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A BRIEF REVIEW OF SALIVA BIOMARKERS AS A DIAGNOSTIC TOOL FOR AUTISM SPECTRUM DISORDER (ASD)

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ABSTRACT

The diagnosis of autistic spectrum disorder (ASD) remains clinical to this date. Immune dysfunction has been a recognized feature in ASD, and several researchers suggest that it can be used as a diagnostic tool by detecting biomarkers as well as an effective route for pharmacological intervention. The molecular biomarkers obtained from biological fluids are gaining relevance because of its lower invasiveness and ease of collection. Patients with autism are characterized by sensory reactivity and behavioral difficulties that can make sample collection problematic and, in this context, saliva appears to be a viable alternative for obtaining relevant biological information, being also especially indicated for children due to its painless and non-invasive sampling characteristics. Also, the saliva represents a valuable resource for studying possible biomarkers of autism. Following is a brief description of the main works published in recent years on saliva biomarkers for the diagnosis of autism.

INTRODUCTION

Autistic spectrum disorder (ASD) is a neurological development disorder characterized by behavioral dynamics characterized by deficits in communication, social interaction, and restricted and repetitive patterns of behavior, interests, and diverse activities (American Psychiatric Association, 2013). One of the most challenging aspects of ASD remains its etiology. The suspected causes of ASD are as diverse as the spectrum itself, and presumably reflect the intrauterine environment, the child's early life, and genetic inheritance (Tchaconas and Adesman, 2013). The accumulated evidence suggests that genetic, environmental, inflammatory, immunological, and metabolic factors play a prominent role in the disorder (Ghaleiha *et al.*, 2015). Traditionally, research in psychiatry has been guided by symptom-based diagnoses in the "Diagnostic and Statistical Manual of Mental Disorders"

(DSM-5) and the selection criteria for clinical trials are based on these groups of symptoms. Although the use of biomarkers is not as reliable or valid due to the wide variety of genetic and epigenetic processes that underlie the diagnosis based on the DSM, studies on biomarkers show that they may help in the early diagnosis, the measurement of ASD development risk, prognosis, characterization of patient subgroups, and definition of subsets of individuals who would respond more favorably to specific treatments (Goldani *et al.*, 2014; Anderson, 2015; Belzeaux, Lin and Turecki, 2017). Molecular biomarkers are generally determined in biological fluids mainly with blood or urine samples. In contrast to these traditional ones, saliva has emerged as an interesting alternative for obtaining biological samples from patients with ASD. Saliva has important advantages: it is cheap and easy to collect, its sampling is painless and non-invasive; it has a less anxiety-provoking effect compared to extracting a blood sample, and is less embarrassing than collecting a urine

sample (Wormwood *et al.*, 2015). Also, saliva samples comprise a valuable source of cells and DNA (Goode *et al.*, 2014), proteins (including cytokines, hormones, peptides, neurotransmitters) (Ratajczak and Sothern, 2015), as well as circulating microRNA (miRNA) (Gallo and Alevizos, 2013). Obtention of clinically reliable markers allows policies that promote greater access to early intervention, resulting in favorable impacts throughout the patient life and providing a promising opportunity to optimize results for the next generations of individuals with ASD (Klin, 2018). In this sense, this work describes the main biomarkers of saliva, as a diagnostic tool for ASD. Initially, we describe a little about history, statistical data, and clinical manifestations. Subsequently, we discuss the types of classic biomarkers with an emphasis on saliva.

METHODOLOGY

For this review, a wide search was carried out on the main platforms for publishing scientific articles such as PubMed, Google Scholar, Science Direct, and others. The following have been used: "autism", "saliva" and "biomarkers". The most emphasized articles in this review were primarily from the years 2010 to 2020. For this research, the criteria of original research studies that compared saliva samples from children with ASD versus control children were considered.

Data Compilation

The expression "autism" was first used in 1911 by Swiss psychiatrist Eugen Bleurer to designate the loss of contact with reality ("a definitive withdrawal from the outside world"), which he considered one of the main symptoms of schizophrenia, a term that he also co-authored. But it was Leo Kanner in 1943, who described autism or ASD (autism spectrum disorder) as an etiologically and clinically heterogeneous group of disorders, diagnosed exclusively by the complex behavioral phenotype (Marchezan, 2018). Through the reports of eleven children with what was initially called "innate affective contact disorder", that is, the children had no habitual interest in other people and in contact with the social environment, Kanner mentioned that these children exhibited "resistance to change" and identified them as having "resistance in the same things" which, in other words, are purposeless motor behaviors (stereotypes) such as body swaying, tiptoe walking and shaking hands (Kanner and Others, 1943; Kanner, 1968). After Kanner's description, the first studies showed great interest in autism, where the lines of work aimed at the potential of parental psychopathology as a cause of autism. Some schools at the time proposed the hypothesis that autism was a psychological reaction to a disturbance in early relationships, placing the mother as the etiological nucleus of the pathology, and proposing analytical treatments. The story also reports a strong movement that pointed to the disorder as the first childhood manifestation of psychosis or schizophrenia. However, some lines of research confronted these statements (Parellada *et al.*, 2014). In 1964 the pioneering work of Bernard Rimland, founder of the Autism Society of America, focused on new approaches to diagnosis and provided a hypothetical neurobiological mechanism for autism. As early as 1972, phenomenological studies by Kolvin and Rutter made it clear that autism was different from schizophrenia in terms of onset, clinical features, and family history. And only in the late 1970s, the first twin studies suggested a strong genetic basis for the

condition (Kolvin, 1972; Rutter, 1972; Greydanus and Toledo-Pereyra, 2012; Volkmar and McPartland, 2014; Marchezan, 2018).

From the 1980s, research converged to include "Child Autism" with a diagnosis officially recognized in the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III), which in 1987 was revised and instituted diagnostic criteria with a development perspective under the term "Invasive (or Global) Developmental Disorders": (1) Autism; and (2) Invasive (or Global) Developmental Disorder, unspecified. In 1994 the fourth edition of DSM-IV already included 5 subgroups within Invasive Development Disorders: Autism, Rett Syndrome, Childhood Disintegrative Disorder, Unspecified Invasive Development Disorder, and Asperger's Syndrome. At the beginning of 2013, the fifth edition of DSM-5 chose to join all subgroups under the common term of ASD and classify them according to the intensity of symptoms into mild, moderate, and severe. DSM-5 excluded Rett's Disorder and Childhood Disintegrative Disorder from the group because they are neurological disorders with a different etiology (Gadia, Tuchman and Rotta, 2004; Tchaconas and Adesman, 2013; Volkmar and McPartland, 2014; Gottfried *et al.*, 2015).

Current statistical data: Since 1942, autism records have mentioned it as a rare condition, affecting around 1 child per 25,000. In 1966, studies carried out by Victor Lotter already reported a prevalence of 4.5 out of 10,000 children aging 8 to 10 years in the town of Middlesex, a county in northwest London. In the 1980-1990s, it was 4-5 / 10,000 inhabitants. Between the years 1990-2000, cases of autism increased from 30-60 per 10,000 children. In 2012, cases of ASD already reached 1% of the population in all countries in Asia, Europe, and North America. In 2018, the Center for Disease Control and Prevention (CDC) estimated the prevalence of ASD with approximately 1 in 59 children aging 8 years, affecting approximately 3/100 boys and 1/100 girls, meaning an approximate increase of 150% between 2000 and 2014 (Baio, Wiggins and Christensen, 2018). Currently, it is estimated that, worldwide, one in 160 children has autism spectrum disorder. This estimate represents an average value and the reported prevalence varies substantially between the studies, because, according to Chiarotti and Venerosi (2020), the studies differ in the diagnostic category and criteria, age in the prevalence assessment, extension of the targeted geographical area, and source of data on diagnostics. These methodological differences, together with the large time interval (almost 50 years from the first to the last study included in the review), are at least partially responsible for the large differences observed in the estimated prevalence. Overall, estimates ranged from 0.19 / 1000 to 11.6 / 1000 (Chiarotti and Venerosi, 2020). Some well-controlled research reports that the cases are significantly higher. The prevalence of ASD in many low- and middle-income countries remains unclear (Autism spectrum disorders, no date; Marchezan, 2018). There are many possible explanations for this apparent increase, including increased awareness of the topic, mainly by health and education professionals, the expansion of diagnostic criteria, better diagnostic tools, and the improvement of reported information (Autism spectrum disorders, no date; Chiarotti and Venerosi, 2020).

Clinical manifestations and diagnosis: The variety of symptoms in the cognitive, emotional, and neurobehavioral

areas of individuals with autism makes their recognition challenging. Covering heterogeneous phenotypes, mainly in the milder cases of the spectrum (Johnson, Myers and American Academy of Pediatrics Council on Children With Disabilities, 2007; Stanković, Lakić and Ilić, 2012; Sharma, Gonda and Tarazi, 2018). The most well-known manifestations of the spectrum are deficits in interaction and social communication, restricted and repetitive patterns of behavior, interests, and activities. Even though these symptoms can occur in a variety of psychiatric disorders, it is the combination of them in the same individual that makes ASD unique and may vary according to age. During childhood, there are more language deficits and a lot of agitation. In adolescence, the most important symptoms may be relationship problems and mood modulation (Nazeer and Ghaziuddin, 2012; Sharma, Gonda, and Tarazi, 2018). ASD patients also have deficits in executive functioning and in the mental capacity to solve problems, in addition to the difficulty of integrating information to produce meaning. Changes in sensory processing are frequent. These children and adolescents may present both hypo and hypersensitivity to sensory stimuli of the same modality or in multiple sensory domains (visual, auditory, olfactory, palatal, and tactile) (Olivié, 2012; American Psychiatric Association, 2013). Hyperactivity, diverse aggressiveness, and self-aggressiveness occur in more than half of these children. Self-aggressive behaviors are more common among patients with cognitive impairment, and the triggers for these behaviors can be predictable (frustration, anxiety, arousal) or seemingly random. Lack of understanding or inability to communicate, or total frustration, can lead to outbursts of aggression (Klin, 2006). The diagnosis of autism and the demarcation of its limits remain a clinical decision, as there is no specific biological marker. Assessment instruments such as interviews with parents/caregivers, interviews with patients, direct observation of patients, and a full review of family history are the main ones used in the diagnosis of ASD and other neurodevelopmental disorders (Gadia, Tuchman and Rotta, 2004; Sharma, Gonda, and Tarazi, 2018).

Biomarkers for autism: Numerous biomarkers have been proposed for ASD, including biochemical, morphological, immunological, hormonal, neurophysiological, neuroanatomical, and neuropsychological markers (Ruggeri *et al*., 2014; Heunis, Aldrich and de Vries, 2016; Inga Jácome *et al*., 2016; Ahmad *et al*., 2017; Li, Karnath and Xu, 2017; Masi *et al*., 2017; Fulceri *et al*., 2018). The term "biomarker" has been defined as a biological measure that differs between groups or is associated with some aspect of a condition. Biomarkers could assist in early diagnosis, in measuring the risk of developing ASD, in prognosis, in characterizing subgroups of patients, and in defining subsets of individuals who would respond more favorably to specific treatments (Anderson, 2015; Zwaigenbaum and Penner, 2018). According to a review study by (Galiana-Simal *et al*., 2018), biomarkers can be grouped into five groups based on their application purpose. Prenatal: from preconception to the gestation period, biomarkers have the potential to stratify pregnancies, which can be of high risk for the offspring to develop ASD; Pre-symptomatic: during pre-symptomatic stages, biomarkers can identify high-risk populations to determine who may require more diagnostic tests, early intervention or increased surveillance; Diagnosis: once the symptoms are obvious, biomarkers can confirm the diagnosis; Subgroup: biomarkers can be used to divide individuals with ASD into biological subgroups; Treatment:

biomarkers can be used to select the ideal therapy, predicting the response to treatment or measuring a physiological index of response to treatment. Even with a variety of proposed biomarkers, to analyze profiles in gene expression, proteomics, metabolomics, brain size, structure and connectivity, and oculomotor measurements (Ecker, Marquand and Mourão-Miranda, 2010; Yap *et al*., 2010; Bosl *et al*., 2011; Griffin and Westbury, 2011; Scherer and Dawson, 2011; Schwarz *et al*., 2011; Freedman and Foxe, 2018), were considered non-universal because of the low levels of sensitivity and failed to positively identify most of the various samples studied. One of the most reported examples, candidates for ASD, is the measurement of serotonin in the blood (Anderson, 2015; Muller, Anacker and Veenstra-VanderWeele, 2016) which is increased in ASD, but also in other neuropsychiatric and non-psychiatric conditions, such as congenital heart disease and cancer (Homsy *et al*., 2015). The clinical management of individuals with ASD remains the domain of specialist doctors who use symptom-based approaches (Frye *et al*., 2019). Genotypic heterogeneity and complexity continue to challenge progress in finding genetic and genomic signatures for the disease; likewise, phenotypic heterogeneity remains a formidable challenge for mechanistic biological studies dependent on more homogeneous samples (de Belen *et al*., 2020). However, the impact of the short-term biomarkers on efforts to optimize clinical outcomes is a reasonable expectation, given the latest, economical, and viable tools for the community to assist in the screening, identification, and early diagnosis of ASD. These, in turn, can strongly allow policies that promote greater access to early intervention, which is known to have a favorable impact. In this case, this effort provides us with a promising opportunity to optimize results for the next generations of individuals with ASD (Klin, 2018).

Salivary proteins markers: Recently, there has been an intense effort in the search for biological markers that can assist in early diagnosis, prognosis, and response to treatment. Proteomic analysis using mass spectrometry can lead to the discovery of biomarkers in human biological fluids, including saliva. Studies by (Ngounou Wetie, Wormwood, Russell, et al., 2015) in an attempt to optimize the search for a saliva proteomic biomarker, analyzed the saliva proteome of individuals with ASD using the mass spectrometry (MS) technique and compared with neurotypical control individuals. The results showed statistically significant differences in several salivary proteins, including high prolactin-induced protein, lactotransferrin, Ig kappa chain C, Ig chain 1 region C, Ig lambda - 2 chain C, neutrophil elastase, and polymeric immunoglobulin receptor. The same research team published another similar study in which proteins involved in oxidative stress and lipid metabolisms, such as apolipoproteins A1 and A4 and Zn alpha2 glycoprotein, were also deregulated in ASD samples compared to control children (Ngounou Wetie, Wormwood, Charette, et al., 2015). These findings are consistent with the possibility of a change in immunity, oxidative stress, and lipid metabolism in patients with ASD (Scott and Dhillon, 2007), which can be detectable in saliva samples measuring the aforementioned biomarkers (Ngounou Wetie, Wormwood, Charette, et al., 2015).

Salivary hormone markers: Although many authors criticize the assessment of cortisol levels in children with ASD, limiting it on their circadian cycle, where the values can vary throughout the day and consequently hinder its reliability and

usefulness as a biomarker, other authors suggest that the collection is mainly in the afternoon, a period in which the variation in cortisol levels is minimal (Sharpley et al., 2016). Some results are reported in the literature about elevated levels of daytime salivary cortisol correlated to stress and anxiety in children with ASD compared to control children, where threatening events end up dramatically increasing salivary cortisol levels in children with ASD (Corbett et al., 2006; Tordjman et al., 2014). This increase in cortisol may be related to cognitive dysfunction (Ogawa et al., 2017). Another study showed that salivary cortisol levels increased significantly and correlated with poor behavior in children with ASD during non-invasive dental procedures, compared to control children (Abdulla and Hegde, 2015). Considering that ASD is more prevalent in men being somehow related to sex, such as sex steroid hormones (Werling and Geschwind, 2013), a survey was conducted to measure testosterone and estradiol levels in children of 18 to 24 months (prenatal-amniotic and postnatal-salivary). The results showed that amniotic testosterone, but not salivary levels, was the only variable that predicted autism on the Q-CHAT scale compared to the control group (Auyeung et al., 2012). Another study reported that salivary androgen levels, specifically androstenediol and dehydroepiandrosterone, as well as androgen-derived androsterone and the androgen precursor pregnenolone, were increased in ASD compared to the control population, predicting early puberty. Given that most of the positively regulated hormones in this study are known to be neuroactive (modulating GABA, glutamate, and opioid neurotransmission), the authors concluded that they could have affected brain development and functioning, contributing to the development of ASD (Majewska et al., 2014). As far as we know, these are the only studies published today that quantify sex hormones in the saliva of children confirmed or suspected of having ASD (Galiana-Simal et al., 2018).

Salivary miRNAs markers: Post-transcriptional mechanisms such as microRNAs (miRNAs) regulate gene expression without changing the genome. They are abundant and can be differently expressed in the brain, blood, olfactory precursor cells, and saliva of individuals with ASD, making them an ideal candidate for the discovery of diagnostic biomarkers. Currently, there are few studies on miRNA present in individuals with ASD. Today it is known that saliva contains sufficient miRNA (Zendjabil et al., 2017; Salloum-Asfar, Satheesh, and Abdulla, 2019) and that these are epigenetic regulators of important neurodevelopment processes (Zendjabil et al., 2017; Anitha, 2015). A recent survey collected saliva from children with ASD to analyzing miRNA expression. Of the total, 14 miRNAs were expressed differently in children with ASD compared to the control group being significantly relevant on the Vineland scale. The authors stated that the specificity of these miRNAs for the diagnosis of ASD is almost double that of M-CHAT-R, which is currently considered the most widely used ASD screening tool. The most promising salivary biomarkers detected were miR-628-5p, miR-335-3p, miR-127-3p, and miR-27a-3p, all of them deregulated in the ASD group compared to the control group (Hicks et al., 2016). In another study, the authors investigated the usefulness of salivary microRNAs to differentiate 443 children (2–6 years) with ASD from their peers with typical development (TD) and developmental delay without autism (DD). The result showed that fourteen microRNAs exhibited differential expression between the ASD, TD, and DD groups. A panel of 4 microRNAs

differentiated children with ASD better from children without ASD (Hicks et al., 2020). In a review study of 12 publications that compared miRNA from ASD patients to miRNA profiles of healthy controls in different tissues and biofluids, including the brain, peripheral blood, saliva, olfactory precursor cells, and cultured lymphoblasts, in the saliva, the miRNAs were found deregulated as well as the miRNAs of the other tissues (Hicks and Middleton, 2016). On other tissues besides saliva, the research by Abu-Elneel et al. (2008) tracked the expression of 466 human miRNA in the postmortem cerebellar cortex of 13 individuals with ASD and an equal number of neurotypical individuals. A total of 28 miRNA showed different expressions in at least one of the samples from individuals with ASD compared to individuals with normal development (Abu-Elneel et al., 2008). However, no specific miRNA has been uniformly deregulated throughout this set of post-mortem samples. A 2008 study evaluated the profile of 470 miRNA in lymphocytes from 6 individuals with ASD compared to paired neurotypical individuals. The authors describe the altered expression of nine miRNA in samples from individuals with ASD (Talebizadeh, Butler, and Theodoro, 2008). Another study also using lymphocytes found 43 miRNAsdifferently expressed in patients with ASD compared to individuals with typical development (Sarachana et al., 2010). A recent study using serum samples from 55 individuals with ASD and 55 control subjects matched for age and sex identified 13 miRNA that was differentially expressed in individuals with ASD (Mundalil Vasu et al., 2014). Chinese study with 15 patients and 15 controls found a significant difference in the expression of 5 microRNA with the increased expression about the controls (HUANG et al., 2015). Another study of 30 children with ASD of both sexes and 30 healthy controls found differences between the serum microRNA expression profile (Kichukova et al., 2017).

CONCLUSION AND PERSPECTIVES

Saliva is one of the human biological fluids with a promising area of research and applications, consequently acquiring potential value as a source of markers for early diagnosis, considered non-invasive. In this review, the data support the concept that saliva is a body fluid suitable for measuring important biomarkers for neuropsychological diseases, including autism. However, few studies of ASD salivary biomarkers have been performed. Through these data, we will be able to help health professionals in the current diagnosis and treatment of ASD, which is quite necessary today.

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