Determination of oxidative stress biomarkers and cortisol in saliva samples of children with Autism Spectrum Disorders (ASD) for the evaluation of cognitive damage and response to psycological stress.

Final Degree Project

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TÍTOL: Determinació de biomarcadors d'estrès oxidatiu i cortisol en mostres de saliva de xiquets amb Trastorns d'Espectre Autista (ASD) per a l'avaluació del dany cognitiu i la resposta a l'estrès psicològic.

RESUM: Els trastorns de l'espectre autista (TEA) són un conjunt d'alteracions del neurodesenvolupament. Es caracteritzen per la dificultat en la comunicació i les relacions socials, i la presència de patrons estereotipats del comportament. En els últims anys han augmentat els casos d'aquests trastorns en la població infantil. L'any 2000 es va estimar que 1 de cada 150 xiquets patien TEA. Actualment, el nombre de casos és de 1 de cada 68. Malgrat la creixent incidència d'aquests trastorns, el coneixement dels principis biològics és molt limitat, tot i que en alguns estudis s'ha establert una relació directa entre TEA i estrès oxidatiu (desequilibri fisiològic entre les espècies oxidants i antioxidants que provoca dany a nivell tissular i cel·lular). A més dels problemes cognitius, els nens amb TEA presenten una baixa tolerància a l'estrès psicològic, a causa d'una reactivitat anormal de l'eix hipotàlem hipòfisi adrenal, provocant un augment en els nivells de cortisol. Per tant, l'objectiu principal d'aquest estudi preliminar, és l'avaluació del dany cognitiu i la tolerància a l'estrès en xiquets amb Trastorn de l'espectre autista (TEA) mitjançant la detecció de compostos de peroxidació lipídica (procés desencadenat per estrès oxidatiu) i cortisol en mostres de saliva amb la finalitat de determinar potencials biomarcadors de forma no invasiva, que permeten un diagnòstic objectiu dels danys associats al trastorn i que ens ajuden a definir un grup de població. L'estudi d'aquests biomarcadors d'estrès oxidatiu en mostres de saliva es va a realitzar mitjançant l'aplicació de diferents mètodes d'anàlisi prèviament validats, i l'avaluació de la tolerància a l'estrès es durà a terme mitjançant una tasca de temps de reacció i la determinació dels nivells de cortisol abans i després de la inducció de frustració. Experimentalment, la determinació dels biomarcadors d'estrès oxidatiu (isoprostans, neuroprostans, isofurans, neurofurans, dihomoisoprostans, dihomoisofurans) i cortisol, es realitzarà mitjançant un mètode analític basat en cromatografia-espectrometria de masses. Els resultats que s'obtinguen seran significatius a nivell internacional, atenent a les necessitats psicosocials actual a causa de la creixent incidència del TEA. La manca de coneixement biològic restringeix el diagnòstic, que actualment es basa en observacions del comportament i eines psicomètriques, ja que no existeixen biomarcadors generals validats per al diagnòstic del trastorn. Per tant, la determinació d'aquests marcadors d'estrès oxidatiu i cortisol, permetrà detectar el dany neurològic de forma primerenca, l'avaluació del grau del mateix a nivell molecular, i la quantificació de la capacitat de regulació de l'estrès en aquests xiquets.

PARAULES CLAU: Trastorns del Espectre Autista (TEA); biomarcadors; estrés oxidatiu; peroxidació lipídica; cortisol; saliva; espectrometria de masses.

TÍTULO: Determinación de biomarcadores de estrés oxidativo y cortisol en saliva de niños con Trastornos del Espectro Autista (TEA) para la evaluación del daño cognitivo y la respuesta al estrés psicológico.

RESUMEN: Los Trastornos del Espectro Autista (TEA) son un conjunto de alteraciones en el neurodesarrollo. Se caracterizan por la dificultad en la comunicación y las relaciones sociales, y la presencia de patrones estereotipados del comportamiento. En los últimos años ha aumentado los casos de estos trastornos en la población infantil. En el año 2000 se estimó que 1 de cada 150 niños padecían TEA. Actualmente, el número de casos asciende a 1 de cada 68. A pesar de la creciente incidencia de estos trastornos, el conocimiento de los principios biológicos es muy limitado, aunque en algunos estudios se ha establecido una relación directa entre TEA y estrés oxidativo (desequilibrio fisiológico entre las especies oxidantes y antioxidantes que provoca daño a nivel tisular y celular). Además de los problemas cognitivos, los niños con TEA presentan una baja tolerancia al estrés psicológico, debido a una reactividad anormal del eje hipotálamo hipófisis adrenal, provocando un aumento en los niveles de cortisol. Por lo tanto, el objetivo principal de este estudio preliminar, es la evaluación del daño cognitivo y la tolerancia al estrés en niños con Trastorno del Espectro Autista (TEA) mediante la detección de compuestos de peroxidación lipídica (proceso desencadenado por estrés oxidativo) y cortisol en muestras de saliva con el fin de determinar potenciales biomarcadores de forma no invasiva, que permitan un diagnóstico objetivo de los daños asociados al trastorno y que nos ayuden a definir un grupo de población. El estudio de estos biomarcadores de estrés oxidativo en muestras de saliva se va a realizar mediante la aplicación de diferentes métodos de análisis previamente validados, y la evaluación de la tolerancia al estrés se llevará a cabo mediante una tarea de tiempos de reacción y la determinación de los niveles de cortisol antes y después de la inducción de frustración. Experimentalmente, la determinación de los biomarcadores de estrés oxidativo (isoprostanos, neuroprostanos, isofuranos, neurofuranos, dihomoisoprostanos, dihomoisofuranos) y cortisol, se realizará mediante un método analítico basado en cromatografía-espectrometría de masas. Los resultados que se obtengan serán significativos a nivel internacional, atendiendo a las necesidades psicosociales actuales debido a la creciente incidencia del TEA. La falta de conocimiento biológico restringe el diagnóstico, que actualmente se basa en observaciones del comportamiento y herramientas psicométricas, ya que no existen biomarcadores generales validados para el diagnóstico de trastorno. Por lo tanto, la determinación de estos marcadores de estrés oxidativo y cortisol, permitirá detectar el daño neurológico de forma temprana, la evaluación del grado del mismo a nivel molecular, y la cuantificación de la capacidad de regulación del estrés en estos niños.

PALABRAS CLAVE: Trastorno del Espectro Autista (TEA); biomarcadores; estrés oxidativo; peroxidación lipídica; cortisol; saliva; espectrometría de masas.

TITLE: Determination of oxidative stress biomarkers and cortisol in saliva samples of children with Autism Spectrum Disorders (ASD) for the evaluation of cognitive damage and response to psycological stress.

ABSTRACT: Autism Spectrum Disorders (ASD) are a set of complex developmental brain disorders. They are characterized by difficulties in communication and social skills, and the presence of stereotyped patterns in behavior. In the last few years, the number of cases of these disorders in child population have increased notably. In 2000, it was estimated that 1 out of 150 children had ASD. Currently, the number of cases is 1 out of 68 children. Despite the raising incidence of these disorders, the knowledge of biological principles is very limited, although in some studies a direct relationship has been established between ASD and oxidative stress (physiological imbalance between oxidizing and antioxidant species that causes tissue and cellular damage). Besides the cognitive problems, children with ASD have low tolerance to psychological stress, due to an abnormal reactivity of the hypothalamic pituitary adrenal axis, causing a raise in cortisol levels. Therefore, the main objective of this preliminary study is the assessment of cognitive damage and tolerance to stress in children with Autism Spectrum Disorder (ASD) by the detection of lipid peroxidation compounds (process triggered by oxidative stress) and cortisol in saliva samples in order to determine potential biomarkers in a non-invasive way, allowing an objective diagnosis of the damages associated with the disorder and helping us to define a population group. The study of these biomarkers of oxidative stress in saliva samples will be performed through the application of different analysis methods previously validated, and the assessment of stress tolerance will be carried out by means of a task of reaction times and the determination of cortisol levels before and after the induction of frustration. Experimentally, the determination of the biomarkers of oxidative stress (isoprostanes, neuroprostanes, isofurans, neurofurans, dihomoisoprostanes, dihomoisofurans) and cortisol, will be carried out by means of an analytical method based on chromatography-mass spectrometry. The results obtained will be significant internationally, attending to the current psychosocial needs due to the increasing incidence of ASD. The lack of biological knowledge restricts the diagnosis, which is currently based on behavioral observations and psychometric tools, since there are no validated general biomarkers for the diagnosis of the disorder. Furthermore, the determination of these markers of oxidative stress and cortisol will allow the early detection of neurological damage, the evaluation of its degree at molecular level, and the quantification of the stress regulation capacity in these children.

KEY WORDS: Autism Spectrum Disorder (ASD); biomarkers; oxidative stress; lipid peroxidation; cortisol; saliva; mass spectrometry.

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ABBREVIATIONS

AdA: Adrenic acid

AA: Arachidonic acid

ASD: Autism Spectrum Disorders

ADHD: Attention Deficit or Hyperactivity Disorder

BSA: Bovine serum albumin

CS: Caesarean Section

CSF: Cerebrospinal fluid

DHA: Docosahexaenoic acid

GSHPx: Glutathione peroxidase

GSSG/GSH: ratio of reduced / oxidized glutathione

IS: Internal standard

IsoP: Isoprostane

IQR: Interquartile range

LLE: Liquid-liquid extraction

NeuroP: Neuroprostanes

PGE: Prostaglandine

PTB: Preterm Birth

PUFAs: Polyunsaturated fatty acids

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RT: Rett Syndrome

SD: Standard deviation

SOD: Superoxide dismutase

UPLC-MS: Ultra Performance Liquid Chromatography coupled to Mass Spectrometry system

WR: working reagent

INDEX OF UNITS

º C: degrees centigrade

kV: kilovolts

L h^{-1}: litre/hour

min: minutes

mm: millimeters

mL min^{-1}: mililitres/minute

ng/mg prot: nanogrames per mg of protein

nm: nanometres

nmol L^{-1}: nanomols/litre

rpm: revolutions per minute

v/v: volume (of solute) per volume (of solvent)

µL: microlitres

µm: micrometers

µmol L^{-1}: micromol/litre

1. INTRODUCTION

Autism Spectrum Disorders (ASD) are a set of complex developmental brain disorders. They are characterized by difficulties in communication and social skills, as well as the presence of stereotyped patterns in behavior (El-Ansary et *al*., 2012; Eapen et al., 2017). Also, recent studies also report a deficit in other domains of cognitive functions, such as stress tolerance, working memory, behavioral flexibility and facial emotion recognition. However, these findings are quite controversial (Ogawa et *al*., 2017). Other remarkable features of the disease include poor eye contact and disruption in motor and cognitive development. The clinical symptoms usually appear at the age of 3 years old, but the deficits in communication, social responses and play might show at 6 or 12 months of age (Khemakhem et *al*., 2017).

The disorder was identified in the decade of the 1940s. But it wasn't until 1980, that ASD was formally recognized as a particular clinical diagnosis entity. Research was performed in small participants until the 1990s, when new techniques and methods were developed. Nowadays, the criteria to obtain a diagnosis of ASD and its relation to Asperger's and Rett syndrome are based on methods of cognitive and social variables, such as genomics, eye-movement tracking or electrophysiology. Recently, neuroimaging of the activity and structure of the brain is also used for the study of ASD (Just et *al*., 2012; Ziats et *al*., 2016). Nevertheless, the current diagnosis of ASD is based on phenotypic studies, rather than biochemical tests. The early identification of the disorder is beneficial for children diagnosed with ASD, since it often reduces the symptoms and associated behaviors, improving outcomes. Regarding therapies, they are more likely to succeed when applied in the early stages of childhood. However, this fact might be delayed depending on the severity degree of the symptoms (Khemakhem et *al*., 2017). The incidence of Autism Spectrum Disorders has increased over the past 30 years, and the prevalence shows differences between countries. The number of cases in Europe is 1 out of 150 children according to the Spanish Autism Confederation. The prevalence in males is 4 to 5 times higher than in females. It can be justified as a group of biological conditions in which genetic and hormonal influences should be examined, to confirm and understand the autism pathobiology (Mayewska et *al*., 2014).

The physiopathology of this multifactorial and heterogeneous disease is still unclear. According to some studies, the first symptoms could be due to the accumulation of environmental toxicants in brain, or to an early exposure to these compounds, which will alter the normal development of the metabolic pathways of the brain. Also, this might be influenced by the genetic predisposition and sensitivity of the individual, causing a dysregulation of the immune

response, oxidative stress enhancement and alteration of the mitochondrial metabolism, which are thought to be some of the most common molecular processes at neurodevelopmental disorders (El-Ansary and Al-Ayadhi, 2012). There have also been observed abnormalities in the white matter of children affected with ASD, altering the normal connectivity of the brain and generating an unusual communication between the cortical centers, caused by a variation of the myelination (formation of insulating white matter) of axons (Figure 1). The cortical centers are responsible of carrying the information between the different regions of the brain (Just et *al*., 2012, Keller et *al*., 2010). The development of a complex disease is often directly related to a metabolic imbalance in the microenvironment of the cell. This might be influenced by different factors, such as genetic, epigenetic and environmental, resulting in a characteristic metabolic profile/footprint, which is an important source of information of the pathophysiology and pathogenesis of the disorder (James et *al*., 2008). An imbalance of reactive species caused by an alteration of the metabolism promotes cellular damage in membranes, proteins, lipids or DNA, causing functional alterations. The role of oxidative stress in ASD has not been deeply investigated, in spite of the contrasted knowledge of the vulnerability of brain in developmental stages to damage caused by oxidative stress. The brain is particularly susceptible to oxidative stress, as it has a limited antioxidant capacity. Its high lipid composition and its requirement of energy, and oxygen is higher than any other organ (Frye et *al*., 2014; Geier et *al*., 2009). Nevertheless, the physiopathology knowledge is very limited, and there is a lack of reliable biomarkers for early ASD detection.

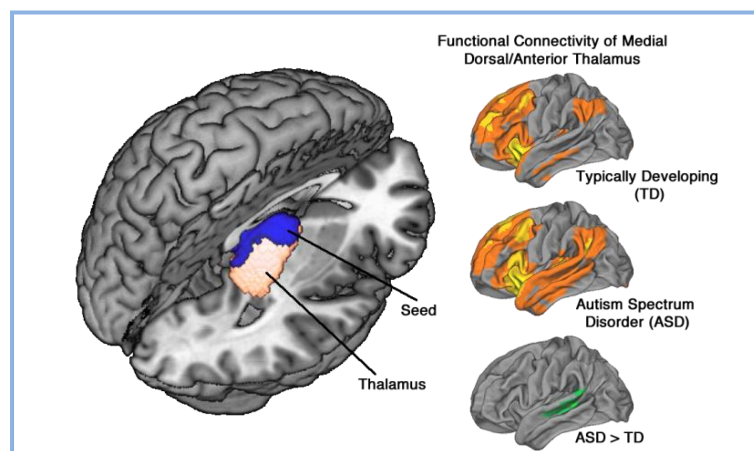


Figure 1: Differences in basic sensorimotor processing and cortical excitability between individuals with ASD and Typically Developing (TD) individuals. *(Woodward et al. 2017)*

Currently, oxidative stress is considered an important molecular mechanism of the disorder being involved in brain damage and inflammation. El-Ansary et *al*. reviewed some works that are focused on red-ox species GSSG/GSH, glutathione peroxidase (GSHPx), catalase and superoxide dismutase (SOD) as markers of the antioxidant system impairment (El-Ansary et *al*., 2017). Nevertheless, few studies have reported clarifying results on autism, most of them based on the discovery of plasma biomarkers by means of redox proteomic approaches. Feng et *al*. carried out a comparative study between children with autism and healthy subject, obtaining an increased carbonyled proteins in plasma samples from autistic children (C8A and IGKC), specifically those proteins involved in complement system and immunoregulation (Feng et *al*., 2017). Qasem et *al*. related the brain vulnerability to oxidative stress during the first developmental stages of childhood with the high ROS species levels, low antioxidant capacity and high levels of lipid content. This was reflected in a higher level of lipid peroxidation products in autistic children compared to control cases (Qasem et *al*., 2016). Based on previous studies about other neurodevelopmental diseases, lipid abnormalities in brain tissue of patients were described. The lipid changes are related to neuropathological alterations, which alter lipid and ganglioside patterns in different areas of the central nervous sytem. Molecules generated by lipid peroxidation derive from four poly-unsaturated fatty acids (PUFAs) attacked by reacive species, which are potential biomarkers for ASD diagnosis: arachidonic acid (AA) (F2-isoprostanes (F2-IsoPs)), docosahexaenoic acid (DHA) (F4-neuropros-tanes (F4-NeuroPs)) and adrenic acid (AdA) (F2-dihomo isoprostanes (F2- dihomo-IsoPs)) (Figure 2) (De Felice et *al*., 2013). Previous studies performed on ASD patients, have detected altered levels of substances in different types of biological samples. The assays have been performed mainly in serum, whole blood and cerebrospinal fluid (CSF). The most significant results were obtained in plasma samples (El-Ansary et al., 2012).

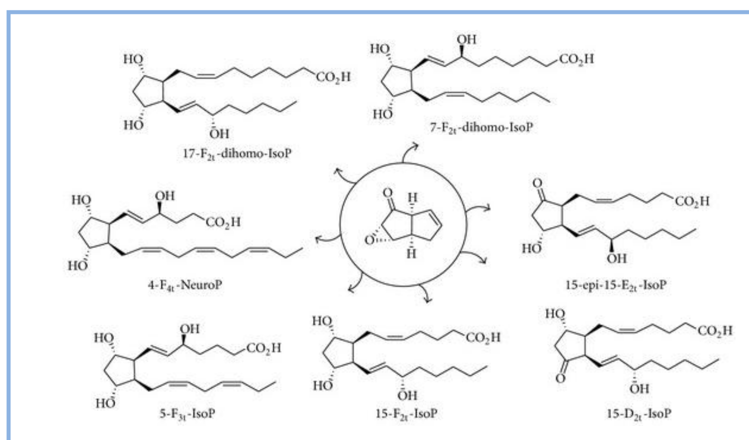


Figure 2: Oxygenated metabolites derived from AA, DHA and Ada. *(Signorini et al., 2016)*

Biomarkers play an important role in ASD diagnosis, as they provide us with significant and objective data for a clear and accurate diagnosis. According to El-Ansary et *al*. biomarkers that measure neurological brain damage are desirable, and specially in autism, for initiating interventions at the earliest age (El-Ansary et *al*., 2016). Feng et *al*. affirm that the demand for finding ASD biomarkers is increasing, as they might help to identify the affected children as soon as possible (Feng et *al*., 2017). However, specific biomarkers for ASD have not been validated yet for the diagnosis of the disorder. In this sense, it is fundamental in order to advance in the knowledge of the disease and to improve the current treatments to define a group of distinctive biomarkers, specially in cases that are not clear during the presymptomatic stage of the disorder (Khemakhem et *al*., 2017). Specifically, previous studies identified reactive species in plasma samples of autistic children (folate, tetrahydrobiopterin, glutathione, reactive oxygen species (ROS) and reactive nitrogen species (RNS)), that are directly involved in cellular homeostasis. We decided to obtain saliva from participants because it is a painless and non-invasive process, which simplifies the sample collecting step. Previous clinical trials have been performed in our laboratory with Alzheimer's Disease patients, which purpose was detecting F2-IsoPs, F4-NeuroPs and F2- dihomo-IsoPs in saliva samples (Table 1). Promising results were obtained.

Table 1: Lipid peroxidation products.

COMPOUND	CHEMICAL FORMULA	MOLECULAR WEIGHT (g/mol)	TRANSITION
8-iso-15(R)-PGF2α	$C_{20}H_{34}O_5$	354.5	353>193
1a1b-dihomo-PGF-2α	$C_{22}H_{38}O_5$	382.5	381>337
2,3-dinor-iPF$_{2\alpha}$-III	$C_{18}H_{30}O_5$	326.4	325>237 325>137
8-iso-15-keto-PGE$_2$	$C_{20}H_{30}O_5$	350.5	349>113 349>161
8-iso-15-keto-PGF$_{2\alpha}$	$C_{20}H_{32}O_5$	352.5	351>315 351>289
PGE$_2$	$C_{20}H_{32}O_5$	352.5	351>271 351>189
8-iso-PGE$_2$	$C_{20}H_{32}O_5$	352.5	351>189
5-iPF$_{2\alpha}$-VI	$C_{20}H_{34}O_5$	354.5	353>115
8-iso-PGF$_{2\alpha}$	$C_{20}H_{34}O_5$	354.5	353>193 353>165
PGF$_{2\alpha}$	$C_{20}H_{34}O_5$	354.5	353>193 353>165
PGF$_{2\alpha}$-D$_4$	$C_{20}H_{30}D_4O_5$	358.5	357.5>197.3
Isoprostanes	-	-	353.2>115
Isofurans	-	-	369.2>115
Neuroprostanes	-	-	377>101
Neurofurans	-	-	393>193

On the other hand, children diagnosed with ASD usually cope with psychological stress due to their lack of capacity to deal with change, anticipation, environmental stimuli and other events in their daily life and activities. Many studies have already demonstrated this fact, by measurements of the level of acute stress hormones of patients. Both, level and accumulation of these type of hormones, is altered in ASD patients as they have low tolerance to psychological stress, due to an abnormal reactivity of the hypothalamic pituitary adrenal axis, which is regulated by cortisol. If stress is maintained, the cortisol secretion may affect negatively to physiological functions (metabolic, immunological and physiological, among others), and in some cases it might aggravate the disease. Moreover, chronic stress may promote a physiological deregulation of the mechanisms involved in the stress response, causing coronary and mental diseases. Salivary cortisol is the most common acute stress hormone measured on autistic patients, as it is a reliable indicator of stress. It has been demonstrated that salivary cortisol works as an important psychological stress biomarker (Tomarken et *al*., 2015; Herman et *al*., 2016).

2. HYPOTHESIS

The determination of lipid peroxidation biomarkers in saliva samples can enable the early detection of neurological damage in ASD patients.

The determination of cortisol in saliva samples will allow the stress dysregulation detection in ASD patients.

3. OBJECTIVES

The main objective of the present study is the evaluation of oxidative stress and psychological stress in Autism Spectrum Disorders (ASD) patients by means of specific salivary biomarkers. For this, a new set of metabolites obtained from lipid peroxidation were determined in saliva samples to assess the cognitive damage, and the determination of salivary cortisol supported the monitoring of the psychological stress. The determination of these lipid peroxidaton biomarkers and cortisol will allow the early detection of neurological damage and the stress regulation impariment in these children.

The secondary objectives are:

i) The development of an analytical method to determine biomarkers of different nature (lipid peroxidation compounds and cortisol) in saliva samples.

ii) The identification of compounds as potential biomarkers of neurological damage or stress dysregulation in ASD patients.

The results obtained will be significant, specially attending to the current psychosocial needs since there is an increasing incidence of ASD. The lack of physiopathological knowledge in ASD development and validated biomarkers to detect early ASD restricts the objective diagnosis. In fact, current diagnosis is based on behavioral observations and psychometric tools.

4. MATERIALS AND METHODS

4.1. PARTICIPANTS

In this study, 84 participants with age from 6 to 18 years old, were classified into children diagnosed with Autism Spectrum Disorders (n= 41) and healthy control children (n=43). Participants with ASD were recruited from Infant Mental Health Unit in the University and Polytechnic Hospital La Fe (Valencia, Spain). The healthy children were recruited from a secondary school (Valencia, Spain). Subjects with language and/or intellectual impairments were not included in the study. The study protocol was approved by the Ethics Committee (CEIC) of the Health Research Institute La Fe (Valencia, Spain). Parental informed consent was obtained for all participants.

4.2. REAGENTS AND SOLVENTS

The solvents used were acetic acid, ethyl acetate, methanol and Milli-Q water. Cortisol standard was purchased from Sigma-Aldrich. The isoprostane standards 15(*R*)-15-F_{2t}-IsoP, 2,3-dinor-15-*epi*-15-F_{2t}-IsoP, 5-F_{2t}-IsoP, 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 15-E_{2t}-IsoP, 15-F_{2t}-IsoP and prostaglandins PGE_2, 1a,1b-dihomo-$PGF_{2\alpha}$ and $PGF_{2\alpha}$, and deuterated internal standard (IS) $PGF_{2\alpha}$-d_4 were purchased from the Cayman Chemical Company (Ann Arbor, Michigan, USA). The standards 7(*RS*)-*ST*-Δ^8-11-dihomo-IsoF, 10-*epi*-10-F_{4t}-NeuroP, d_4-10-*epi*-10-F_{4t}-NeuroP, 4(*RS*)-4-F_{4t}-NeuroP, 17-*epi*-17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF, *ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP, and 14(*RS*)-14-F_{4t}-NeuroP were synthesized by Professor Durand's team at the Institute of Biomolecules Max Mousseron (IBMM) (Montpellier, France).

The calibration curves were prepared by serial dilutions in H_2O (pH 3):CH_3OH (85:15 v/v) with CH_3COOH 0.01%, in concentrations from 150 nmol L^{-1} to 0,07 nmol L^{-1} of each analyte.

For protein measurement, Pierce™ BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, Illinois, USA).

4.3. MATERIALS

The resources used for the samples storage and treatment were: freezers of -20ºC and -80ºC, miliQ water system, centrifuge, vortex mixer, ultrasonic bath, vacuum concentrator, thermoblock and spectrophotometer for 96 well plates.

4.4. INSTRUMENTATION

The chromatographic system consisted of a Waters Acquity Ultra Performance Liquid Chromatography system coupled to a Xevo Triple Quadrupole system mass spectrometry system (Waters, United Kingdom) (Figure 3). The conditions used were: ionization in negative mode (ESI-), capillary tension 2.0 kV, temperature of the source 150 °C, desolvation temperature of 395 °C, gas flow of the nitrogen cone, and desolvation of 150 and 800 L h^{-1}, respectively.

The liquid chromatography conditions were selected to achieve appropriate chromatographic retention and resolution by using an Acquity UPLC BEH C_{18} column (2.1 x 100 mm, 1.7 µm) from Waters Mobile phases consisted of water with 0.01% v/v acetic acid as mobile phase A and acetonitrile with 0.01% v/v acetic acid as mobile phase B. The temperatures of the column and the autosampler were set at 55 °C and 4 °C, respectively. The injection volume was 8 µL and the flow rate was set to 0.45 mL min^{-1}. The elution gradient consisted of 0.5 min with 80 % A and 20% B, which was linearly changed to 55 % A and 45 % B from 0.5 to 6 min; then the proportion of B was increased to 95 % in the next 0.2 min and kept constant for 0.8 min until minute 7. Finally, the initial conditions were recovered and maintained for 1.3 min for column conditioning (Peña-Bautista et *al.*, 2018).

Figure 3: Waters Acquity Ultra Performance Liquid Chromatography system coupled to a Xevo Triple Quadrupole system. *(Image from Waters)*

4.5. SAMPLES COLLECTION AND TREATMENT

Saliva samples (n = 84) were collected in sterile bottles and stored at -80 °C until analysis. Then, they were treated following the optimum procedure established in a previous work (García-Blanco et *al*., 2016). It was based on liquid-liquid extraction (LLE). In order to separate biochemical compounds from a biological matrix. For this, a liquid solvent phase with suitable polarity was added to the original sample, in order to achieve the transfer of the analytes from one phase to the other (Figure 4).

Therefore the sample treatment consisted of thawing them on ice briefly. A centrifugation step was carried out (3500 rpm, 5 minutes). After that, 150 µL of the supernatant were taken, and 5 µL of internal standard solution (IS) (PGF2α-d4 (10 µmol L^{-1}) and d4-10-epi-10-F4t-NeuroP 6 (µmol L^{-1})) were added to the sample, as well as 450 µL of H_2O (HAc 0,01%). Then, a cleaning and pre-concentration phase was carried out by liquid-liquid extraction (LLE) by adding 600 µL of ethyl acetate. After that, samples were subjected to sonication for 10 minutes, followed by a centrifugation step (3500 rpm, 5 minutes). Then, the samples were placed on the freezer (-20 ºC, 15 minutes) in order to improve the separation of the two phases. The organic phase containing the analytes (600 µL) was placed in a new tube. A second extraction was performed to the remaining aqueus phase. By the end of the LLE, a total volume of 1200 µL of organic solvent containing the analytes of interest was evaporated by using a vacuum system (Speed Vacuum). Then the samples were reconstituted in 100 µL of H2O (pH 3):CH3OH (85:15 v/v) containing 0.01% (v/v) CH3COOH. Finally, the samples were injected into the chromatographic system (UPLC-MS/MS).

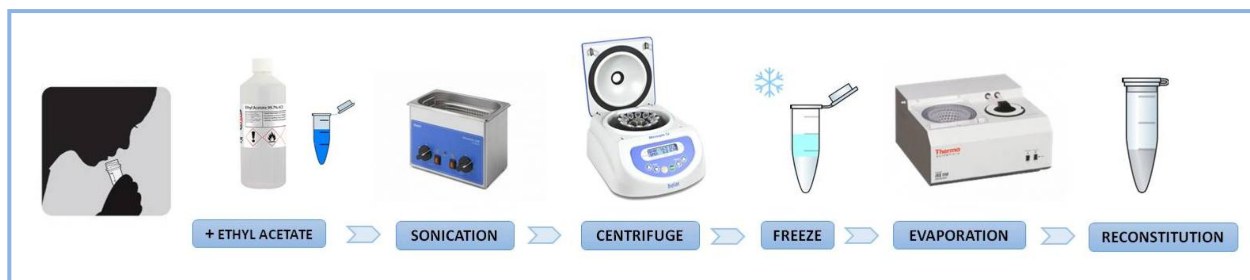


Figure 4: Scheme of sample collection and saliva treatment.

Protein concentration measurement was performed following the protocol of the Pierce ™ BCA Protein Assay Kit. First, the diluted albumin (BSA) standards and BCA working reagent (WR) (50:1, Reagent A:B) were prepared. 5 µL sample were diluted in 20 µL of H_2O (1:4, sample:H_2O) and deposited in a 96 well plate.

Then the WR was added (200 µL). An incubation step (30 min, 37ºC) was carried out by thermoblock. Finally, the absorbance was measured by a spectrophotometer (λ=562 nm).

4.6. STATISTICAL ANALYSIS

The results were statistically analyzed by means of SPSS software. The demographic and clinical variables considered were gender, age, gestational week, birth weight, type of delivery, current treatment and age of diagnosis (Table 1). The significant results were obtained considering *p value* < 0,05. The data were represented as mean ± standard deviation (SD), median (inter-quartile range, IQR) or number of cases (n) (percentage (%)) (Table 2). The comparison tests performed were t-test for mean results and Mann-Whitney for median results.

5. RESULTS

5.1. DEMOGRAPHIC DATA

The demographic data and clinical variables have been described in Table 1 according to the incidence pattern and characteristics of ASD patients. The study participants were between 6 and 18 years old, 13 (11-16) (median(IQR)) for ASD patients, and 13.50 (11.75-16.25) (median(IQR)) for healthy participants. The participants were selected in this age range because the psychological evaluation required some capacities that do not appear in earlier stages of childhood. The age limit was 18 years old, as the neurodevelopment is usually stabilized at this age. The gender was also taken into account when choosing the healthy participants, as the prevalence is 4-5 times higher in males than in females. In fact, the 87.5% of ASD participants (n=41) were males.

Among clinical variables that could be related to the appearance of the disease, it is important to highlight the gestational age at birth, since preterm birth is associated to an increasing ASD incidence. The causes are not clear, but the preterm birth might cause different alterations or even pathologies in further stages of the children's development (García-Blanco et *al*., 2016). According to this, the ASD participants were classified into 2 groups: preterm births (<37 weeks of pregnancy, 35.7%) and term births (≥38 weeks of pregnancy, 64.3%). The birth weight is directly related to the gestational age, so it was also taken into account. In this sense, ASD participants were distributed into three different groups: low weight (<2500 g., 21.4%), low to medium weight (2500-3000 g., 17.9%) and normal weight (>3000 g., 60.7%). Moreover, attending to the type of labor, the ASD participants were classified into 4 groups: natural (55.4%), induced natural (60.7%), caesarean section (11.3%) and by planned caesarean section (18.5%).

Despite the fact that many patients (41.4%) were not having any treatment, it was consulted for the rest of the ASD participants, and they were classified attending to the different treatment they received. A set of the whole medicaments was registered, and the active principle was checked for performing the classification: antidepressant treatment (3.4%) and antipsychotic treatment (18.8%). For those children affected with Attention Deficit or Hyperactivity Disorder (ADHD), which is often related to ASD, a psychostymulant treatment is given, corresponding to the 13.8% of the total of the cases. Also, 13.8% of the participants received two or more drugs. When the drugs were classified into the subtypes, it was observed that ASD patients also consumed antiepileptic drugs. However, none of the participants had this type of treatment. When the database was reviewed, it was noticed that this medication

was prescribed combined with antipsychotics, so these participants were included in those having two or more drugs.

Finally, the age at diagnosis was 7 (3.5-9.5).

Table 2: Clinical variables considered for the participants with ASD.

VARIABLES		CASES
Age (years) (median (IQR))		13 (11-16)
Gender (n=%)	Male	87.5
Gestational age (n=%)	Preterm (<37)	35.7
	Term (38-40)	64.3
Birth weight (n=%)	<2500 g	21.4
	2500-3000 g	17.9
	>3000 g	60.7
Type of labor (n=%)	Natural	55.4
	Induced natural	14.8
	Caesarean section	11.1
	Planned caesarean section	18.5
Treatment (n=%)	None	41.4
	Antiepileptic	-
	Antidepressant	3.4
	Antipsychotic	13.8
	Psychostimulant	13.8
	Melatonin	13.8
	Two or more	13.8
Age diagnosed (years) (median (IQR))		7 (3.5-9.5)

5.2. SAMPLE TREATMENT OPTIMIZATION

To determine lipid peroxidation biomarkers and cortisol in saliva, a previous optimization of the sample treatment and procedure was performed. The internal standards were selected by similarity to the analytes of interest. Retention time, polarity and structure were taken into account, thus PGF2α-d4 (similar to F2-IsoPs) and d4-10-*epi*-10-F4t-NeuroP (similar to F4-NeuroPs) were selected. For cortisol determinations, sulfadimethoxine was selected as internal standard. All internal standards were added in the first step of the treatment procedure to minimize experimental errors (García-Blanco et *al*., 2016; Peña-Bautista et *al*., 2018). Then, the sample volume was also assayed between 25 and 150 µL. The results showed that the optimal sample volume providing quantifiable signals was 150 µL. After that, several assays were conducted in order to elucidate the best working conditions for the calibrate. Better

results were obtained applying the same sample treatment. Finally, the parameters of the vacuum concentrator were also modified. For saliva, the equipment was programmed at minimum temperature (45 ºC), and the evaporation concluded at 30-40 minutes.

5.3. STATISTICAL RESULTS

5.3.1. OXIDATIVE STRESS

The statistical analysis performed in the study is based on univariate analysis. Non-parametric methods were used as the variables were not normally distributed. Quantitative variables were expressed as mean (standard deviation, SD) or median (inter-quartile range, IQR), and qualitative variables were expressed as n (%).

First a comparison of each analyte between groups (controls and cases) using Mann-Whithney test indicated the differences between medians. Subsequently, the lipid peroxidation biomarkers showing statistically significant differences between groups were: PGE2 (p=0.014), 15R-15-F_{2t}-IsoP (p=0.013), Total IsoPs (p=0.000). Figure 4 depicts the box plots for PGE2 (a), 15R-15-F_{2t}-IsoP (b) and Total IsoPs (c). While PGE2 showed a higher expression in the group controls compared to cases, the median of 15R-15-F_{2t}-IsoP and total IsoPs appeared higher in cases than controls, and they can be considered as potential ASD biomarkers. The variable gender was also compared, observing that the medians for Total IsoPs showed statistically significant differences between males and females, meaning that these biomarkers tend to appear more in the first group (Figure 5). Therefore, the appearance of IsoPs might be related to gender and not to the disease. This result will be discussed below. No statistically significant differences were obtained for the other compounds.

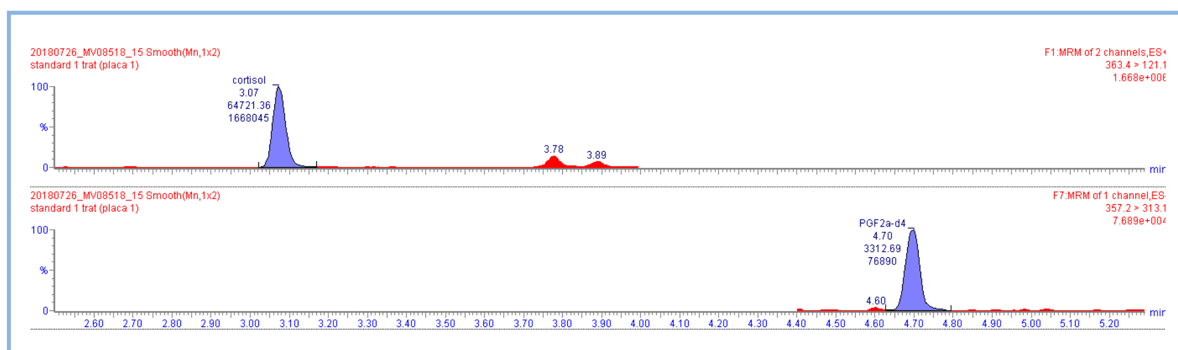
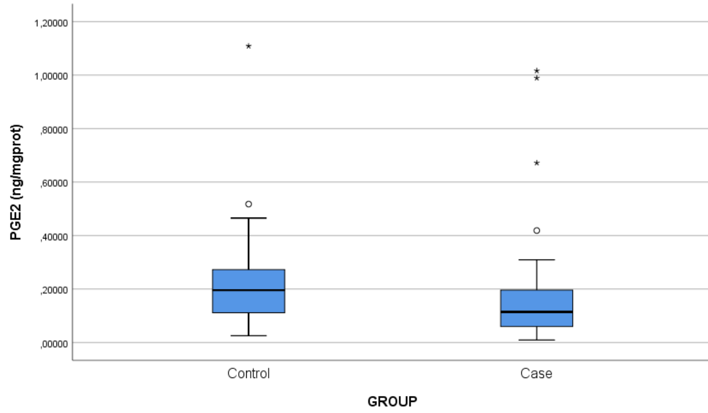
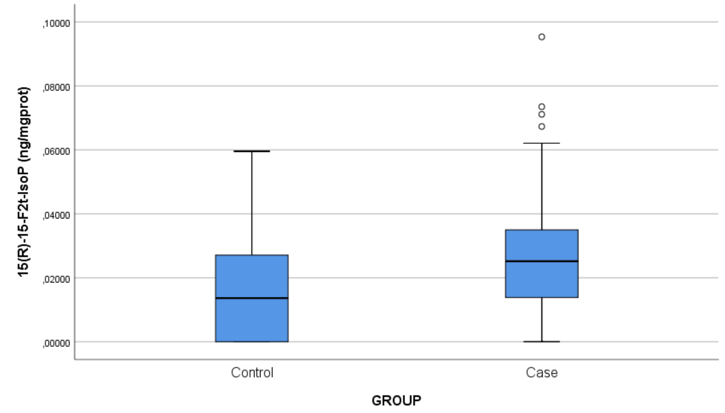


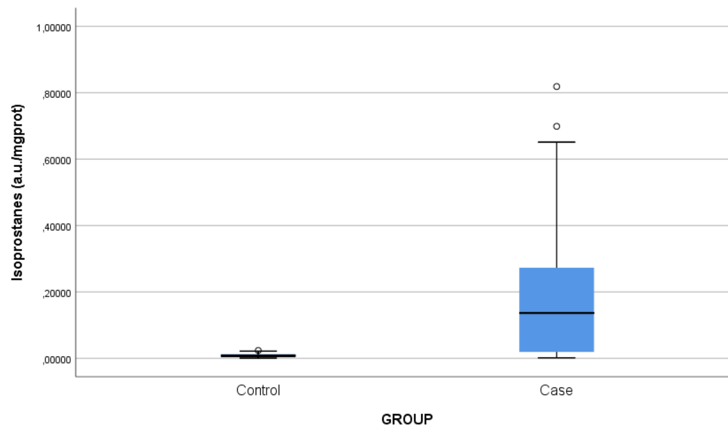
Figure 5: Example of chromatogram: cortisol peak appears at 3.07 min. PGF2α-d4 (deuterated IS) peak appears at 4.70 min.



(a) **Box plot of PGE2 levels for each group.**



(b) Box plot of 15(R)-15F_{2t}-IsoP for each group.



(c) Box plot of total IsoPs for each group.

Figure 6: Box plots for PGE2 (a), 15R-15-F_{2t}-IsoP (b) and total IsoPs (c) showing significant differences between group control and group case.

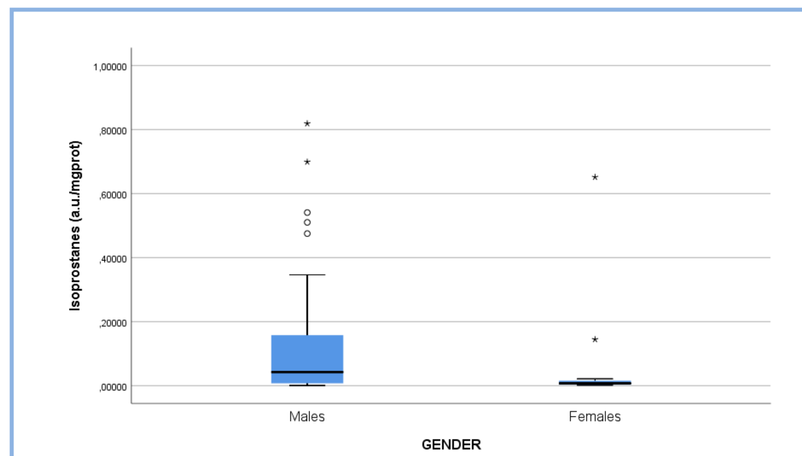


Figure 7: Box plots for total IsoPs showing significant differences between males and females.

5.3.2. PSICOLOGICAL STRESS

The same statistical analysis was performed with cortisol. By means of Mann-Whitney test, cortisol (p=0,031) showed statistically significant differences between group (Table 2). In Figure 6 we can see that the control group showed higher levels than the case group. The cortisol levels did not show any significant differences depending on the gender.

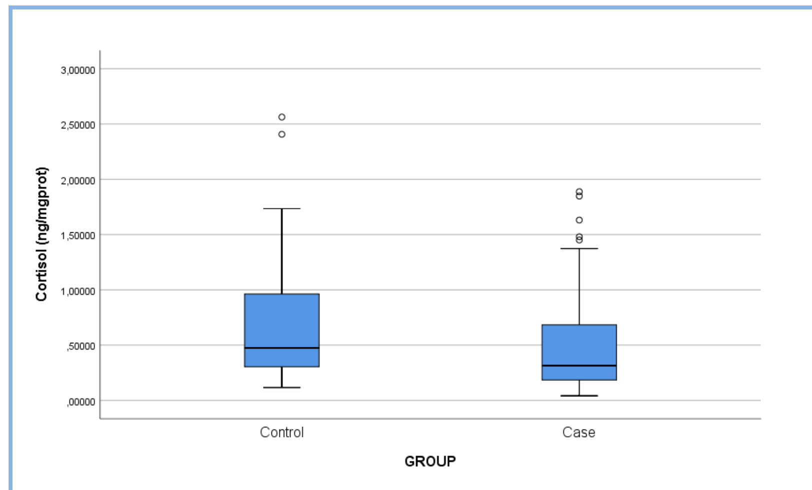


Figure 8: Box plots for cortisol showing significant differences group control and group case.

Table 3: Statistical results obtained by SPSS software: p value, mean, median (inter-quartile range, IQR), and qualitative variables expressed as n (%).

	MEAN±SD (ng/mg prot)		MEDIAN (IQR) (ng/mg prot)		
Compound	**Control (n=43)**	**Case (n=41)**	**Control (n=43)**	**Case (n=41)**	**p value**
15-keto-15-E_{2t}-IsoP	0.06±0.09	0.39±1.66	0 (0.00-0.10)	0.03 (0.00-0.11)	0.429
15-keto-15-F_{2t}-IsoP	0.00±0.01	0.00±0.00	0 (0.00-0.00)	0 (0.00-0.00)	0.308
PGE2	0.23±0.09	0.18±0.22	0.20 (0.11-0.27)	0.12 (0.06-0.20)	0.014
15-E_{2t}-IsoP	0.06±0.03	0.06±0.05	0.06 (0.04-0.07)	0.04 (0.03-0.07)	0.092
5-F2t-IsoP	0.02±0.04	0.03±0.04	0 (0.00-0.03)	0 (0.00-0.04)	0.299
15(R)-15-F_{2t}-IsoP	0.02±0.02	0.03±0.02	0.02 (0.00-0.03)	0.03 (0.01-0.04)	0.013
15-F2t-IsoP	0.01±0.01	0.01±0.01	0.02 (0.01-0.02)	0.01 (0.00-0.02)	0.202
PGF2α	0.15±0.09	0.15±0.09	0.17 (0.08-0.24)	0.13 (0.08-0.19)	0.616
4(RS)-14F_{4t}-NeuroP	0.07±0.20	0.06±0.21	0 (0.00-0.00)	0 (0.00-0.00)	0.696
14(RS)-14-14_t-NeuroP	0.55±0.58	0.61±0.79	0.60 (0.00-0.88)	0.50 (0.00-0.84)	0.814
Ent-7(RS)-7-$F2_y$-dihomo-IsoP	0.02±0.06	0.06±0.10	0 (0.00-0.00)	0 (0.00-0.11)	0.113
7(RS)-ST-Δ8-11-dihomo-IsoF	0.22±0.26	0.24±0.38	0 (0.00-0.46)	0 (0.00-0.40)	0.748
17-epi-17-F_{2t}-dihomo-IsoP	0.11±0.16	0.25±0.63	0.07 (0.04-0.10)	0.06 (0.04-0.10)	0.863
17-F2t-dihomo-IsoP	0.25±0.13	0.23±0.14	0.23 (0.18-0.29)	0.20 (0.15-0.26)	0.083
1a,1b-dihomo-PGF2α	0.22±0.40	0.09±0.26	0 (0.00-0.58)	0 (0.00-0.00)	0.106
Cortisol	0.72±0.58	0.55±0.52	0.47 (0.30-0.98)	0.31 (0.18-0.70)	0.031
Isoprostanes*	0.01±0.01	0.19±0.21	0.01 (0.01-0.01)	0.14 (0.00-0.40)	0
Isofurans*	0.01±0.01	0.01±0.01	0.00 (0.00-0.01)	0.01 (0.02-0.30)	0.757
Neuroprostanes*	0.17±0.29	0.15±0.18	0.08 (0.04-0.18)	0.08 (0.04-0.20)	0.909
Neurofurans*	0.03±0.05	0.02±0.02	0.02 (0.01-0.03)	0.02 (0.01-0.02)	0.560
Dihomoisofuranes*	0.00±0.01	0.00±0.01	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.702
Dihomoisoprostanes*	0.01±0.02	0.02±0.04	0.01 (0.01-0.02)	0.01 (0.01-0.01)	0.274

* (arbitrary units/mg protein)

6. DISCUSSION

In this work, an analytical method have been developed to determine lipid peroxidation and cortisol levels in saliva samples from ASD patients.

One of the first questions that may arise from the clinical variables that were exposed is the fact that the incidence of Autism Spectrum Disorder is higher in males than in females. The present study shows this fact clearly. From the total number of patients (n=41), 87.5% of the population were males. The male bias of ASD incidence can be considered one of the most extreme of all neurodevelopmental disorders. Several theories have been constructed. One of the possible explanations is a hypothetic vulnerability of males to genetic mutations. However, the most significant theory relates to the levels of testosterone, which generates estradiol under the action of neuronal aromatase enzyme. The combination of these steroids modulate brain development by unknown cellular mechanisms in neuropsychiatric disorders. Thus, the challenge for the future is determining the cause of the dysregulation of the genes that regulate normal development in males, and the identification of therapies for reversing the effects (McCarthy et *al*., 2017).

On the other hand, some obstetrical and delivery factors were considered, as several studies correlate perinatal adverse events with ASD, so the next clinical variable taken into account was the gestational age at birth. Associations between preterm birth and ASD have been reported lately. The presence of inflammatory mediators in the maternal, fetal and neonatal blood may interfere during pregnancy in brain development and cause an atypical development of the children, and may initiate the preterm labor and delivery process. The inflammatory proteins (cytokines, chemokines…) are present in maternal, placental and fetal compartments. Their main function in the brain is recruiting cells to defend and repair tissue damage, but their prolonged presence in brain tissue may lead to neurological and behavioral abnormalities. Some studies have reported that inflammatory protein imbalances during the first stages of brain development might make the brain vulnerable to subsequent insults. Moreover, inflammatory markers in brain tissue and CSF of ASD patients have been detected in several researches (Erdei et *al*., 2014). Regarding the type of labor, induced natural labor, caesarean section (CS) and programmed caesarean section were considered. Many epidemiologic researches on possible risk conditions for ASD have focused on these factors, and suggest that occur more often in autistic patients compared to neurotypical individuals. According to Gialloreti et *al*., the majority of studies were performed comparing case and control looking for odd ratios of ASD children whose birth was by CS. Moreover, planned CS

delivery is usually considered, but not emergency/urgent CS. Furthermore, studies should be performed for defining population subgroups based on the genetic susceptibility of children with ASD, as undergoing CS does not mean that CS is playing a significant role in the disorder (Giarotelli et *al*., 2014). In relation with labor induction, oxytocin (OT) plays an important role. A high percentage of women undergoing labor induction receive exogenous oxytocine. The exposure to synthetic OT is associated to adverse effects in the mother and offspring. Recent studies have also suggested that the development of the oxytocinergic system is sensitive to gender (Weisman et *al*., 2015; Schieve et *al*., 2016). Finally, the birth weight was evaluated. Numerous studies have documented associations between preterm birth and low birth weight with different developmental disabilities and disorders, such as ASD, Attention Deficit Hyperactivity Disorder (ADHD) and Intellectual Disability (ID). However, there are not etiologic subgroups defined for being able to correlate a specific developmental disorder to specific preterm conditions or birth weight. In the present study, 9.75% of the participants with ASD, were also diagnosed with ADHD. From this subgroup, 25% were born with low birth weight. No definitive correlations can be established based on this data.

The treatment of ASD patients was also consulted and included in the database. The heterogeneity of ASD is mainly due to the occurrence of comorbidities. The most significant psycopathologies in autism are anxiety, depression, ADHD, intellectual disabilities, epilepsy, schizophrenia and sleep difficulties. From the results obtained in the present study, it has been observed that a high number of ASD patients with comorbid epilepsy or schizophrenia, also show less severe comorbidities, as anxiety or social impairments, whereas patients diagnosed with ASD only, do not show any other psychopathologies. So it can be affirmed, that the presence of one or more of moderate comorbidities may be associated with severe autism symptoms. Furthermore, the behavioral dysregulation in ASD patients is correlated with anxiety, depression and sleep difficulties (Masi et *al*., 2017).

The age of the diagnosis of ASD was checked as well, because it is important to know when this event occurred. If this did not happen when the first symptoms of the disorder appeared, the patient might not have received the most adequate treatment. It is impossible to establish the borderline age for ASD diagnosis, because every children developmental phases are different. Moreover, the etiology of the disorder is not clear as well, thus the environmental factors might also have a role on the appearance of the first symptoms. These conditions are clearly unpredictable, so it is impossible to define an appropriate age for the diagnosis.

As it has been said previously, saliva has emerged as an alternative matrix biological sample in ASD patients studies, since it is easy to collect and non-invasive. Moreover, the procedure of sample collection does not promote anxiety compared to blood extraction. However, some saliva biomarkers might be present in very low concentrations compared to other biological fluids as plasma or urine, but this issue can be solved by using highly sensitive analytical techniques. In this case, UPLC-MS/MS was the analytical technique used, being able to detect several compounds. Once they were compared by statistical analysis, PGE2, 15R-15-F2t-IsoP, total IsoPs showed significant differences between the groups of participants. PGE2 is a membrane lipid derived molecules that function as signaling compound. PGE2 derives from the conversion of arachidonic acid to the precursor prostaglandin H2 (PGH2) by membrane-bound cyclo-oxigenase enzyme (COX). The researches of Brigandi et *al*. quantified plasma levels of PGE2 of control subjects and autism patients by means of LC/MS. The control samples were under the limit of detection, whereas PGE2 levels were detected in some ASD individuals. Our findings do not show these results. In fact, saliva samples from control group showed slightly higher levels of PGE2 than in autistic children. One possible explanation for this fact based on the work of Dean et al., is that the prostaglandins present a short half life, and consequently, the analysis of PGE2 might be more accurate if it is performed with urine samples. Moreover, PGE2 levels can be influenced by external factors, such as diet (Brigandi et *al*., 2015; Dean et *al*., 2012). Isoprostanes are prostaglandin-like compounds generated from different PUFAs. The clinical relecanve of the different classes of IsoPs hinges on the precursor location in the organism. F_2-Isoprostanes derive from AA, which is equally distributed throughout the organism. These prostanoids are less reactive than other lipid peroxidation products and consequently, they are easily detected in plasma and urine. According to Signori et *al*., F_2-IsoPs have become the biomarker for assessing oxidative stress due to their chemical stability. Moreover, elevated IsoPs levels in plasma and urine have been reported in several diseases (Signorini et *al*., 2013). In the present study, total IsoPs showed significant differences between groups. However, when a gender-matched analysis was conducted between the autistic and control groups, we obtained total IsoPs increased in males, meaning that the appearance of isoprostanes levels were linked to the gender and not to the disease. For confirming this fact, a more representative control population should be selected, considering the incidence pattern of ASD. In the present study, 87.5% of the cases were males, but the percentage of males in controls was lower. So the results obtained for total IsoPs are not conclusive. The last significant compound obtained was 15R-15-F_{2t}-IsoP, and it showed higher levels in saliva samples from ASD patients. Isomers of F_2-Isoprostanes are considered standards for oxidative stress and potential biomarkers in neurodevelopmental pathologies,

such as ASD and Rett Syndrome (RS). RS is considered a genetic model for ASD. This syndrome is included in the set of ASD, and affects only females, as the causative mutation is linked to the X chromosome and affects to the methyl-CpG binding protein 2 gene (MECP2). The majority of cases show a similar clinical picture: loss of cognitive, social and motor skills, development of autistic behavior and acquisition of a stereotyped hand movement. The genetic mechanism of this disease has been highly studied, but recent studies have considered the role of the altered redox homeostasis. RS is characterized by increased levels of IsoP at every stage of the disease. High plasma levels of F_2-dihomo-IsoPs are detectable at the first stages of the disease, whereas F_2-IsoPs do not increase with the same raise. Consequently, plasma isoprostanes might be considered as an index of generalized of lipid peroxidation (Signorini et *al*., 2013). Furthermore, interesting conclusions were obtained by Karamouzi et *al*. in their study about salivary levels of 15-F_{2t}-Isoprostane. It was obtained an elevation of saliva levels of 15-F_{2t}-Isoprostane in individuals with autism compared to controls, indicating an increased oxidative stress. Also, it was demonstrated that oxidative stress and lipid peroxidation may play a role in autism after the observation of altered glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase activities, total gluthatione (TGSH), GSH/GSSG and cysteine levels in autistic children compared to control children. Moreover, in the study children with ASD were found to have increased environmental toxins in their body that may generate oxidative stress and lipid peroxidation (Karamouzi et al., 2017). These results support the bibliographic evidences, and confirm that 15R-15-F_{2t}-IsoP might be considered as a potential biomarker for Autism Spectrum Disorder, and sustain as well the theory that oxidative stress might be one of the etiological causes of the disorder.

Cortisol has been defined as one of the most important molecular markers for psychological stress. Many studies reported clear results when cortisol levels of children diagnosed with ASD compared to healthy children were tested. Another objective of our study was comparing the levels of cortisol in saliva of the participants. Surprisingly, it was obtained that the cortisol level was higher in control children than ASD cases. A possible interpretation for this result could be that the majority of participants are high school students that might suffer from psychological stress during weekdays. However, further research is required to confirm this result.

7. CONCLUSIONS

The development of an analytical method to determine biomarkers of different nature has been carried out successfully. However, the objective proposed in this study has not been fully accomplished.

The main conclusions are:

1. 15R-15-F_{2t}-IsoP was identified as a potential biomarker, showing higher levels in ASD patients than control participants.
2. The optimized analytical method allowed the identification and quantification of biomarkers of different nature.
3. PGE2 and cortisol showed higher salivary levels in control participants than patients with ASD.
4. Total isoprostanes cannot be considered significant biomarkers, because a linkage between gender and Total IsoPs was observed.

The limitations of the study can be easily overcome. Performing the assay with a higher number of participants, and selecting the control individuals following the prevalence of ASD could be one of the sort-term objectives. The period when the samples are collected can be tested as well, to corroborate if this fact has any influence on the cortisol levels detected when it is performed on the control group.

Autism Spectrum Disorders are still underestimated, in spite of its increasing incidence. The ASD physiopathology remains unknown, but the present study has brought some light into what seems one of the most important factors that cause ASD, oxidative stress. Further studies are required to find more potential biomarkers for the disease, and define the specific biomarkers that can be detected in biological samples. It is important to highlight the convenience of saliva samples for the diagnosis of autism, as it is a non-invasive sample whose collection does not cause anxiety to the patient. Therefore, this study can be an interesting starting point for future research about Autism Spectrum Disorders.

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