Open Access Research Journal - ONLINE **Medical and Health Science Journal, MHSJ**
www.mhsj.pradec.eu ISSN: 1804-5014 (ONLINE)
www.academicpublishingplatforms.com VOLUME 18, ISSUE 1, 2017, PP.2-13
The primary version of the journal is the online version **DOI:** http://dx.doi.org/10.15208/mhsj.2017.01

HETEROGENEITY NEUROCHEMISTRY IN CINGULATE CORTEX IN ADULTS WITH AUTISM SPECTRUM DISORDERS: A PROTON MR SPECTROSCOPY STUDY

Abnormal functional responses and reductions in functional connectivity associated with functional abnormalities of the posterior cingulate cortex (PCC) is one of the most consistent findings in individuals with autism spectrum disorders (ASD). However, the significance of such findings for the pathophysiology of autism is unclear. In this study, we investigated cellular neurochemistry with proton magnetic resonance spectroscopy imaging (1H-MRS) in both, anterior (ACC) and posterior (PCC) cingulate cortices, brain regions associated, with networks sub serving alerting and executive control of attention in patients with ASD. Compared to typical development (TD) group, the ASD group showed significantly higher N-acetyl-aspartate/choline (NAA/Cho) ratio in PCC and demonstrated the metabolic differences between anterior and posterior cingulated cortices as a contribution to the pathogenesis of autism. Furthermore, provide the first direct evidence of the relationship between abnormal metabolic activity and posterior cingulate cortex dysfunction in ASD.

CARMEN DOLORES JIMENEZ-ESPINOZA,
FRANCISCO MARCANO,
JOSE LUIS GONZALEZ-MORA

Campus Ciencias de La Salud,
University of La Laguna, Tenerife, Spain

Keywords: Proton Magnetic Resonance Spectroscopy, autistic spectrum disorder, cingulate cortex, brain metabolism

Email: carmen(dot)jimenez(dot)87[ats]ull(dot)edu(dot)es

Corresponding contact: Department of Physiology, School of Medicine, Campus of health,University of La Laguna, 38071, Tenerife, Spain

Citation: Jimenez-Espinoza, C. D., Marcano, F., Gonzalez-Mora, J. L., 2017. "Heterogeneity neurochemistry in cingulate cortex in adults with autism spectrum disorders: A proton MR spectroscopy study", Medical and Health Science Journal, Vo.18(1), pp.2-13,
DOI: http://dx.doi.org/10.15208/mhsj.2017.01

Introduction

Autism spectrum disorders (ASDs) are neurodevelopmental disorder with early life onset and variable developmental trajectory and is primarily affecting high-order integration processes, such as complex social interactions, associative thinking, and appropriate emotional reactions. The posterior cingulate cortex (PCC) is part of the *cingulate gyrus* which is a major part of the anatomical limbic system and according to classic accounts, is involved in emotion (Vogt, Finch, & Olson, 1992), but despite the importance of PCC in health and disease, there is no clear consensus about its function (Leech, Braga, & Sharp, 2012). ASD has been associated too with functional abnormalities of the PCC, specifically abnormal functional responses and reductions in functional connectivity. Recently (Rudie & Dapretto, 2013) reported a reduction in connectivity between the PCC and medial prefrontal cortex in a mixed group of children and adolescents young children with the disorder. Furthermore, have shown that the abnormalities in cingulate responses during interpersonal interaction correlate with the severity of autistic symptoms, and the failure to show task dependent deactivation in the PCC correlates with overall social function

(Leech & Sharp, 2014). In the present study, we have examined two regions of interest, the ACC and PCC, likely significant substrate responsible for some autistic behavior.

Cingulated gyrus

The anterior cingulate cortex (ACC) which is concerned with vocalizing, emotional and motoric functioning involving the hands, and regulating autonomic and endocrine activities is cytoarchitectonically agranular, defined term denoting the type of heterotypic cortex that is distinguished by its relative thickness and lack of granule cells. On a cellular level, the ACC is unique in its abundance of specialized neurons called spindle cells (Watson, Jones, & Allman, 2006). In contrast, the posterior cingulate cortex (PCC) forms a central node in the default mode network (DMN) of the brain, which is a network of interacting brain regions known to have activity highly correlated with each other and distinct from other networks in the brain. The DMN is most commonly shown to be active when a person is not focused on the outside world and the brain is at wakeful rest (Buckner, Andrews-Hanna, & Schacter, 2008). The PCC has been shown it is communicating with various brain networks simultaneously and is involved in various functions involved in visual-spatial and tactile analysis as well as motor output and memory, it is cytoarchitectonically granular. The name granular cells has been used by anatomists for a number of different types of neuron whose only common feature is that they all have very small cell bodies, have a structure typical of a neuron consisting of dendrites, a soma and an axon. Furthermore, the ACC exhibited greater dendritic complexity than the posterior cingulate (Schüz & Miller, 2003). This anterior advantage may reflect functional differences between the two regions as well as differences in interconnectivity (Vogt et al., 1992; Vogt, Rosene, & Pandya, 1979), with the anterior cingulate pyramidal cells receiving diverse synaptic input of both an effective and a cognitive nature (Devinsky, Morrell, & Vogt, 1995). The ACC region was characterized by a lower cell packing density than the posterior portion, thus indicating an inverse relationship between cell packing density and dendrites arborization, at least in homotypical isocortex (Schüz & Miller, 2003), directing our attention to deepen understanding of the variation in metabolic activity intro ACC and PCC.

Magnetic resonance spectroscopy as a tool for assessing metabolites activity in vivo

The magnetic resonance spectroscopy (^{1}H-MRS) is a non-invasive neuroimaging technique that estimates specific chemical metabolite measures in vivo, of different metabolites in specific cerebral regions. One of the most important contributions of ^{1}-H-MRS to clinical neurology is its ability to quantify neuronal loss and to demonstrate reversible neuronal damage (Rudkin & Arnold, 1999). Metabolites that can be detected in the human brain through ^{1}H-MRS include N-acetyl-aspartate (NAA), for instance, is used as a marker of neurons density and/or mitochondrial function, which is present in neurons and is actively involved in myelin synthesis; Choline-containing compounds (Cho) is a primarily related to the synthesis of membrane phospholipids. Furthermore, cholinergic nuclei in the human brain has an extensive direct and indirect influence on the cortical and limbic activity; creatine plus phosphocreatine (Cr) is related to cellular energy metabolism production and storage; and myo-inositol (mI) which is a regulator of brain osmotic balance, a precursor for phosphoinositide involved in the second messenger system and a glial marker as well as to reflect processes associated with the breakdown of myelin (Fayed & Modrego, 2005; Friedman et al., 2006; Zeegers, van der Grond, van Daalen, Buitelaar, & van Engeland, 2007).

Previous MRS findings in ASD

MR spectroscopy has many advantages in developmental psychiatry because provides chemical information on metabolites and can be useful to explaining the aberrant trajectories of brain development in ASD have not yet been elucidated. As brain structural studies show an aberrant trajectory of neurodevelopment, it was reasonable to predict that the degree of neurochemical abnormalities indexed by ^{1}H-MRS may also change according to the developmental stages and brain regions in ASD (Aoki et al., 2012). This recent meta-analyses of all available ^{1}H-MRS data conclude that metabolic abnormalities as measured with ^{1}H-MRS provide some evidence that ASD is characterized by age-dependent fluctuations in metabolite level across the whole brain and at the level of specific regions thought to underlie ASD-associated behavioral and affective deficits. Although specific changes in NAA metabolism have been reported in the ACC, we focused on this region of interest and chose the PCC as another comparator region of interest, which has frequently been implicated in the pathophysiology of ASD. As previous studies had not been reported increased as well as decreased metabolites signals in the PCC region, the working hypothesis was not directed, but we expected different signals of metabolites in patients with ASD.

Taking all presented evidence, the aim of this study was to investigate the posterior cingulate cortex using ^{1}H-Magnetic Resonance Spectroscopy, with the purpose to detecting changes in neuron density, glial density, cell-membrane processes, and energetic metabolism using of metabolites changes as markers present in brain of subjects with ASD. This study to used proton magnetic resonance spectroscopy (^{1}H-MRS) in youth with ASD and age-matched healthy control subjects to investigate potential between-group differences in metabolite concentrations in cerebral anterior cingulate cortex (ACC) and posterior cingulate cortex (PCC). Based on the research outlined above and prior work, we hypothesized that anterior cingulate cortex and posterior cingulate cortex would demonstrate abnormalities in neurons density as measured using N-acetyl aspartate (NAA) as a marker by in vivo ^{1}H-MRS in subjects with ASD.

Materials and Methods

Subjects

These study was approve by the Research Ethics Comities and Animal Welfares (CEIBA). Registration Number: CEIBA2013-0056 of University of La Laguna, and all subjects gave written informed consent. Participants in the study included 12 youth (2 female, 10 male) aged 17-23 years (mean, 20.2 $\pm$ 1.4 years) with high-functioning autism and 12 (3 female, 9 male) healthy control youth aged 19-24 years (mean, 21.8 $\pm$ 2.8 years). All 24 subjects right handed (Table 1, Appendix), ASD group age – and Autism Quotient test (AQ) (Baron-Cohen, 2001), matched typical development (TD). The diagnosis of autism was established by Psychiatrist and Psychologist in every case, was verified in each through your medical history.

MR Imaging and 1H-MRS Examination

All patients underwent MRI and ^{1}H-MRS at a 3T Signal HD MR scanner (GE Healthcare, Waukesha, WI, USA). The single-voxel acquisition used a spin-echo sequence recorded within the following parameters: echo time (TE) =23ms, repetition time (TR) =1070ms, 2 NEX, flip angle=90º, 256 acquisitions with point-resolved spectroscopy (PRESS) technique. During obtained acquisitions, the same experienced neuroradiology and blind to the clinical data placed the voxels (20 x 20 x 20 mm3) at bilateral anterior cingulated cortex and bilateral posterior cingulated cortex. The main metabolite resonances were

limited to 2.02 ppm (ppm) for NAA, 3.02 ppm for Cr, 3.20 for Cho, and 3.56 ppm for (mI). We use a TE-23ms because it's known that myo-inositol is readily detected in short-TE ^{1}H-MRS spectra of the brain due to its high concentration of 4-8 miliMolar (mM), (De Graaf, 2013). The voxel were positioned excluding contamination of signal from the skull and subcutaneous fat. Morphological examination enabled us to exclude other pathologies, such as congenital abnormalities, lesions in cerebral palsy, tumors and hydrocephalus.

1H-MRS post processing

The ^{1}H-MRS data sets were collected using single voxel acquisition techniques was performed using the SAGE software platform and LC Model. The post processing procedure consisted of the following steps: Zero filling, filtering (Hanning), 2D fast Fourier transformation, frequency shift correction, base line correction, and phase correction with constant phase angle.

Data Analysis

The volume of interest (VOI) was located in the anterior and posterior cingulated cortex regions (Figure 1, Appendix) and, we collected data on four metabolites from two subjects group, in two brain regions ACC and PCC. The spectra for each location were investigated separately and averaged. The results were processed with LC Model 6.2 (Provencher, 2001) to obtain concentration estimate for the following metabolites in each location in each subject: Cr, NAA, Cho and mI. The use of ratios does not necessarily assume one of the metabolites to be constant (Miller, 1991; Nery et al., 2009; Soares and Law, 2009). Furthermore, ratios account for all non-metabolite-specific differences, and these ratios therefore can be reasonably compared across all participants scanned at the same institution with the same protocol.

Statistical Analysis

In the statistical analysis were performed metabolite concentrations and ratios to the signal of creatine (Cr) considering its level as an inner standard of the examination, of N-acetyl aspartate (NAA), choline (Cho), and myo-inositol (mI) were analyzed. We used the nonparametric Mann-Whitney U test (P) with the assumption that normal distribution could not be achieved and, student's t-test (t) to determine if the two sets of data are significantly different from each other. Statistical significance was defined as $p<0.05$

Results

The demographic characteristics of the participating subjects summarized in (table 1), shown that the groups did no differ significantly neither gender nor age, but were differ significantly AQ between the groups ($p<0.0001$, two-tailed). Conventional MR images of all subjects showed no significant abnormalities. Concentrations of brain N-acetyl-aspartate (NAA), creatine + phosphocreatine (Cr+PCr), Choline + phosphocholine-containing compounds (Cho + PCho) and myo-inositol (mI) were determinate by 3T ^{1}H-MRS examinations in 12 high-functioning medication-free adults with a diagnosis of ASD and 12 age- and AQ-matched typical development (TD) in the ACC and PCC.

1. In 1H-MRS study, we found differences in metabolite absolute concentrations (Table 2, Appendix) in anterior and posterior cingulate cortex between ASD and TD groups. The four major resonances in the spectra (NAA, Cho, Cr, and mI) were curved-fitted and absolute concentrations were obtained from all voxel (Figure 2, Appendix). Our findings indicate that the group with ASD had a significantly lower ($p=0.003$) myo-inositol and

Choline concentration (p=0.01) in posterior cingulate cortex, compared with the anterior cingulate cortex in TD group. Taking into account the above, we consider that the calculation of NAA/Cr, NAA/mI and NAA/Cho ratios provide us added value in terms of severity of autism.

2. The NAA, Cho, Cr and mI absolute concentrations were used to calculate metabolite ratios of NAA/Cr, Cho/Cr, mI/Cr, NAA/mI and NAA/Cho (Table 3, Appendix). Ratios relative to Cr were calculated in order to control for the potential effects of differences in brain water content, T1 and T2 relaxation times of water.

3. We showed significant lower NAA/Cr (p=0.04), and mI/Cr (p=0.05) ratios (Figure 3, Appendix) in anterior cingulate cortex, conversely, NAA/Cho ratio that was significant increase (p=0.05) in posterior cingulate cortex in ASD group compared with TD group.

4. Furthermore, our findings shown significant lower NAA/Cr (p=0.002) and NAA/Cho (p=0.001) in ACC compared with PCC in ASD group, while TD group showed no significant difference between ACC vs. PCC (Figure 4, Appendix).

5. In ASD group NAA/Cho was significantly (p = 0.05) increased on PCC than TD group while NAA/Cr ratio in ACC was decreased significantly (p = 0.04) (a). Conversely, NAA/mI (p=0.03), NAA/Cho (p=0.007) was higher significance on (PCC) in ASD group compared with (ACC) in TD group, while NAA/Cho (p=0.02) was significantly decreased on (ACC) in ASD group than (PCC) in TD group (b) (Figure 5, Appendix).

Discussion

We investigated neuronal integrity in cortical regions implicated in the neuropathology of autism using single-voxel proton magnetic resonance spectroscopy. We evaluated the metabolites peaks in anterior and posterior cingulate cortices in adults with autism spectrum disorders comparing with healthy control group and, we considered reporting absolute concentrations properly required account for additional factors related to tissue-type, and relaxation times, using the reference scan without water suppression acquired in the LC Model. Furthermore, as discussed in review (Soares & Law, 2009), we consider that the use of ratios does not necessarily assume one of the metabolites to be constant. This study extended prior work in this posterior cingulate cortex area by establishing that metabolic abnormalities, which have been identified using ^{1}H-MRS.

The significant differences in the NAA/Cr and NAA/Cho ratios between ACC and PCC in the ASD group compared with ACC and PCC in the TD group, as well as, between ACC (ASD) vs PCC (TD) and PCC (ASD) vs ACC (TD) may be associated with disturbed brain energy metabolism in ASD. Furthermore, the abnormal increase of NAA/Cho ratio on posterior cingulate cortex compared with healthy group which not previously described in patients with ASD, suggests that the effects are characteristic of local posterior cingulate cortex and not anterior cingulate cortex.

- *N-Acetyl Aspartate*

Our findings show increasing levels of N-Acetyl aspartate (NAA) on the PCC in ASD, according to increased neuronal attenuation or metabolic abnormality, and/or abnormal synaptic pruning observed in adults with Asperger syndrome (Murphy et al., 2002). Furthermore, other studies has suggested the NAA was expressed by oligodendrocytes (Bjartmar, Battistuta, Terada, Dupree, & Trapp, 2002), and is less distributed in glia (Urenjak, Williams, Gadian, & Noble, 1993).

Also, local elevations of NAA could result from multiple mechanisms, including faster astrocyte-membrane decomposition of N-acetyl-aspartyl-glutamate (NAAG) into glutamate (Glu) and NAA (Cassidy & Neale, 1993), slower oligodendrocyte membrane degradation of NAA into acetate and aspartate (Baslow, 2000, 2010), slower intra-neuronal synthesis of NAAG out of NAA and Glu (Cangro, Namboodiri, Sklar, Corigliano-Murphy, & Neale, 1987), and/or faster intra-neuronal NAA synthesis out of

aspartate and acetyl-CoA (Patel & Clark, 1979). On the other hand, the excess of NAA concentration may reflect increased mitochondrial metabolism (Fayed & Modrego, 2005).

- *Creatine*

Another metabolite detected in our study is the creatine, which is an important indicator of energy production and creating ATP, and is used as internal reference to calculate the concentration of the other metabolites in ^{1}H-MRS, to be less concentration variable. The creatine also was increased in previous studies in other brain regions in adults with ASD (Brown, Singel, Hepburn, & Rojas, 2013; Page et al., 2006). Consistent, with our finding of elevated creatine in ACC suggests, that the overall cellular energy metabolism may be elevated in the ASD group by contrast, on PCC where the creatine was diminished. About this, It has been shown in vitro that glial cells contain a two-to fourfold higher concentrations of creatine than do neurons (Urenjak et al., 1993). On the other hand, higher levels of creatine may indicate that the glial/neuron ratio is higher in the ASD group than in the control group.

- *Choline and myo-inositol*

On the one hand, the choline is generally used as the marker of the cellular density and membrane turnover, so it reflects the damage in cholinergic neurons and, on the other, the myo-inositol is primarily located in glial cells and interpreted as a marker of gliosis and glial cells numbers, as it exists in a considerably higher concentration within glial cells. This suggested that although choline and myo-inositol are both linked to glial cells and its membrane, we might not fully understand the mechanism of these metabolites. Another reports include increased Cho/Cr, mI/Cr ratios (Gabis et al., 2008), in the amygdala hippocampal region in the ASD group, in contrast to our results of lower Cho/Cr, mI/Cr ratios in anterior cingulate cortex in ASD, which is concerned with vocalizing, emotional and motoric functioning involving the hands, and regulating autonomic and endocrine activities (Devinsky et al., 1995), and would explain the shortcomings of these skills in people with ASD.

The social behavioral deficits in the ASD group were specifically linked to aberrant connectivity of the PCC (Lynch et al., 2013) and it is plausible that altered connectivity of this system caused by higher NAA/Cho ratio in PCC may contribute to deficits in the social domain in ASD. Our study hypothesis, that patients with ASD would have different signals of metabolites anterior and posterior cingulated cortices by contrast with TD group, was supported by the results obtained. Although others previous studies have investigated brain metabolites of ASD patients (Aoki et al., 2012) it is difficult to consider these results once all the ^{1}H-MRS studies had different population characteristics, evaluated distinct brain regions, and used different ^{1}H-MRS parameters. However, our findings showed the differences of NAA/Cho and NAA/Cr ratios between anterior and posterior cingulated cortices as a contribution to the pathogenesis of autism. The data suggest that each group of individuals has a characteristic metabolic pattern that can be discriminated by ^{1}H-MRS.

Study limitations

Only few cases were examined in these investigations and therefore more neuropathology studies will be need to examine neural cell density, organization and synaptic properties and their relation to changes in metabolite concentration on the posterior cingulated cortex in ASD. Furthermore, have to consider more brain areas potentially implicated in autism etiology. A larger sample will increase substantiate results in low functioning ASD adults, and may reveal more significant changes in metabolic levels and association with symptomatology.

Conclusion

This study provides evidence of abnormalities in neurotransmission related to networks sub-serving executive control and alerting of attention, functions which have been previously implicated in ASD pathogenesis. The metabolic differences between the two regions ACC and PCC are evident (decreased metabolite ratios in the ACC and increased metabolite ratios in the PCC) in the ASD group, although each region ACC and PCC has different functions, the TD group reveals maintain a metabolic balance, absent in ASD group. The ASD group has a higher metabolic activity in the posterior cingulate cortex (neuronal density/turnover membrane) compared with the anterior cingulate cortex. The increase in the posterior cingulated of N-acetyl-aspartate in ASD group has not been described above; however there is evidence of elevated NAA in the brain as the cause of other pathologies. Furthermore, provide the first direct evidence of the relationship between abnormal metabolic activity and posterior cingulated cortex dysfunction in ASD. The next step in our research is elucidating within the multiple causes of a local elevation of NAA cellular mechanisms, which is linked to all our findings in the PCC. Finally, the magnetic resonance spectroscopy is a tool which provides important information about the abnormal brain metabolism and metabolic pathways involved in autism, so that understanding the cause and consequences of this dysfunction is likely to be important part of developing biomarkers that suggest new therapeutic avenue.

Acknowledgements

The authors wish to acknowledge the generous bequest of patients, who without the precious collaboration no research into the neurochemicals underpinnings of autism would have been possible. We also thank the organizations of persons with ASD (APANATE, ASPERTEN, and ALDIS) who assisted us to make this study, and MRI service for Biomedical Research (SRMIB).

Preliminary results of this study were presented to International Congress for Autism Research IMFAR 2014.

Funding

The preparation of this paper was supported, in part, by ROVIRALTA Foundation and Neurochemistry and Neuroimaging Laboratory. Ministerio de Ciencia e Innovación [PTA2011-4995-I, TIN2011-28146]; Ministerio de Industria, Turismo y Comercio [TSI-020100-2011-189, TSI-020100-2010-346]; Ministerio de Economía y Competitividad [IPT-2012-0961-300000].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

C.D.J-E, designed the experiment, oversaw its implementation, critical analysis of the results, and wrote the final manuscript. F.M.S performs the spectrometry analysis. J.L.G-M, assisted in the development paradigm.

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Appendix

TABLE 1. DEMOGRAPHIC DATA ASD & TD GROUPS

	ASD (n = 12)		TD (n = 12)		
Characteristic	**M**	**SD**	**M**	**SD**	**P**
Age (years)	20.2	1.4	21.8	2.8	0.645
AQ	32.3	7.8	10.3	5.9	< 0.0001
Sex					
Males	10		9		
Females	2		3		

Source: Own elaboration
Note: M = mean, SD = Standart Desviation, P = p value

TABLE 2. METABOLITE ABSOLUTE CONCENTRATION ACORDING TO BRAIN REGION

Absolute concentration [mM]	**NAA**			**Cr**			**Cho**			**mI**		
Regions	**M**	**P**	**t**	**M**	**P**	**t**	**M**	**P**	**t**	**M**	**P**	**t**
Anterior cingulate cortex												
ASD	9.18	0.15	0.07	5.85	0.30	0.18	2.07	0.60	0.48	6.12	0.34	0.30
TD	10.32			5.08			2.08			6.54		
Posterior cingulate cortex												
ASD	9.97	0.83	0.38	4.60	0.17	0.05*	1.41	0.14	0.04*	5.08	0.21	0.04*
TD	9.77			5.68			1.71			6.06		

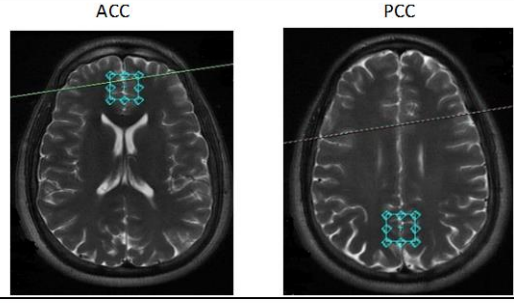
Source: Own elaboration
Note: : ASD vs. TD values for N-acetyl aspartate (NAA), creatine (Cr), Choline (Cho), and myoinositol (mI) absolute concentrations are group mean. P < 0.05. M = mean, P = p value, t = t Student

TABLE 3. CASE-CONTROL ANALYSIS OF METABOLITES RATIOS ACCORDING BRAING REGIONSACCORDING BRAING REGIONS

Ratios	**NAA/Cr**			**mI/Cr**			**Cho/Cr**			**NAA/mI**			**NAA/Cho**		
Brain Regions	**M**	**P**	**t**	**M**	**P**	**t**	**M**	**P**	**t**	**M**	**P**	**t**	**M**	**P**	**t**
Anterior Cingulate cortex															
ASD	1.64	0.04*	0.02*	1.09	0.05*	0.04*	0.36	0.25	0.08*	1.65	0.50	0.47	5.11	0.17	0.42
TD	2.33			1.53			0.45			1.64			5.31		
Posterior Cingulate cortex															
ASD	2.26	0.05*	0.08*	1.17	0.64	0.38	0.31	0.67	0.43	2.02	0.52	0.13	7.25	0.05*	0.01*
TD	1.90			1.13			0.32			1.76			5.96		

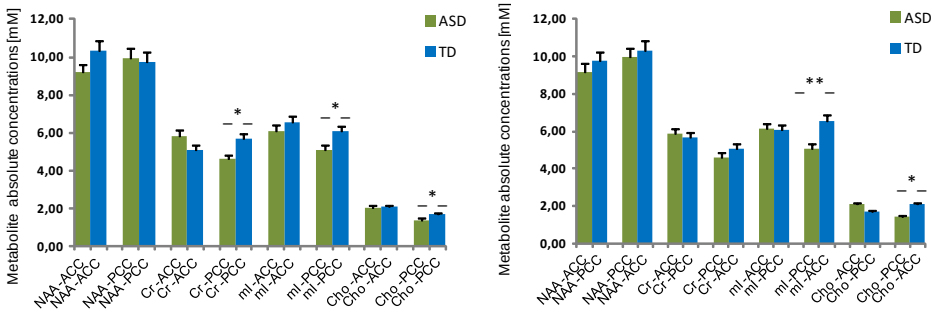
Source: Own elaboration
Note : ASD vs. TD values for N-a NAA/Cr, mI/Cr, Cho/Cr, NAA/mI and NAA/Cho ratios are group mean . P< 0.05. M = mean, P = p value, t = t Student

FIGURE 1. LOCALIZATIONS OF VOXELS (ROI).



Source: Own elaboration

FIGURE 2. MEAN METABOLITE ABSOLUTE CONCENTRATION (ACC VS.PCC)

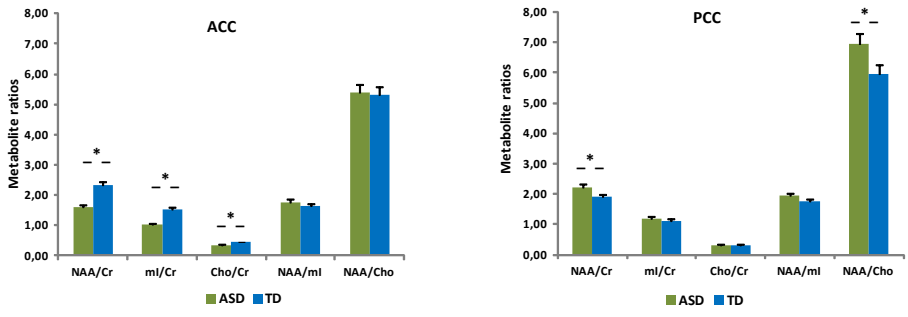


Source: Own elaboration

Note: Mean N-acetyl aspartate (NAA), creatine (Cr), choline (Cho), and myoinositol (ml) metabolite concentrations between ASD vs. TD, in (ACC) and (PCC).

P<0.05 Mann-Whitney test and t-student.

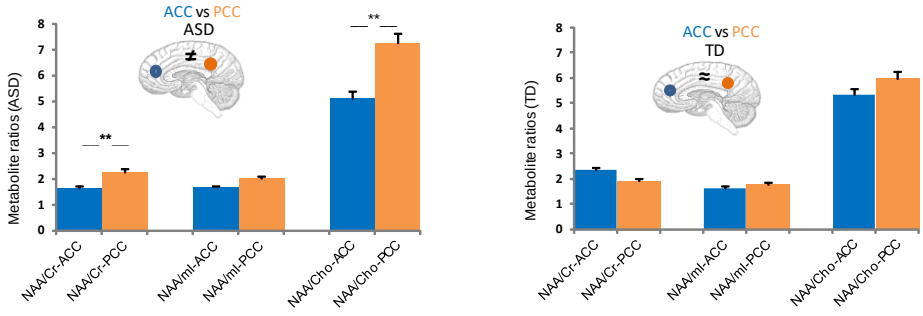
FIGURE 3. MEAN RATIOS (ASD vs. TD), ACC & PCC



Source: Own elaboration

Note: Mean NAA/Cr, ml/Cr, Cho/Cr, NAA/ml, NAA/Cho ratios between ASD vs. TD, in ACC and PCC. P< 0.05 Mann-Witney test and t-student.

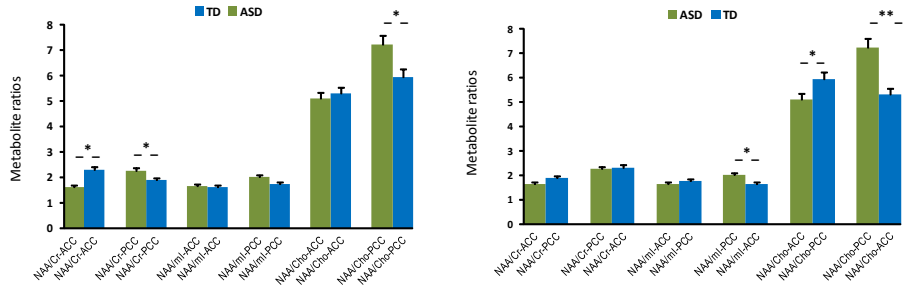
FIGURE 4. MEAN RATIOS (ACC vs. PCC), ASD & TD



Source: Own elaboration

Note: Mean NAA/Cr, NAA/mI and NAA/Cho ratios between (ACC) vs (PCC) in ASD and TD. P< 0.05 Mann-Whitney test and t-student.

FIGURE 5. MEAN RATIOS (ASD)$_{ACC}$ vs. (TD)$_{ACC}$; (ASD)$_{PCC}$ vs. (TD)$_{PCC}$; (ASD)$_{ACC}$ vs. (TD)$_{PCC}$; (ASD)$_{PCC}$ vs. (TD)$_{ACC}$



Source: Own elaboration

Note: Mean NAA/Cr, NAA/mI and NAA/Cho ratios in ACC (ASD) vs. ACC (TD) and PCC (ASD) vs. PCC (TD) by contrast ACC (ASD) vs. PCC (TD) and PCC (ASD) vs ACC (TD). P< 0.05 Mann-Whitney test and t-student.