized in a table for better understanding.

3. Results

The study selection process is shown in Figure 1. Firstly, there were 564 selected studies in PubMed and 185 in Google Scholar, by only screening the titles. After screening the abstracts from all identified studies, we excluded 647 articles, either due to irrelevance or because they did not fulfil the inclusion criteria.

The remaining 102 original studies were evaluated in full and 83 were excluded beacuse they evaluated biomarkers without previous studies or had inconclusive results. Thus, nineteen complete articles met the inclusion criteria and, therefore, were included in the qualitative analysis of the systematic review. Relevant articles published until November 2019 were included and the IF overall mean of the chosen articles was 4.19 ± 4.21, ranging between 0.678 (minimum) and 16.193 (maximum).

Figure 1. Flowchart of the literature search and study selection.

Descriptors: "Autism", "Saliva" and "Plasma", "Biomarker" and "Diagnostic"

(Present in the title or summary)
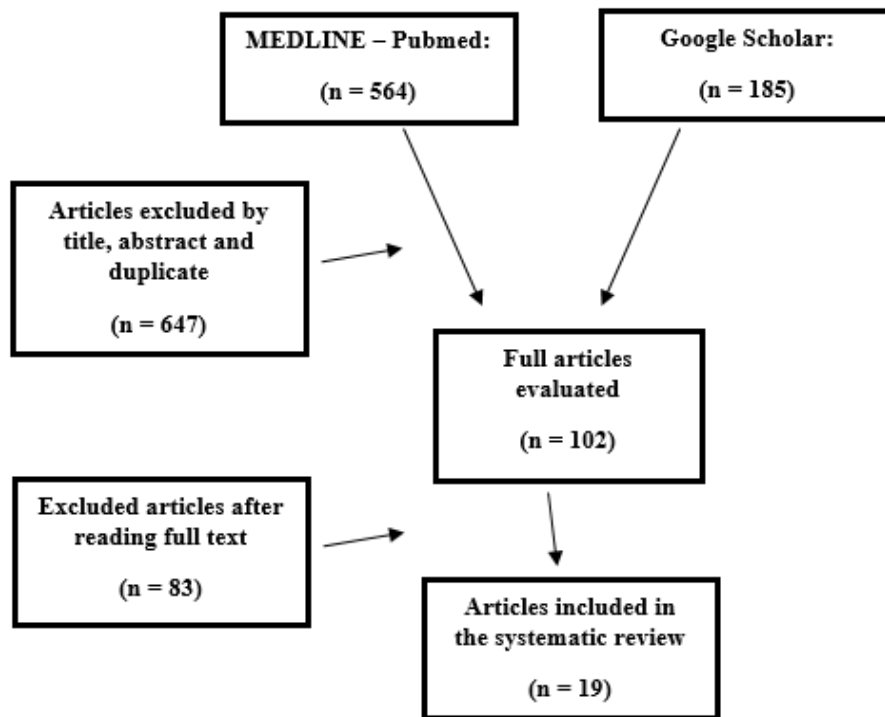
Source: Authors (2020).

After a careful analysis of the articles found, nineteen publications were selected and organized by the main points of the study, following the lines of reasoning of the authors, in order to allow greater understanding of the content, as shown in the following Table 1.

Of the nineteen articles included, fourteen studies analysed plasma samples, four analysed saliva samples and one analysed both. In total, we included 679 individuals with ASD in this review. The mean age of participants ranged from 3.3 to 15.4 years old (range: 2–19 years old), except one studied that ranged between 24-72 years old. The male percentage ranged from 50% to 100%. Only seven studies reported race or ethnicity of the subjects, with the majority being caucasians.

All autistic children were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders (DSM) after extensive interviews with multiple professionals, including psychiatrists, neurologists, psychiatrists or pediatricians. Other diagnostic methods and severity assessment used were Autism Diagnostic Interview-Revised (ADI-R), Autism Behavior Checklist (ABC), Autism Diagnostic Observation Schedule (ADOS), Childhood Autism Rating Scale (CARS) and questionnaires develop by authors.

The sampling and processing methods varied among studies. Seventeen articles required fasting prior to collecting plasma and saliva samples, ranging between 1 to 12 hours, and among them, two studies with saliva also required not brushing teeth. One study required rinsing the mouth before saliva sample collection. In eleven of them, the saliva (3) and blood (8) were sampled in specific times of the day. The volume of saliva sample collected varied from 1 mL to around 6 mL and the mean volume of blood sample collected was 10 mL.

The saliva collection was performed as unstimulated in all of the studies. The techniques used vary between spitting directly into a tube, passive drooling, and Swab-based sampling.

Table 1. Characterization of the articles selected for the study.

Authors and year of publication	Article Title	Journal name	Objective	Sample	Biomaker	Conclusion (in individuals with ASD)
Warren et al., 1994	Decreased Plasma Concentrations of the C4B Complement Protein in Autism	Archives of Pediatrics and Adolescent Medicine (JAMA Pediatrics)	To determine complement C4 protein concentrations in the plasmas of autistic subjects and their family members.	**Plasma**	C4b-binding protein	Decreased C4b concentration.
Modahl et al., 1998	Plasma Oxytocin Levels in Autistic Children	Biological Psychiatry	To measure oxytocin levels in autistic children and normal controls, and to examine the association of OXT levels and social behaviors in both groups.	**Plasma**	Oxytocin (OXT)	Lower oxytocin levels in individuals with ASD.
Dhossche et al., 2002	Elevated plasma gamma-aminobutyric acid (GABA) levels in autistic youngsters: stimulus for a GABA hypothesis of autism	Medical Science Monitor	Pilot study to measure plasma GABA levels in youngsters with Attention-Deficit Hyperactivity Disorder (ADHD) and Autistic Disorder.	**Plasma**	Gamma-aminobutyric acid (GABA)	Increased plasma levels of GABA.
Al-Gadani, El-Ansary, Attas, & Al-Ayadhi, 2009	Metabolic biomarkers related to oxidative stress and antioxidant status in Saudi autistic children	Clinical Biochemistry	To measure oxidative stress and antioxidant-related parameters (enzymatic and non-enzymatic) in Saudi autistic children.	**Plasma**	Lipid peroxidation, vitamin E, vitamin C, glutathione, glutathione peroxidase (GSH-Px), catalase, and superoxide dismutase (SOD)	Increased lipid peroxidation, glutathione peroxidase, and SOD. There was a decrease in vitamin E and glutathione. The catalase remained unchanged.
Geier et al., 2009	A Prospective Study of Transsulfuration Biomarkers in Autistic Disorders	Neurochemical Research	To evaluate transsulfuration metabolites in participants diagnosed with ASD.	**Plasma**	Reduced glutathione (GSH), oxidized glutathione (GSSG), cysteine, taurine, sulfate, and free sulfate	Decreased levels of GSH, cysteine, taurine, sulfate, and free sulfate. Increased GSSG levels.
Croonenberghs et al., 2010	Serum testosterone concentration in male autistic youngsters	Neuroendocrinology letters	To study morning concentrations of serum testosterone in a very homogenic group of postpubertal youngsters with autism and a group of normal controls.	**Plasma**	Testosterone	Decreased plasma testosterone concentration.

Naushad et al., 2013	Autistic children exhibit distinct plasma amino acid profile	Indian Journal of Biochemistry and Biophysics	To investigate the plasma amino acids in a new cohort of autism cases and controls with increased sample size, in order to provide better understanding of the metabolic basis for autism.	**Plasma**	Amino Acids	High levels of excitatory amino acids (glutamate and asparagine) and decreased levels of essential amino acids (phenylalanine, tryptophan, and methionine) and neurotransmitter precursors (tyrosine and tryptophan).
El-Ansary & Al-Ayadhi, 2014	GABAergic/glutamatergic imbalance relative to excessive neuroinflammation in autism spectrum disorders	Journal of Neuroinflammation	To elucidate the relationship between glutamatergic/ GABAergic imbalance and neuroinflammation as two recently discovered autism-related etiological mechanisms.	**Plasma**	Glutamate, GABA, and glutamate/GABA ratio as an excitotoxicity marker along with TNF-α, IL-6, IFN-γ, and IFI16 as neuroinflammation markers	Higher concentrations of glutamate and GABA and lower levels of the glutamate/GABA ratio. TNF-α and IL-6 were significantly lower, while IFN-γ and IFI16 were notably higher.
Yang et al., 2015	The roles of cortisol and pro-inflammatory cytokines in assisting the diagnosis of autism spectrum disorder	Research in Autism Spectrum Disorders	To evaluate diurnal variation of cortisol (cortisol VAR), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-a) as potential biomarkers for ASD.	**Saliva (cortisol) and Plasma (IL-6 e TNF-α)**	Diurnal variation of cortisol (Cortisol VAR), Interleukin 6 (IL-6), and Tumor Necrosis Factor alpha (TNF-α)	Decreased salivary levels of cortisol VAR and increased plasma levels of IL-6 and TNF-α.
Ratajczak & Sothern, 2015	Measurement in saliva from neurotypical adults of biomarkers pertinent to autism spectrum disorders	Future Science OA	To measure biomarkers pertinent to autism in saliva from humans	**Saliva**	CD26, IL-12, carnitine, C4b, reduced glutathione (GSH) and oxidized glutathione (GSSG), MT-2, testosterone, IFN-γ, cysteine, glutamine, glutamic acid, GABA, serotonin, melatonin, and cortisol	Lower levels of CD26, C4b, carnitine, glutamine, glutamic acid, GABA, melatonin, cortisol, reduced glutathione (GSH), MT-2, and cysteine were found, as well as high levels of IL-12, total glutathione (GSSG), IFN-γ, serotonin, and testosterone.
Ngounou Wetie et al., 2015	A Pilot Proteomic Analysis of Salivary Biomarkers in Autism Spectrum Disorder	Autism Research	To optimize methods for salivary proteomic biomarker discovery and to identify initial putative biomarkers in people with ASDs.	**Saliva**	Salivary proteins	Increased levels of prolactin-inducible protein, lactotransferrin, Ig kappa chain C region, Ig gamma-1 chain C region, Ig lambda-2 chain C regions, neutrophil elastase, and polymeric immunoglobulin receptor.

Yang et al., 2015	The combined role of serotonin and interleukin-6 as biomarker for autism	Neuroscience	To evaluate the alterations of 5-HT and IL-6 levels in autism and their roles as potential biomarkers for autism.	**Plasma**	Serotonin (5-HT) and interleukin-6 (IL-6)	Increased levels of 5-HT and IL-6.
Cai et al., 2016	Elevated plasma levels of glutamate in children with autism spectrum disorders	NeuroReport	To assess the clinical significance of plasma glutamate levels in ASD.	**Plasma**	Glutamate	Increased plasma levels of Glutamate. Higher levels with increasing severity of ASD.
Cortelazzo et al., 2016	Expression and oxidative modifications of plasma proteins in autism spectrum disorders: interplay between inflammatory response and lipid peroxidation	Proteomics – Clinical Applications	To detect plasma protein changes in subjects with ASDs, in order to explore possible changes in expression and/or oxidative status of potential biomarkers of key physiopathological mechanisms.	**Plasma**	Plasma proteins	Alterations in 12 proteins, 10 of which are involved in the acute inflammatory process. Significant changes in 2 immunoglobulins alpha and gamma chains. Increased lipid peroxidation.
El-Meshad et al., 2017	The plasma zinc/serum copper ratio as a biomarker in children with autism spectrum disorders	Menoufia Medical Journal	To assess the plasma zinc (Zn)/serum copper (Cu) ratio as a biomarker in children with ASD.	**Plasma**	Zinc, Copper and the Zinc/Copper ratio	Decreased zinc concentration, increased copper concentration and decreased zinc/copper ratio.
Khemakhem et al., 2017	Novel biomarkers of metabolic dysfunction is autism spectrum disorder: potential for biological diagnostic markers	Metabolic Brain Disease	To examine a wide variety of plasma biomarkers of mitochondrial metabolism and the related abnormalities of oxidative stress and apoptosis in ASD.	**Plasma**	Pyruvate, creatine kinase, complex 1, glutathione S-Transferase, glutathione, Caspase 7, and lactate dehydrogenase	Alterations in the mentioned biomarkers. Changes in complex 1 and glutathione S-Transferase were observed only in the most severe cases.
Bakker-Huvenaars et al., 2018	Saliva oxytocin, cortisol, and testosterone levels in adolescent boys with autism spectrum disorder, oppositional defiant disorder/conduct disorder and typically developing individuals	European Neuropsychopharmacology	To compare levels of oxytocin, cortisol, and testosterone in adolescents with ASD or oppositional defiant disorder (ODD)/conduct disorder (CD), and in typically developing individuals (TDI), and relate hormone levels to severity and subtype of aggression and callous-unemotional (CU) traits.	**Saliva**	Oxytocin (OXT), Cortisol and Testosterone	Lower oxytocin concentration and unchanged cortisol and testosterone concentrations.

Deshpande et al., 2019	Comparative evaluation of salivary zinc concentration in autistic and healthy children in mixed dentition age group-pilot study	Indian Journal of Dental Research	To compare and evaluate salivary zinc concentration in saliva samples of autistic and healthy children in mixed dentition age group.	**Saliva**	Zinc	Decreased salivary zinc concentration.
Alzghoul et al., 2019	The association between levels of inflammatory markers in autistic children compared to their unaffected siblings and unrelated healthy controls	Turkish Journal of Medical Sciences	To identify a possible association between interleukin-6, -8, -9, and -10 and tumor necrosis factor alpha (TNF-α) in autism among Jordanian children by comparing the plasma levels of these cytokines with their unaffected siblings and unrelated healthy controls.	**Plasma**	Interleukin -6, -8, -9, and -10 and TNF-α	Increased concentrations of IL-8 and TNF-α. Unchanged IL-9 and IL-10 levels. Increase in IL-6, which is not a specific biomarker.

Source: Direct research (2020).

4. Discussion

The purpose of this systematic review was to analyze the existing literature on plasmatic and salivary biomarkers for ASD, considering that it is crucial to find an easy and safe method to perform, also inexpensive, preferably non-invasive, and able to detect biomarkers in the early stage of ASD. Although specific biomarkers of ASD in plasma and saliva are still unknown, they have proven to be valid candidates for that purpose.

Probably, the great variety of ASD biomarkers researched in the evaluated studies is due to their pathophysiology and etiology still unknown. In addition, there was a high prevalence in the use of plasma samples. On the other hand, studies carried out with saliva samples aimed to detect the presence of hormonal changes, mainly cortisol and proteins, as specific biomarkers for this disorder.

Some studies suggest a link between ASD and dysfunctions of inflammatory metabolic factors, especially those related to neuroinflammation and hormonal stress, as well as to enzymatic, mitochondrial, oxidative and toxic changes (Boris et al., 2007; Ghaleiha et al., 2015; Marchezan et al., 2019).

Al-Gadani et al. (2009) suggested that oxidative stress would play a relevant role in the interaction between environmental, genetic, and immunological factors. The authors assessed parameters related to oxidative stress and antioxidant enzymes in children diagnosed with ASD as possible biomarkers for the disorder. The results showed that, differently from the neurotypical individuals, those with ASD presented increased expression of superoxide dismutase (SOD) associated with decreased of glutathione (GSH), while catalase level was unchanged.

Geier et al. (2009) studied the metabolites of transulfurization present in individuals with ASD and neurotypicals, including glutathione and its derivatives. The authors observed increased oxidative stress and decreased detoxification capacity, especially of mercury, in ASD individuals, validating these metabolites as possible diagnostic biomarkers.

As reported in several studies, low levels of glutathione may be associated with many of the autistic symptoms, resulting from several changes such as: increased oxidative stress, lipid peroxidation and inflammatory processes (Kern & Jones, 2006; Marí et al., 2013); immune dysfunctions, such as impaired or altered immune response and dysregulation of inflammatory cytokines (Cohly & Panja, 2005; Kern & Jones, 2006); and reduced gastrointestinal integrity, with increased permeability in this tissue (Furlano et al., 2001; Kern & Jones, 2006; Sen, 1997); and toxicity (Kern & Jones, 2006).

Futhermore, Al-Gadani et al. (2009) and other studies suggest that the formation of reactive oxygen species could act as a second messenger in the activation of several brain injury-inducing reactions, as Purkinje cell loss and increased brain volume, inducing the autistic disorder (Costa, Carvalho, & Bezerra, 2020; Kern & Jones, 2006).

Al-Gadani et al. (2009) also noticed that the increased reactive oxygen species and lipid peroxidation could be correlated to mitochondrial dysfunction, once the loss of mitochondrial membrane potential and impairment of electron transport chain activity (ETC) are secondary to the peroxidation. They observed a decrease of vitamin E level, which is an antioxidant that protects membranes from oxidation. Since the brain contains high levels of oxidizable lipids, a vitamin E supplementation for autistic patients may permit a better prognosis.

In turn, Khemakhem et al. (2017) analyzed biomarkers for dysfunctions of mitochondrial metabolism, oxidative stress and apoptosis in individuals with ASD, demonstrating changes in pyruvate, creatine kinase, complex 1, glutathione S-transferase, glutathione, caspase-7, and lactate dehydrogenase. Only the most severe ASD cases showed alterations in complex 1 and glutathione S-transferase. The authors also stated that caspase-7 is sensitive and specific to differentiate individuals diagnosed with ASD and neurotypical individuals, suggesting the use as of this biomarker as a possible tool for early diagnosis.

Ratajczak and Sothern (2015) evaluated a wide and varied range of possible biomarkers involving gastrointestinal, immunologic, neurologic, and toxicologic systems. The markers included CD26, IL-12, carnitine, C4b, reduced glutathione (GSH), oxidized glutathione (GSSG), MT-2, testosterone, IFN-γ, cysteine, glutamine, glutamic acid, GABA, serotonin, melatonin, and cortisol. This study demonstrated the effectiveness of using saliva sample seeking specific metabolic markers for ASD. The results showed that ASD present high levels of oxidative stress biomarkers, which associated with a glutamatergic immune response and a pineal gland dysfunction, will possibly provide biomarkers for the disorder.

The pineal gland dysfunction is associated with alterations of the melatonin production, one of the most important endogenous antioxidants found in brain tissues and in the body. Lower melatonin levels can lead to oxidative stress and neuronal damage (Shomrat & Nesher, 2019), affecting cognition and behavior. Therefore, the administration of melatonin has great potential in the treatment of sleep disorders in ASD individuals (Ratajczak & Sothern, 2015; Schwichtenberg & Malow, 2015; Tilford et al., 2015).

In addition, reduced levels of the C4b binding protein have been detected in saliva (Yang et al., 2015) and plasma (Warren et al., 1994) samples from individuals with ASD, which may have implications for the immune system. Serotonin (5-HT) levels were also increased in saliva, similar to the results observed with plasma samples (Yang et al., 2015). Along with increased Interleukin 6 (IL-6), these results are associated with greater severity in cases of ASD (Yang et al., 2015).

The results of the selected studies demonstrated a wide variety of biomarkers that are altered in both plasma and saliva samples. The composition of a panel with the biomarkers that stood out most in these studies can be a proposal for the laboratory diagnosis of ASD, where altered levels in the results obtained would indicate the diagnosis of ASD (Gabriele et al., 2014; Ratajczak & Sothern, 2015).

The immune system and pro-inflammatory cytokines, especially IL-6, may play an important role in the pathogenesis of autism. One theory points out that immune activation, caused by severe infections during pregnancy, increases the risk of developing ASD in the child, since maternal immunoglobulins cross the placenta and disturb brain development by targeting fetal brain tissue (Frye et al., 2019).

Yang et al. (2015) observed an increase in plasma levels of IL-6 and Tumor Necrosis Factor (TNF-α) and a decrease in anti-inflammatory cytokines. IL-6 is normally expressed at low levels in the brain; however, in the presence of brain injury or inflammation, IL-6 is elevated (Gadient & Otten, 1997; Wei et al., 2011). Chronic overexpression of IL-6 may cause neuroanatomical and neurophysiological alterations associated with the pathogenesis of neurological diseases, such as impairment in adhesion and migration of neural cells and imbalance of excitatory and inhibitory synapses (Gruol & Nelson, 1997; Wei et al., 2011).

Alzghoul et al. (2019) also reported an IL-6 increase in the plasma sample of ASD individuals, but as a non-specific biomarker. Additionally, the authors observed an increase in IL-8 and TNF-α. In contraposition, El-Ansary and Al-Ayadhi (2014) reported results from a study where the neuroinflammatory markers Tumor Necrosis Factor (TNF-α) and Interleukin 6 (IL-6) were significantly lower in plasma in individuals with ASD, while Interferon-gamma (IFN-γ) was notably higher.

Testosterone hormone also presented an increased in its concentration, diverging from other studies that either related no change or observed a decrease in the plasma testosterone concentration (Bakker-Huvenaars et al., 2018; Croonenberghs et al., 2010). These conflicting results could be justified by variable levels of testosterone according to age range and sex (Bakker-Huvenaars et al., 2018; Ratajczak & Sothern, 2015).

Cortisol is another hormone intensively studied. It is associated with stress control and inflammatory processes, besides contributing with the immune system regulation (Caetano Júnior, Castilho, & Raniero, 2017), also acting in the emotional

development and functioning (Ratajczak & Sothern, 2015). A decrease in salivary cortisol levels was observed in the studies by Ratajczak and Sothern (2015) and Yang et al. (2015), indicating a hypothalamic-pituitary-adrenal axis (HPA) dysfunction. However, Bakker-Huvenaars et al. (2018) found unchanged values of salivary cortisol in individuals with ASD, demonstrating an inconsistency of cortisol as a biomarker.

Oxytocin (OXT) is another relevant hormone avaliated in few studies with ASD individuals. The action of oxytocin was initially associated with the contractions for childbirth and breastfeeding. Modahl et al. (1998), using plasma, and Bakker-Huvenaars et al. (2018), using saliva samples, have reported that autistic children had significantly lower OXT levels. The recognition of OXT as an important regulator of human social behaviors has increased in recent times (Cochran et al., 2013). Studies have shown that nasal oxytocin can act as an important regulator of human social behavior, showing improvement in social interaction in patients with ASD (Aita et al., 2019). The detection of oxytocin in both plasma and saliva samples confirm that it is possible to use a non-invasive source, as saliva, for monitoring central neuroendocrine function in ASD (Carter et al., 2007).

Another research pathway evaluates the detection of neurotransmitters and amino acids as biomarkers for diseases diagnosis. Glutamate is the most abundant free amino acid in the brain and is the main excitatory neurotransmitter in the central nervous system (Zhou & Danbolt, 2014). Glutamate actively participates in several neurological development processes, along with gamma-aminobutyric acid (GABA), which acts as an inhibitory neurotransmitter. These processes are mainly related to memory and learning modulation, activities in which individuals with ASD have deficiencies, indicating changes that make the analysis of this neurotransmitter relevant (Cai et al., 2016).

Naushad et al. (2013), El-Ansary and Al-Ayadhi (2014), and Cai et al. (2016) evaluated plasma samples from individuals with ASD and reported an increase in glutamate levels, which may induce hyperexcitability in post-synaptic neurons until cytotoxicity. On the other hand, Dhossche et al. (2002) and El-Ansary and Al-Ayadhi (2014) observed an increase in plasma GABA levels in a study with saliva samples, diverging from the data presented by Ratajczak and Sothern (2015), who observed a decrease in the salivary concentration of GABA. These studies also demonstrated a dysfunction between the gabaergic and glutamatergic systems early in development, leading to a severe excitatory / inhibitory imbalance in neuronal circuits that may be responsible for the behavioral deficits seen in patients with autism (El-Ansary & Al-Ayadhi, 2014).

Zinc has aroused interest as a possible biomarker for autism. This is one of the most prevalent metal ions in the brain and participates on the regulation of neurogenesis, neuronal migration, and differentiation, shaping cognitive development and maintaining brain function healthy (Deshpande et al., 2019). Zinc deficiency can lead to neurophysiological changes, having a role in the pathogenesis of autism. The zinc (Zn) and copper (Cu) metabolism is important for detoxification of heavy metals, including mercury (Hg), which its toxicity may be a major cause of metallothionein dysfunction in ASD children.

In individuals with ASD, there is a decrease in salivary (Deshpande et al., 2019) and plasma (El-Meshad et al., 2017) concentrations of this ion, in addition to an increase in copper concentration, which may cause toxicity, and a consequent decrease in the zinc/copper ratio. Thus, this is the reason zinc/copper can be considered a potencial ASD diagnostic biomarker, but is not a promising severity marker (El-Meshad et al., 2017).

Finally, there is an increase in studies using proteomic analysis, mainly in saliva, in order to detect markers for early ASD diagnosis. Ngounou Wetie et al. (2015) reported an increase in several proteins related to changes in the immune system, oxidative stress and lipid and cholesterol metabolism. In turn, Cortelazzo et al. (2016) reported changes in 12 plasma proteins, 10 of which were involved in the acute inflammatory process, besides the increase in lipid peroxidation, also observed in the studies by Al-Gadani et al. (2009).

We observed that the results of studies searching biomarkers for the early diagnosis of ASD demonstrate a scenario in which the absence of consensus on the feasibility of the immediate application of these data prevails, regardless of the fluid analyzed. The reduced knowledge on the etiopathogenesis and pathophysiological mechanisms of ASD may be some of the reasons. There are countless divergences, and many of them are related to the detected concentration of the different studied markers. Therefore, all alternatives presented must be carefully evaluated and new biomarkers researched, considering that late diagnosis of ASD leads to developmental losses in these individuals, many of which are irreversible.

5. Conclusion

This systematic review identified Cortisol, glutamate/GABA, glutathione, Lipid peroxidation, markers of oxidative stress, mitochondrial dysfunction, and pro-inflammatory cytokines, especially IL-6, as the main plasma and salivary biomarkers currently studied for the early diagnosis of ASD. However, considering that several results were controversial and inconclusive, further studies are needed to validate specific biomarkers as diagnostic tools. The current findings encourage studies that are controlled, multicentric, prospective and of greater diagnostic precision.

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