PhD Course in Basic and Developmental Neuroscience

Head: Prof. Giovanni Cioni

"Autism Spectrum Disorders: from clinical identification to neuroimaging detection of brain abnormalities"

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Contents

Abstract

This thesis collects doctoral studies about early autism spectrum disorders (ASD) clinical identification and early ASD detection of brain magnetic resonance imaging (MRI) abnormalities. The work has been organized in five parts. In particular, the first report regards a screening population program for early ASD identification, purposely suited by Prof. Muratori and its research group and carried out by pediatricians of ASL 1 in all children of Massa-Carrara district twice, *i.e.* at 12 and 18 months of age. The second work is a retrospective study about growth of head circumference (HC) during the first 14 months of age, in children with ASD compared to typical developing children. Respect to anthropometric measurement of control group, courtesy provided by pediatricians Dr. Becattini and Dr. Soldateschi, children subsequently diagnosed as ASD show in the first six months of life significantly excessive growth of HC. Nevertheless, the mechanism for ASD brain enlargement remains to be elucidated and it is unknown whether brain enlargement is a cause or consequence of ASD. The third report analyzes the capacity of CBCL parent-report questionnaire to discriminate between ASD patients, subjects with other psychiatric disorders and typical children and investigates on its possible use as a ASD screening instrument for children between 18 and 60 months of ages. The fourth research is implemented in cooperation with the Natbrainlab laboratory (Institute of Psychiatry, King's College Hospital London), directed by Dr. Marco Catani, with the aim of detecting structural connectivity differences between ASD patients and control subjects by means diffusion tensor imaging (DTI) measurements. The fifth and last study stems from the strong collaboration with the Istituto Nazionale di Fisica Nucleare (INFN) and concerns a structural MRI investigation on female children with ASD, a population poorly investigated in ASD neuroimaging studies and, for this reason, considered as "research orphan".

Riassunto

Questa tesi raccoglie gli studi effettuati nel corso del dottorato riguardanti il riconoscimento clinico precoce dei disturbi dello spettro autistico (DSA) e l'identificazione precoce tramite risonanza magnetica (RM), delle anomalie cerebrali nei pazienti DSA. Il lavoro è stato organizzato in cinque parti. In particolare, il primo resoconto riguarda un programma di screening per l'identificazione precoce dei DSA, messo a punto dal Prof. Muratori e dal suo gruppo di ricerca e condotto dai pediatri di libera scelta della ASL 1 con una duplice valutazione, effettuata a 12 e a 18 mesi di vita in tutti i bambini della provincia di Massa-Carrara. Il secondo lavoro è uno studio retrospettivo sulla crescita della circonferenza cranica (CC) nei primi 14 mesi di vita in bambini con DSA confrontati con bambini con uno sviluppo tipico. Rispetto alle misure antropometriche del gruppo di controllo, cortesemente fornite dai pediatri Dott.ssa Becattini e Dott. Soldateschi, i bambini successivamente diagnosticati come DSA mostrano nei primi sei mesi di vita una crescita significativamente maggiore della CC. Tuttavia, il meccanismo alla base dell'aumento cerebrale e il suo ruolo nell'eziopatogenesi dei DSA rimangono argomenti da chiarire. La terza ricerca analizza la capacità del questionario CBCL compilato dai genitori di discriminare tra pazienti con DSA, soggetti con altri disturbi psichiatrici e bambini con sviluppo tipico e indaga inoltre il suo possibile utilizzo come strumento di screening per i DSA nei bambini di età compresa tra i 18 e i 60 mesi. Il quarto lavoro è stato progettato in collaborazione con il laboratorio Natbrainlab (Institute of Psychiatry, King's College Hospital London), diretto dal Dott. Marco Catani, ed ha lo scopo di individuare eventuali differenze nella connettività strutturale tra i pazienti DSA e i soggetti di controllo attraverso misure derivate dall'imaging del tensore di diffusione (DTI). Il quinto e ultimo studio nasce dalla forte collaborazione con l'Istituto Nazionale di Fisica Nucleare (INFN) e riguarda un'indagine di RM strutturale focalizzata sulle bambine con DSA, una popolazione scarsamente presa in considerazione dagli studi di neuroimmagine nei DSA e

pertanto considerata “research orphan”.

Introduction

Autistic Disorder –AD-, Asperger Syndrome –AS- and Pervasive Developmental Disorder Not Otherwise Specified –PDD-NOS- (referred to hereafter as Autistic Spectrum Disorders, -ASD-) are a class of neurodevelopmental pathologies characterized by a triad of early symptoms: qualitative impairments in social interaction and in social communication, alongside unusually restricted or stereotyped interests and behaviours (American Psychiatric Association, 2000). The pathogenesis remains unclear in more than 90% of cases (Fombonne, 2003), in which the pathology is defined as "idiopathic", i.e. triggered by interactions between multiple, unknown genes and environmental factors (Bailey et al. 1996; Trottier et al. 1999). ASD is a relative common disability, with a prevalence of 1:110 children in the U.S (Centers for Disease Control and Prevention -CDC-, 2009) increasing almost ten-fold from studies published fifty years ago. Remains to be elucidated if it is a true increase in the prevalence of ASD or a consequence of: a) changing criteria (inclusion of milder cases, such individuals with PDD-NOS – first appeared as a new category in the *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised DSM-III-R*- and AS -included for the first time in the *DSM-IV*) b) changing characteristics (enhanced inclusion of ASD subjects without mental retardation, i.e. high-functioning autism); c) diagnostic substitution (inclusion of children that were diagnosed as having conditions such as idiopathic mental retardation, speech impairment and learning disability -Shattuck 2006-); d) increased early recognition (reconductable to higher public awareness and parents knowledge of ASD symptomatology and, on the other hand, to employment of screening tools and more reliable evaluation instruments by health care professionals).

Currently, ASD is only defined on the basis of a select set of behavioral abnormalities that map onto specific functional circuitry of the brain. The difficulties with social reciprocity,

communication, and restricted and repetitive behaviors and interests that occur with ASD suggest that the syndrome affects a diverse and widely distributed set of neural systems.

There is converging evidence that ASD is associated with structural and/or functional brain abnormalities. In particular, several volumetric MRI studies, in agreement with clinical measurement of head circumference, have demonstrated that ASD brain undergoes atypical patterns of growth during early postnatal life and thus it is accompanied by subtle and spatially distributed differences in brain anatomy in comparison with typical development subjects. In particular, differences in regional grey matter (GM) and white matter (WM) volumes have been found by several neuroimaging laboratories, according to voxel-based morphometry (VBM) studies of structural T1 brain MRI data. Theoretic models suggest that one consequence of an increased brain size in ASD patients will be an alteration of neural connectivity that in its turn could determine a distorted information transfer characterized by local over-connectivity and long-range under-connectivity. New procedures for studying brain connectivity include diffusion tensor imaging (DTI), a non-invasive magnetic-resonance method that can identify the presence of subtle WM abnormalities in the absence of volumetric changes detectable by conventional MRI. Recent studies on DTI data have reported abnormal connectivity patterns in the brains of ASD subjects that may be accompanied by decreases in white matter integrity. On the other hand, functional magnetic resonance imaging (fMRI) has shed some light on which brain regions show functional abnormality in ASD, revealing, for example, altered functional activity in the brain region that strongly supports language and communication abilities, as well as social perception and social cognition.

However, the considerable heterogeneity of results between existing studies requires further work to better characterize both the GM and the WM abnormalities. Several studies on this topic are actually affected by the limiting factor of an exiguous sample size and/or

heterogeneity investigated age range that disturbs clear hypothesis about brain developmental trajectories.

Our research can be subdivided into two main domains:

1) clinical studies on early ASD diagnosis by means a population screening program, a parent-report questionnaire and a seriate measurement of early head circumference;

2) MRI studies on brain volumetric and structural connectivity (DTI) data in ASD children.

In particular, the following aspects will be trated in the thesis:

Chapter 1 focuses on the challenge of early ASD identification and presents the preliminary data of a pilot ASD screening study.

Chapter 2 presents the final data of a doctoral study on the identification of preschoolers with ASD through a care-giver-completed behavioral checklist, the Child Behavior Checklist for Ages 1½-5 (CBCL 1½-5).

Chapter 3 shows the final data of a doctoral study on abnormal head circumference (HC) growth during the first months of life in children with ASD.

Chapter 4 describes neuroimaging techniques (diffusion tensor imaging –DTI-, voxel based morphometry –VBM-) applied to the in vivo study of the brain in children with ASD.

Chapter 5 illustrates the final data of a doctoral study on VBM in female children with ASD.

Chapter 6 reports the preliminary data of an ongoing doctoral study on DTI in children with ASD.

Chapter 1

Screening for Autism Spectrum Disorders

1.1 Early ASD identification

Early diagnosis is a crucial step to ameliorate outcome of children with Autism Spectrum Disorders (ASD); in fact a timely, intensive and specialized treatment can have a beneficial impact and achieve encouraging results for both language and cognitive skills as well as social functioning (Bristol, 1996; Dawson, 2010; Harris and Handleman, 2000; Landa, 2008; Matson, 2007; National Research Council, 2001; Reichow, 2009; Rogers and Lewis, 1989; Rogers, 2008; Stone, 1999-2001; Szatmari, 2003): this assumption has led the American Academy of Pediatrics (AAP; 2006) to recommend a screening for ASD in all 18- and 24-month-olds children.

In clinical practice, in order to realize a standardized diagnostic evaluation in young children referred for possible ASD, the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000) and the Autism Diagnostic Interview – Revised (ADI-R; Rutter et al., 2003) are widely used and accepted as a “gold standard" diagnostic instrument. However, these tools were not developed for the purpose of an initial screening of autism during a well-being check-up, because they are lengthy, require extensive training, skill and experience on the part of the examiner to be administered and appropriately interpreted (Scambler et al., 2001); for these reasons they can be considered second level instruments (Filipek et al., 2000).

In the absence of a standardized, validated and reliable screening protocol for ASD (for a review see Barbaro et al., 2009 and Zwaigenbaum et al., 2009), different studies have proposed behavior checklists and questionnaires for detect ASD during the first years of life, searching for good sensitivity and specificity (Baron-Cohen et al., 1992; Dietz et al., 2006; Gray et al., 2008; Oosterling et al, 2009; Pierce et al., 2009; Robins et al., 2001; Reznick et al., 2006; Wetherby et al., 2004). In fact, even if most parents of children with ASD first become

concerned about their child's development between 12 and 23 months of age (Baghdadli et al., 2003; Chawarska et al., 2007; Coonrod et al., 2004; Cox et al., 1999; De Giacomo et al., 1998; Frith and Soares, 1993; Lord, 1995; Ozonoff et al., 2009), several months may elapse before they discuss it with their child's physician and there may be a further delay between the paediatric visit and ASD diagnosis. This implies that these toddlers are not professionally diagnosed until approximately 3 years or older (Howlin et al., 1997; Howlin and Asgharian, 1999; Maestro et al, 1999; Mandell et al., 2002; Mandell et al., 2005; Sivberg et al, 2003; Wiggins et al., 2006).

The implications of this gap extend far beyond developmental gains associated with early intervention; in fact, most parents of children with autism experience considerable amounts of stress owing to the fact that they parenting a child with atypical development, and the uncertainty of diagnosis accentuates parental anxiety (Doussard-Roosvelt et al., 2003).

Since parents are for the most part reliable informants, checklists and questionnaires can help clinicians to point out the actual risk for ASD, provide a prompt assessment procedure, and reduce the lag between parental concerns and treatment (Wiggins et al., 2006).

Some screening instruments are developed in order to identify very young children with ASD (for a review, see Robins et al., 2006; Johnson et al., 2007 and Pinto-Martin et al., 2008), while others provide informations about general developmental and behavioral disorders (Briggs-Gowan et al., 2006; Carter et al., 1999; DeGangi et al., 1995; Doig et al., 1999; Gadow & Sprafkin 1997, 2000; Glascoe et al., 1997; Matson et al., 2010).

Difficulty in early recognize ASD subjects

In the second year of life (Palomo et al., 2006) consistent behavioral differences could be identified between children subsequently diagnosed as ASD and typical subjects. The attempt to diagnose ASD children before the age of 24 months is hampered by the fact that ASD symptomatology usually emerge gradually over time (Ozonoff et al., 2008) and earliest

abnormalities of children subsequently diagnosed as ASD are often non-specific (sleeping, eating, temperament alterations). Moreover, prospective studies on younger siblings of ASD patients (children with an higher genetic risk of developing an ASD) show the absence of group differences during the first year of life (Bryson et al., 2007; Landa et al., 2006; Nadig et al., 2007; Yirmiya et al., 2007; Zwaigenbaum et al., 2005): only by 12 months, developmental differences begin to differentiate ASD from typical subjects.

1.2 Preliminary data from the first Italian screening for ASD.

In the present study, we used two screening programs, at 12 and 18 months, each of them combining a parent-report instrument with a task the child has been submitted to by the paediatrician. The aim is to identify children at risk of ASD in a community-based sample through the application of the presented screening protocol. The first screening program consists in its turn of two different levels. At first level: a) parents fill out First Year Inventory (FYI; Baraneck et al., 2003); b) paediatricians carry out the "response to name" task during medical well-child visits. At second level, children who failed "response to name" and/or met a score above the cut-off in Social-Communication Domain and/or in Total score at FYI were evaluated by an expert child psychiatrist with the Autism Observation Scale for Infants (AOSI; Bryson et al., 2007). The second screening program was realized at 18 months of age through a) the filling out by parents of Modified Checklist for Autism in Toddlers (M-CHAT) b) the "joint attention" task administered by paediatrician.

Methods

Subjects

A preliminary ongoing sample is composed of 180 children recruited in a primary care setting. Chronological (or corrected in preterm) age of 12 months (within two weeks before or after the baby's first birthday), absence of severe sensory or motor impairments and absence of identified genetic disorders were the inclusion criteria.

Measures

First Year Inventory: is a 63-item parent-report questionnaire developed as a general population-screening tool to identify 12-month old infants that might be at-risk for autism or a related developmental disorder. The FYI comprises two broad developmental domains: social communication and sensory regulatory functions. Established cut-off score are 10 for the Social Domain and 8 for the Total score.

*Autism Observation Scale for Infants***:** is a brief set of tasks intended to discriminate children with ASD from other children in infancy and has shown promising results as low as 12 months.

*Modified Checklist for Autism in Toddlers***:** is a 23-item parent-report questionnaire that is used as a screening tool for autism in toddlers between 18 and 30 months.

Results

Among the 180 children participating in first level screening, 8 were considered at risk (in particular: 3 of them failed both tasks, while the remaining 5 passed the response to name task, but went beyond the fixed cut-off at the FYI). The AOSI was employed as second level screening instruments on all 8 subjects, in order to reduce false positive. Only one child failed the task and was therefore referred to a diagnostic and therapeutic assessment. The remaining 7 children will undergo a neuropsychiatric evaluation at 15 months of age to monitor chiefly the socio-communicative skills. In order to minimize the false negative results,

all the 180 children of the sample will be re-evaluated at 18 months (second screening program) and finally at their third year of life.

Conclusions

This double screening program could be a promising instrument to detect children at risk for ASD, but larger samples are necessary in order to define its most effective use.

Chapter 2

The CBCL 1½-5 and the identification of preschoolers with autism spectrum disorders.

2.1 Child Behavior Checklist for Ages 1½-5 (CBCL)

The CBCL 1½-5 (Achenbach & Rescorla, 2000; Frigerio et al., 2006) is a 100 item parent-report measure designed to record the maladaptive behaviour of preschoolers. Each item describes a specific behavior and the parent is asked to rate its frequency on a three-point Likert scale (0 = not true; 1 = somewhat or sometimes true; 2 = very true or often true). The scoring gives summary profiles (including Internalizing, Externalizing and Total problems scores), syndrome profiles (*i.e.* Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems, and Aggressive Behavior), and five different DSM-Oriented scales (Affective Problems, Anxiety Problems, Pervasive Developmental Problems, Attention Deficit/Hyperactive Problems and Oppositional Defiant Problems). A T-score of 63 and above for summary scales, and of 70 and above for syndrome and DSM-Oriented scales, are generally considered clinically significant; values between 60 and 63 for summary scales, or between 65 and 70 for syndrome and DSM-oriented scales, identify the borderline clinical range; values under 60 or under 65 are not-clinical. Scores and profiles for each child were obtained thanks to a computer scoring software. Each profile has an easy-reading layout, which allows to immediately understanding if the scores are in normal, borderline or clinical range. For our research aim we focus on the Pervasive Developmental Problems scale (Table 2.1.), which is composed of 13 item that seemed to best fit with DSM-IV-TR criteria based on clinical judgement.

Among evaluation instruments, CBCL is the most widely used parent report checklist that measures a broad range of behavioral and emotional problems (Bird et al, 1987; Achenbach et

al, 1987; Crijen et al, 1999), displays adequate reliability and validity (Achenbach & Rescorla 2000) and requires little effort (it takes 5-10 min for parents to complete and 5 min to score). Almost twenty-years-ago, Rescorla was the first researcher to use CBCL for preschoolers with autism (Rescorla, 1988). In this study the emergence of an autistic factor suggested that a future use of the CBCL as a possible instrument to recognize children with autism might be fruitful. However, after Rescorla's investigation, only a few studies have applied CBCL to young children with autism (Duarte et al., 2003; Eisenhower et al., 2005; Hartley et al., 2008-2009).

In more recent years the CBCL was reformulated as ASEBA (Achenbach System of Empirically Based Assessment) where the preschool form, the CBCL 1½-5, was identified and used in different settings (Rescorla, 2005). The 100 problem item of the CBCL 1½-5 allows for both empirically based summary and syndrome scales and the new DSM-oriented scales (Achenbach and Rescorla, 2000). To construct these new DSM-oriented scales, the relationship between DSM IV diagnostic criteria for ASD and item of CBCL 1½-5 were studied (Krol et al., 2006). During the last years we have used this CBCL form for preliminary assessment in our second level neuropsychiatric clinic and we assumed, over time, that clinically significant elevations on the PDP scale was in good agreement with clinical ASD diagnosis. Our observation is supported by a recent paper that applied the CBCL 1½-5 to a sample of children referred to a third level autism program (Sikora et al., 2007), and where it was suggested that it can be a useful behavioral checklist for screening ASD. ASD screening tools covers until about the child's first two years of life, but early identification programs couldn't be available in every area (e.g.: in Italy, few regions performs screening ASD survey) or the disorder could be recognized only later, especially if belong to regressive onset (Werner et al., 2005) or if the level of impairment is subtle (Wiggins et al., 2006). In

these cases may be useful an instrument that helps a non-ASD-specialistic clinician to identify developmental anomalies not previously detected.

The overall purpose of the present investigation is to provide more detailed understanding of the predictive properties of the CBCL 1½-5 and in particular the Pervasive Developmental Problems (PDP) scale as an instrument to address a preschooler ASD diagnosis in a non-specialistic setting.

2.2 Methods

Participants

A total of 313 children aged 18-71 months were included in the study. Participants were divided into three groups: 1) an experimental group of 101 children (85 males and 16 females) affected by an ASD; 2) a control group of 95 children (43 males and 52 females) with other psychiatric disorders (OPD); 3) a second control group of 117 pre-schoolers (65 males and 52 females) with Typical Development (TD). Demographic characteristics of patients and controls are summarized in Table 1. All the ASD subjects were consecutively admitted to the Division of Child Neuropsychiatry of the University of Pisa, Scientific Institute 'Stella Maris' (Pisa, Italy) between September 2005 and June 2008 and diagnosed based on DSM-IV-TR criteria coupled with clinical judgments made by a research child psychiatrist and an experienced clinically trained research child psychologist with expertise in autism and confirmed by ADOS-G. Laboratory tests to rule-out medical causes of autism included audiometry, standard karyotyping, fragile X testing, and metabolic screening; brain imaging and EEG were performed when there was a clinical indication.

In the OPD group, diagnostic assessment were made by two experienced child psychiatrists and ASD was clinically eliminated; in order to support the exclusion of an ASD, the Childhood Autism Rating Scales (CARS; Schopler et al, 1986) was applied to this sample and all the children showed a total score less than or equal to 21, i.e. much less than 30, the cut-off point

for an ASD diagnosis. This clinical control group was recruited at the Department of Infant Psychiatry of the same Scientific Institute; final diagnosis of these children, according to the Diagnostic and Statistical Manual of Mental Disorders criteria (DSM-IV-TR; 2000) or Diagnostic and Classification of Mental Health and Developmental Disorders of Infancy and Early Childhood system, Revised Edition (DC: 0-3; 2005) was affective disorders for 59 subjects, oppositional defiant disorder for 25 subjects, and mixed disorders (adjustment disorder, reactive attachment disorder, encopresis, or feeding disorder) for 11 subjects, in the absence of mental retardation. Children with a clinical diagnosis of attention deficit hyperactivity disorder (ADHD), multi-system developmental disorder (MSDD) or regulatory disorder were excluded from this sample in order to avoid a possible, partial overlap with ASD symptoms.

The sample with TD was collected in three urban kindergartens in Pisa, Tuscany (Italy); were excluded subjects with whatever internistic problems or/and some parent or teacher concern about child development. The whole group (ASD, OPD and TD) was composed of Caucasian children of Italian descent belonging mostly to middle/upper middle class families according to the Hollingshead and Redlich criteria (1958). There were no differences in socio-economic status among the three groups of patients.

The study was approved by the research ethics boards of the Stella Maris Scientific Institute.

Table 2.1. Demographic characteristics of 101 autism spectrum disorders (ASD), 95 other psychiatric disorders (OPD) and 117 typical development (TD) children involved in this study.

	ASD (n = 101)	*OPD (n = 95)*	*TD (n = 117)*
Age (months)			
Mean ± SD (range)	44 ± 12.3 (21-71)	40 ± 12.7 (18-70)	47 ± 12.0 (18-63)
Median			
Sex			
Male *n*	85	43	65
Female *n*	16	52	52
M/F ratio	5.31	0.83	1.25

Procedures

Parents (mother when possible) of the 313 children filled the CBCL 1½-5. In ASD and OPD, CBCL 1½-5 was completed at the beginning of a multidisciplinary clinical observation at the Scientific Institute Stella Maris (a suburban public academic hospital providing care to patients of all socioeconomic levels, coming from all over Italy); parents of TD filled the CBCL ½-5 in anonymous way at kindergarten. To avoid any bias related to the fact that caregivers of the clinical groups were subjected to different diagnostic interviews, CBCL was administered before the beginning of the clinical assessment.

2.3 Data Analysis

Chi Square test was used to compare categorical variables among the three groups. One way ANOVA with post-hoc S-N-K or was performed in order to test differences on age and the CBCL scales among ASD, OPD and TD groups, Multivariate Analysis of Covariance (MANCOVA) was used to evaluate differences on CBCL scales, regardless gender and age.

Logistic regression analysis with odds ratios (ORs) was performed to identify CBCL scales discriminating among the three groups. We used separate logistic regression models to

compare ASD with TD and ASD with OPD. In Model 1, the independent variable was CBCL Total score; in Model 2, the independent variables were Internalizing and Externalizing scores; in Model 3 the independent variables were syndrome scales; in Model 4 the independent variables were the five different DSM-Oriented scales.

CBCL scales which were identified as predictors of an ASD diagnosis in the logistic regression analysis at $p<.001$ were used in a receiver operating characteristic (ROC) analysis, in order to determine their optimal cut-offs to differentiate children with ASD from children with TD or OPD.

In the ROC analysis, sensitivity and specificity were plotted over the range of cutoff points. The area under the curve (AUC) represents the accuracy of the instrument in predicting children who will have or will not have ASD. The interpretation of the AUC values is traditionally as follows: an AUC<0.7 suggests "low" diagnostic accuracy; an AUC from 0.7 to 0.9 suggests "moderate" diagnostic accuracy; an AUC≥0.9 suggests "high" diagnostic accuracy (Sweet and Picket, 1982).

Analyses were carried out using SPSS version 15.0 for Windows (SPSS Inc. Chicago, IL, USA).

2.4 Results

Participant characteristics

Overall, 313 subjects were recruited (61% males and 38% females, mean age 43.8±12.5 months).

Chi-square analysis revealed a significant difference between gender distribution among ASD, OPD and TD groups (Chi-square=36.32, $p<0.001$), in particular the percentage of females was significantly lower in the ASD group as compared to the other two groups.

A one-way ANOVA indicated a significant difference in age among the groups ($F[2,310]=5.20$, $p=0.006$); S-N-K post hoc test revealed that the difference was due to the younger age of the

OPD compared to TD group (p=0.005); no differences were found between ASD and TD and between ASD and OPD.

Clinical characteristics

Analysis of Variance comparing ASD, OPD and TD groups revealed that ASD and OPD groups had significantly higher scores in all CBCL scales than TD group, except for Somatic Complaints and Sleep Problems scales that were not significantly different between ASD and TD groups. Moreover, ASD group presented higher scores than OPD group on Withdrawn and Attention Problems scales of the CBCL syndrome profile and on PDP scale of the CBCL DSM-Oriented. OPD group had higher scores compared to ASD on Anxious/Depressed, Somatic Complaints, Sleep Problems, and Aggressive Behavior scales of the CBCL syndrome profile and on Anxiety Problems and Oppositional Defiant Problems of the CBCL DSM-Oriented scales (Table 2.2.).

Using MANCOVA we found that the results were not significantly different after controlling for age and gender.

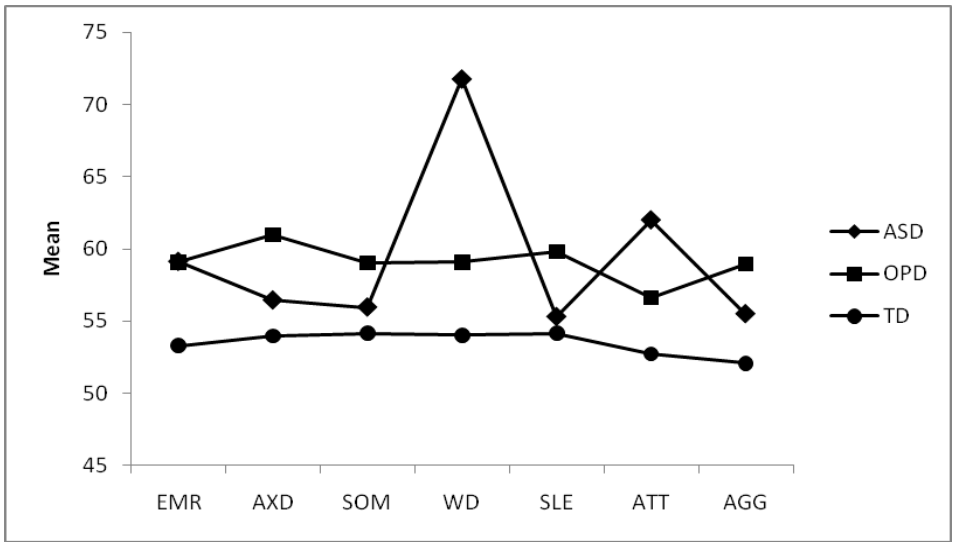
Figure 2.1a and 2.1b show CBCL profiles of the three groups on the syndrome scales and the DSM-Oriented scales, respectively.

Table 2.2 Means and standard deviations of the CBCL T-scores for ASD, OPD and TD groups.

CBCL Scales	ASD (N=101)	OPD (N=95)	TD (N=117)	F	**P** (value)
Total Score	59.97 (8.20)	59.22 (10.70)	47.70 (9.11)[a,b]	58.56	<0.001
Internalizing	62.11 (7.48)	59.67 (10.28)	48.84 (10.56)[a,b]	59.84	<0.001
Externalizing	56.01 (7.60)	56.47 (10.56)	46.70 (8.47)[a,b]	42.01	<0.001
Emotionally Reactive	59.12 (8.21)	59.07 (9.13)	53.31 (5.32)[a,b]	21.29	<0.001
Anxious/Depressed	56.44 (6.51)	60.98 (10.08)[a]	53.97 (5.70)[a,b]	23.02	<0.001
Somatic Complaints	55.95 (6.84)	59.02 (7.72)[a]	54.15 (5.69)[b]	13.85	<0.001
Withdrawn	71.77 (8.40)	59.07 (8.67)[a]	54.03 (5.89)[a,b]	151.18	<0.001
Sleep Problems	55.29 (6.86)	59.81 (11.64)[a]	54.15 (5.51)[b]	13.41	<0.001
Attention Problems	62 (8.15)	56.62 (7.11)[a]	52.72 (4.41)[a,b]	53.09	<0.001
Aggressive Behavior	55.49 (6.11)	58.94 (8.93)[a]	52.07 (4.07)[a,b]	29.18	<0.001
Affective Problems	58.85 (8.09)	60.55 (10.47)	54.03 (5.40)[a,b]	18.96	<0.001
Anxiety Problems	57.22 (7.54)	60.97 (9.72)[a]	53.53 (5.58)[a,b]	24.77	<0.001
PDP	71.59 (7.30)	60.82 (9.40)[a]	54.33 (6.26)[a,b]	138.77	<0.001
ADHD	58.50 (7.02)	57.71 (7.93)	52.97 (4.52)[a,b]	23.04	<0.001
Oppositional Defiant Problems	54.98 (5.63)	56.98 (7.56)[a]	51.69 (3.87)[a,b]	22.93	<0.001

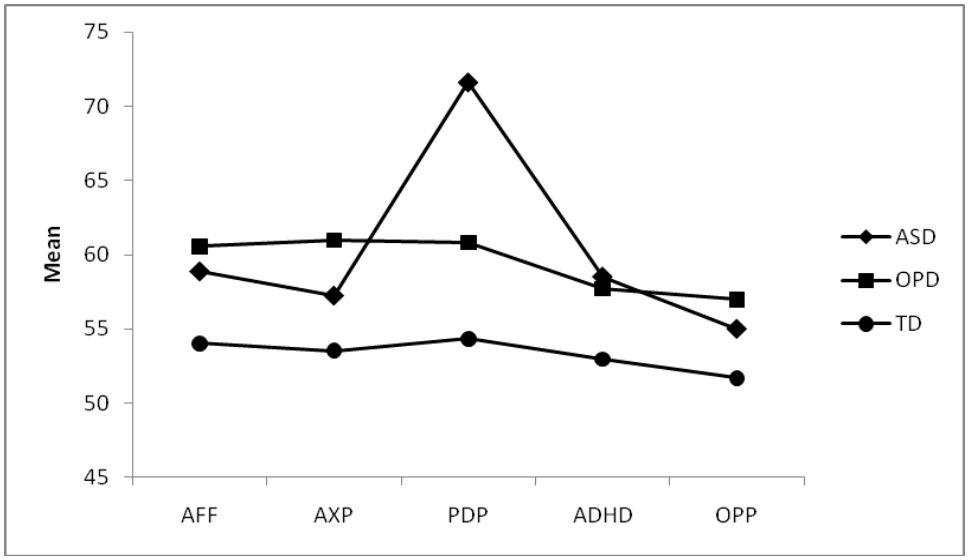
ASD Autism Spectrum Disorder, OPD Other Psychiatric Disorders, TD Typical Development. CBCL Child behavior checklist
a $p<0.05$ vs ASD, b $p<0.05$ vs OPD, according to S-N-K post-hoc tests

Figure 2.1a. Means of CBCL syndrome scales



ASD Autism Spectrum Disorder, OPD Other Psychiatric Disorders, TD Typical Development
EMR Emotionally Reactive, AXD Anxious/Depressed, SOM Somatic Complaints, WD Withdrawn, SLE Sleep Problems, ATT Attention Problems, AGG Aggressive Behavior.

Figure 2.1b



ASD Autism Spectrum Disorder, OPD Other Psychiatric Disorders, TD Typical Development
AFF Affective Problems, AXP Anxiety Problems, OPP Oppositional Defiant Problems.

Table 2.3. reports odds ratios for every CBCL scale in predicting ASD. Comparing ASD to TD or OPD, the CBCL scales predicting the presence of an ASD were the Internalizing scale, the Withdrawn scale, the Attention Problems scale and, among the DSM-oriented scales, the PDP scale. Moreover, the CBCL Total and Internalizing scores were predictors of the presence of an ASD, when comparing ASD with TD, while they did not distinguish between ASD and OPD.

Table 2.3. Odds Ratio and 95% Confidence Interval in ASD vs. OPD and ASD vs. TD

	ASD vs OPD			ASD vs TD		
CBCL Scales	p	OR	95%CI	p	OR	95%CI
Model 1						
Total Score	.447	1.01	0.98 to 1.04	.000	1.17	1.12 to 1.23
Model 2						
Internalizing	.014	1.05	1.01 to 1.10	.000	1.13	1.07 to 1.18
Externalizing	.116	0.96	0.92 to 1	.015	1.07	1.01 to 1.13
Model 3						
Emotionally Reactive	.063	1.10	0.99 to 1.22	.047	1.10	1 to 1.21
Anxious/Depressed	.001	0.85	0.78 to 0.94	.099	0.91	0.82 to 1.01
Somatic Complaints	.024	0.87	0.78 to 0.98	.132	0.92	0.83 to 1.02
Withdrawn	.000	1.32	1.2 to 1.45	.000	1.29	1.19 to 1.39
Sleep Problems	.723	0.98	0.90 to 1.06	.145	0.92	0.82 to 1.02
Attention Problems	.001	1.17	1.06 to 1.29	.000	1.18	1.07 to 1.30
Aggressive Behavior	.000	0.75	0.66 to 0.86	.303	0.92	0.70 to 1.07
Model 4						
Affective Problems	.216	0.96	0.90 to 1.02	.334	0.95	0.87 to 1.04
Anxiety Problems	.000	0.87	0.81 to 0.94	.035	0.91	0.83 to 0.99
PDP	.000	1.27	1.18 to 1.37	.000	1.34	1.23 to 1.45
ADHD	.970	1	0.93 to 1.07	.099	1.08	0.98 to 1.19
Oppositional Defiant Problems	.110	0.93	0.85 to 1.10	.885	0.99	0.86 to 1.13

ROC analyses

Because Withdrawn, Attention Problem and PDP scales have been identified as the best predictors of the probably presence of ASD in the logistic regression analysis, we have used ROC analyses to estimate the best cut-offs for these scales (Figure 2.2). In Table 2.4. sensitivity, specificity, negative and positive predictive values and area under the curve (at the optimal cut-offs for the three scales in discriminating ASD from TD and OPD) are reported.

ASD vs TD

ROC analysis indicated that in discriminating ASD from TD group the optimal compromise between sensitivity and specificity was achieved at a score of 65 both on PDP and Withdrawn scales (PDP scale: AUC=0.947; 95% CI 0.920–0.975; Withdrawn scale: AUC=0.945; 95% CI 0.914–0.977).

For the PDP scale the sensitivity was 0.85, indicating the proportion of actual ASD subjects who were correctly identified as such and the specificity was 0.90 indicating the proportion of actual TD subjects who were correctly identified. The score of 65 yielded a positive predictive value of 0.88 (i.e., the proportion of individuals with a score of 65 or more who were diagnosed in the ASD group) and a negative predictive value of 0.87 (i.e., the proportion of individuals with a score less than 65 who were diagnosed in the TD group).

For the Withdrawn scale the sensitivity was 0.85 and the specificity was 0.92 (PPN=0.90, PNV=0.90).

For the Attention Problems the best cut-off discriminating ASD from TD was 55 (AUC=0.850; 95% CI 0.799–0.902) with a positive predict value and a negative predict value near to 0.80.

ASD vs OPD

In order to discriminate ASD from OPD using the PDP scale the optimal cut-off was 65 (AUC=0.813; 95% CI 0.753-0.873), the proportion of subjects with ASD who were correctly diagnosed was 0.85 (sensitivity) and the proportion of cases with OPD who were correctly diagnosed was 0.60 (specificity) (PPV=0.69, PNV=0.79).

For Withdrawn scale the optimal compromise between sensitivity and specificity was achieved at a score of 62 and for the Attention Problems scale the optimal cut-off was 55.

Table 2.4 Sensitivity, specificity, PPV and PNV at the best cutoff points in the Withdrawn, Attention Problems and PDP scales of the CBCL, discriminating ASD from OPD and TD.

	ASD vs TD			ASD vs OPD		
	Withdrawn (cutoff=65)	Attention Problems (cutoff=55)	PDP (cutoff=65)	Withdrawn (cutoff=62)	Attention Problems (cutoff=55)	PDP (cutoff=65)
Sensitivity	89%	72%	85%	89%	72%	85%
Specificity	92%	80%	90%	65%	55%	60%
PPV	90%	76%	88%	72%	63%	69%
PNV	90%	77%	87%	87%	55%	79%
AUC	0.945	0.850	0.947	0.850	0.704	0.813
Sweet & Picket criteria for AUC interpretation (1982)	High	Moderate	High	Moderate	Moderate	Moderate

Figure 2.2 Receiver operating curve (ROC) for Withdrawn (red), Attention Problems (yellow) and PDP (blu) scales. Under the figures are reported Sensitivity, Specificity, PPV and PNV at the best cutoff points in the three CBCL scales discriminating ASD vs TD and ASD vs OPD.

2.5 Discussion

The lack of medical tests or biological markers for identifying ASD has led researchers to concentrate on behavioural anomalies in order to detect early signs of autism. As recommended by the Practice Parameters of the AACN (Filipek et al., 2000), an appropriate and timely ASD diagnosis requires two different level of investigation: 1) a routine developmental surveillance and 2) an exhaustive evaluation restricted to children identified at risk at level 1. The aim of the present study was to investigate the possible use of CBCL 1½-5 PDP scale as a level 1 tool to support non-specialized professionals (e.g. a paediatrician) in their ability to detect behaviours that are suggestive of an ASD. In fact, in primary care settings it is not possible to provide a thorough evaluation of emotional and behavioural disorders; nevertheless, in this context brief and validated tools must be used in order to redirect families for an in-depth examination by professionals experienced in developmental disabilities and in the administration of the ADOS which is widely accepted as a gold standard diagnostic instrument for autism.

The present study has examined CBCL 1½-5 as one of these level 1 instruments. Results from Odd ratios and ROC analysis to evaluate discriminative ability of CBCL scales suggest the following considerations.

First, we confirm the validity of the PDP scale in differentiating preschoolers with ASD from those with TD. Therefore, we confirm its utility as an effective level 1 tool in individuating children at risk for ASD in the general population. According to Sweet and Picket (1982) interpretation of the Area Under the Curve (AUC) values, the diagnostic accuracy of the PDP scale is high. When ASD is compared to TD, sensitivity, specificity, PPV and NPV of the PDP scale are all above 80% which is the recommended cutoff for first level instruments (Meisels, 1989). Sensitivity, that is the proportion of actual ASD which are correctly identified as such,

is the main value for a good first level tool; high sensitivity, indeed, corresponds to a low percentage of false negatives, so that the possibility of being affected by an ASD and not being properly diagnosed is reduced. Thus the low rates of false negatives indicates that the PDP scale is able to identify preschoolers at risk for ASD and that the majority of these young children can be referred to appropriate services with minimal delay. For this reason our results provide support for the CBCL ½-5 – PDP scale as a screening tool for ASD. Moreover, the PDP scale shows a very high specificity (90%) that means low rates of false positives. This is the second reason to support its use as a screening tool because it limits to families of healthy preschoolers an unnecessary, time-consuming and emotionally exhausting referral to specialty clinics.

As a complementary finding, the Withdrawn scale has shown a power of discrimination that is similar to the PDP scale. The presence of Withdrawn as a discriminative scale for ASD is consistent with Sikora findings (2008) and with the more recent paper on older children conducted in Singapore (Ooi et al., 2010). In both these papers elevation on this scale is reported as a specific behavioral pattern indicative of autism, and it may be pointed out that the Withdrawn cluster of items has to be considered in future research and practice not only as indicative of an affective or mood disorder, as usually it is intended, but also as the expression of social difficulties specific to ASD. Findings from the present research support that a very high value on the Withdrawn scale, associated with a similar high value on the PDP scale could be considered more indicative of autism than of an affective disorder. It should be noted that five out of the eight items in the Withdrawn scale are shared with the thirteen items in the PDP scale: thus, future research has to take in consideration this overlapping of items and to develop clusters of items better fitting in the depressive withdrawn or in a social withdrawn indicative of autism.

Third, the present study adds a contribution to the literature on differentiating ASD from other psychiatric disorders. At the cutoff of 65, the PDP scale obtained an high sensitivity but a too low specificity so that this scale (and, similarly, the Withdrawn scale at the cutoff of 62) has a moderate diagnostic accuracy according to the Sweet and Picket (1982) interpretation of the Area Under the Curve (AUC) values. The high rate of false positives, that is preschoolers having a psychiatric disorder and who are misdiagnosed as having ASD, is only partially unexpected. One interpretation could be linked to the prevalence in our OPD group of young children with internalizing disorders who usually have high scores in the Withdrawn scale. This view is supported by the fact that five out of eight item in the Withdrawn scale are also present in the 13-items PDP scale, so that their positivity on the PDP scale can be due essentially to the high rate in these items. A second explanation concerns the high co morbidity between autistic conditions and internalizing disorders; recent meta-analyses have reported that up to 84% of ASD experience anxiety (White et al, 2009) and up to 34% experience depression (Stewart et al, 2006). Thus we can hypothesize that parents of ASD children answer positively to some item considering the internalizing traits of their autistic child. For both these reasons, that are linked to the overlap of items between Withdrawn and PDP scales, the specificity of the PDP scale in differentiating ASD vs OPD decreases.

Fourth, the elevation on the Attention problem scale in ASD compared to TD or OPD confirms also in preschoolers the frequently observed coexistence of attention problems in older children with autism (Bolte et al., 1999; Sinzig et al., 2009). The overlap between ASD and ADHD is often reported in literature (de Bruin et al., 2007) and sometime it makes hard to distinguish between these two disorders. Our study confirms that attention deficit as an externalizing symptom on the clinical autism phenotype may be important not only as a coexisting symptom but also in identifying some preschoolers with ASD. Nevertheless, according to the AUC, diagnostic accuracy is only moderate when ASD are compared to both

TD and OPD. Thus, while we confirm the frequent association we do not confirm the utility of this scale in screening autism.

2.6 Conclusions

Finally, the high sensitivity and specificity of the CBCL 1½-5-PDP scale (and of the Withdrawn scale), indicate this instrument as a tool that can integrate the pediatric observation maximizing the role of the parents in the detection of the disorder.

Nevertheless, there are some limitations associated with the current study. First of all, CBCL 1½-5 PDP scale is able to differentiate already diagnosed patients with ASD from TD children, but it is to investigate if this high ASD detection is maintained in a broad population without subject selection (i.e. individuals that typically take part in a screening survey). Second, the two control groups (TD and OPD) are composed of subjects without mental retardation, while in the ASD sample an intellectual disability isn't contemplated among the exclusion criteria; as a result, differences in mental age could be a bias of the present findings that prevent us from claiming our data as specific of an ASD and not of a more general developmental delay (Eisenhower et al., 2005). Third, CBCL results are generated from parent surveys: strengths and limitations are associated with this type of informant: in fact, primary caregivers for the most part know very well their child, but their reliability could be invalidated by parental characteristics (Lecavalier, 2006). Individuals with anxiety or mood disorder could over-estimated maladaptive behaviours of their own kid, while others may be reluctant to acknowledge child's problems.

Despite these limitations, the present study opens new rooms for future research on early detection and screening of ASD. The distinctive PDP profile has shown excellent sensitivity and specificity that are better than in other well-know tools for screening autism as CHAT and M-CHAT; thus, the 13-item PDP scale could become a brief, rapid, easy, specific tool for screening ASD in primary settings. Some problems persist for its specificity particularly

towards internalizing disorders and there could be strong argument for some adjustment of the PDP items reducing the overlap of its items with those in the Withdrawn scale.

Chapter 3

Abnormal growth of head circumference in ASD is limited to the first six months of life.

3.1 Introduction

Since the first Kanner's description, macrocephaly is an intriguing finding in children with Autism Spectrum Disorders (ASD). Several retrospective, prospective and postmortem studies have reported increased incidences of macrocephaly (head size greater than 2 standard deviations above the norm) with percentages included between 14% (Lainhart et al., 1997), 18% (Davidovitch et al., 1996), 24% (Stevenson et al., 1997) and 37-42% (Bailey et al., 1993). On the basis of these studies, it has been suggested that macrocephaly may represent a clinical marker for grouping individuals with autism into homogeneous subgroups which can be useful for genetic analysis (Carmichael et al., 1995; Maes et al., 1997; Silventoinen et al., 2000; Losh et al., 2008).

More recently, different studies have signaled that macrocephaly, even if common in autism, is not usually present at birth. For example a recent retrospective fetal ultrasound study of brain size has suggested that head circumference was not abnormal during fetal development in children subsequently diagnosed with an ASD (Hobbs et al., 2007). Because macrocephaly seemed to develop after birth, regular observations of head circumference (HC) and of its growth rate between seriate measurements during early stages of life, has become the object of many studies (Fukumoto et al., 2008-2010; Elder et al., 2008; Gillberg et al., 2002; Hazlett et al., 2005; Mills et al., 2007; Webb et al., 2007).

Courchesne (2003) arrived first in providing retrospective information about the course of brain growth during the first year of life. This author has described the developmental course of HC during infancy of 15 preschoolers with ASD compared to 15 healthy infants and he

found out a smaller HC at birth, followed by an accelerated growth such that by 6-14 months 53% of the sample was revealing macrocephaly. The finding of the early overgrowth, with a subsequent decline in trajectory as from the second year of life, has now been replicated by several independent research groups (Fukumoto et al., 2010; Dementieva et al., 2005 Dissanayake et al., 2006; Dawson et al., 2007; Deutsch et al., 2002). Also recent reports on younger siblings of ASD patients, a population with an higher genetic risk of developing ASD than typical children, have found an association between enlarged HC growth rate and early emerging symptoms (Elder et al., 2008).

Nevertheless, some studies are in contrast with the report of an early increased HC growth in ASD. For example, van Daalen (2007) concludes that in ASD there is a dysregulation of growth in general, rather than a dysregulation limited to brain growth, while Rommelse (2011) describes an accelerated growth of height not only in ASD children, but also in other childhood psychiatric disorders. Other studies do not confirm either the finding of abnormally small head size at birth (Lainhart et al., 1997; Gillberg et al., 2002; Dementieva et al., 2005; Torrey et al., 2004; Mraz et al., 2007) or the positive association of HC overgrowth with measures of autism symptom severity (Dementieva et al., 2005; Torrey et al., 2004). Thus, research seems to indicate that head growth abnormalities are present in only a subpopulation of ASD, both in terms of a larger head circumference and an atypical acceleration of growth. It is possible that for this specific ASD group there is a peculiar growth pattern (Hultman et al., 2002): at birth, they appear to have a normal, or even decreased, head circumference and only after some months there is an increase in the rate of HC growth (Stevenson et al., 1997; Courchesne et al., 2003; Dementieva et al., 2005; Amaral et al., 2008). Nevertheless, cautions are warranted: first of all, we do not know yet how many children undergo this abnormal brain growth trajectory since studies have analyzed differences only in terms of groups; second there have been no published prospective data to assess its potential

predictive validity; third, not always correlation with body length and weight were considered. Finally, there is still no consensus about when HC enlargement is first displayed. Since during the first years of life HC correlates well with brain size, it has been suggested that this phenomenon could reflect an abnormal acceleration of postnatal brain growth processes. If this hypothesis will be confirmed by longitudinal MRI studies examining trajectories of brain development from birth, an accelerated head growth could become an early biological marker for ASD. Currently, the only longitudinal MRI data are related to toddlers from about two years of age (Schumann et al., 2010), but future prospective studies on younger siblings of ASD patients, followed from birth through multiple MRI scans, could shed light on the neuropathological alterations at an early stage. In fact, although some authors have argued that an excess of cortical neurons and/or glial cells (Courchesne et al., 2001) and alteration of cell microcolumns (Casanova et al., 2002) causes early brain overgrowth, the exact pathophysiology of this process remains to be established. Experimental studies have documented that possible consequences of an early overgrowth of the brain could be an alteration of connectivity (Ringo et al., 1991) resulting in an excess of short-distance cortical connectivity and a reduction of long-distance connectivity (Courchesne et al., 2007). This peculiar brain disconnection is hypothesized to be the neural substrate of ASD core symptoms, as confirmed by some diffusion tensor imaging (DTI) studies on altered structural connectivity in individuals with ASD (e.g. Barnea-Goraly et al., 2004; Lee et al., 2007; Sundaram et al., 2008).

The present study aims to describe HC developmental course in the first 14 months of life in a relatively large group of Italian children with ASD and to explore associations of this developmental process with later symptom severity and cognitive impairment of children (Lainhart et al., 1997; Deutsch et al., 2003; Lainhart et al., 2006).

3.2 Methods and Materials

Participants

Fifty preschoolers (mean age: 52 months, SD = 2.1 months) with idiopathic and non-syndromic ASD (40 males and 10 females) were recruited consecutively between November 2007 and November 2009 among patients referred to the second level Centre for Autism at the Stella Maris Scientific Institute in Pisa. Inclusion criteria were 1) normal term birth (gestational age between 37-42 weeks) and 2) pediatric data records reporting head circumference, height (H) and weight (W) measurements at four age periods: birth (T0); 1 to 2 months (T1); 3 to 5 months (T2) and 6 to 14 months (T3). All children met criteria for Autistic Disorder (n=20) or Pervasive Developmental Disorder Not Otherwise Specified (n=30) according to DSM-IV-TR (APA; 2000) and confirmed by the Autism Diagnostic Observation Schedule (ADOS; Lord et al., 2002).

HC, H and W of ASD patients were compared to the same measurements of 100 healthy children (mean age: 52 months, SD = 11.7 months). This sample included 80 males and 20 females and was a subset of an healthy pediatric population living in the metropolitan area of Pisa (Tuscany). A non clinical Child Behaviour Checklist 1½-5 (CBCL; Achenbach et al., 2000) Total Score < 50 was assumed as an index of typical behavioral development for this healthy group. A detailed description of the two samples is presented in Table 3.1.

Table 3.1. Sample description

Total sample	ASD (N=50)	TD (N=100)
Gender (Male/Female)	40/10 (Ratio 4:1)	80/20 (Ratio 4:1)
Age (Months)	Mean: 52 (DS: 2.1)	Mean: 52 (DS: 1.7)
Diagnosis	PDDNOS: n=30 (60%) Autistic Disorder (AD): n=20 (40%)	
Cognitive skills	IQ≥70: n=29 (58%) IQ<70: n=21 (42%)	
ASD way of onset	Regressive onset: n=11 (22%) Early onset: n=39 (78%)	

ASD: Autism Spectrum Disorder; TD: Typical Development

Instruments

Cognitive development was assessed by the Leiter International Performance Scale – Revised (Leiter-R; Roid & Miller, 1997) and/or Wechsler Scales (WWPSI, 1973; WISC-R,1986) according to the patient's age and linguistic level. On the basis of IQ subjects were divided into two groups: IQ <70 and IQ $\geq$70.

Regressive onset of autistic symptoms was evaluated through the Italian version of the Early Development Questionnaire (EDQ; Ozonoff et al., 2005).

This study was approved by the Institutional Review Board of Stella Maris Scientific Institute.

Statistical Analyses

Statistical analyses were performed using SPSS statistical software version 15 (SPSS Inc, Chicago, III). HC, H and W measures were normalized across sex and age by converting to z scores based on the CDC growth charts (National Center for Health Statistics; 2000). ASD group was compared with TD group on HC, H and W at each considered period, using t-test. Repeated-measures analyses of variance were carried out to analyze rate growth in HC, H and W among the two groups. Bonferroni correction was used to control for multiple testing.

In order to analyze the association between HC and autistic symptomatology (on the basis of ADOS-G values) we carried out a linear regression analysis.

In ASD group, t-test was used to compare HC between males and females, Autistic Disorder (AD) and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) diagnosis, IQ level, and type of onset (early *vs* regressive).

3.3 Results

Table 2 shows differences between ASD and TD at the different time points on HC, H and W.

Head Circumference

At birth (T0) there was no significant difference on HC size between ASD and TD groups. At birth the ASD group is composed of 5/50 children (10%) with microcephaly (HC at 5th

percentile); 15/50 (30%) with HC between 5th and 25th percentile; 30/50 (60%) with HC greater than 25th percentile. TD group is composed of 8/100 children (8%) with microcephaly; 27/100 (27%) with HC between 5th and 25th percentile; 65/100 (65%) with HC greater than 25th percentile.

At 1-2 months (T1) no significant difference in HC size was still present between ASD and TD groups. At 3-5 months (T2) HC was significantly greater in ASD compared to TD (mean value was located at 55th percentile in ASD group, and at 43th percentile in TD group).

At 6-14 months (T3) HC was still significantly greater in ASD group compared to healthy infants (mean value was located at 75.8th percentile in ASD group, and at 65.5th percentile in TD group). At T3, macrocephaly (HC>97th percentile) was present in 9/50 (18%) infants with ASD and in 9/100 (9%) infants with TD. This difference doesn't reaches significance (Chi ^{2} = 2.56 p = 0.110).

In ASD group, 10 infants (20%) had a not increasing developmental course of HC: 7 showed a course similar to TD group, and 3 showed a decreasing course with HC values at T3 lower than at T0.

ASD and TD groups did not show significant differences on HC growth when males and females subgroups were compared (F=1.00, p=0.323).

Weight and Height

The height was not significantly different between ASD and TD in all four considered periods, and no significant difference were found between the two groups (F=0.47, p=0.491) for the rate of height growth.

While the weight was similar at T0, it was significantly smaller in ASD group compared to TD at T1 (36.5th percentile *vs* 58th), at T2 (52.5 th percentile *vs* 65 th) and at T3 (51th percentile *vs* 63th) (see Table 3.2). However, no difference was found in the rate of weight growth (F=0.20, p=0.655) between the two groups.

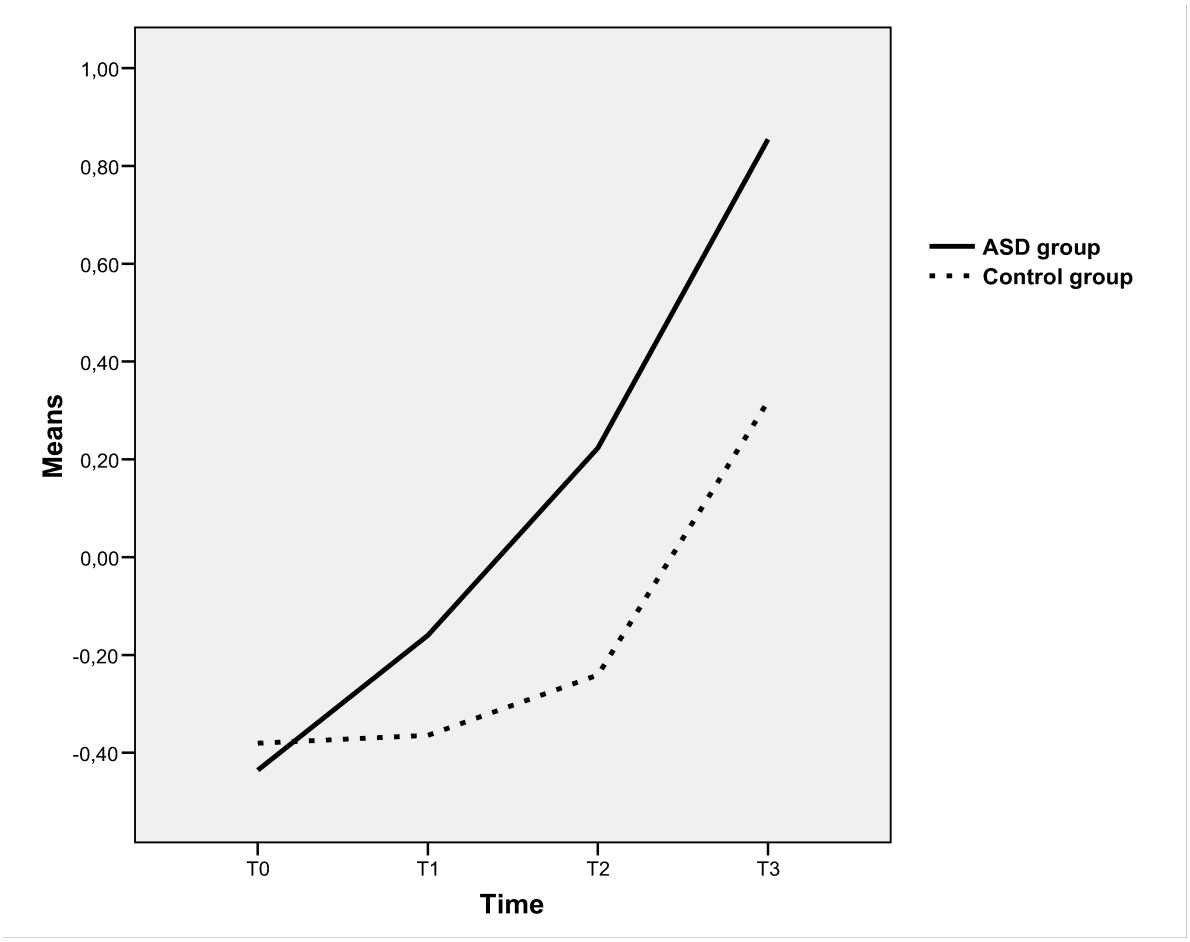
HC growth

Analysis of variance for repeated measures, controlling for weight and height, shows that, over time, the rate of HC increases in both groups ($F=127.42$, $p<0.001$), but the growth was significantly greater in ASD compared to TD group ($F=11.60$, $p=0.001$) (Figure 3.1).

Table 3.2. Differences between ASD and TD at each period on HC, weight and height (values are converted to z scores)

	ASD (n=50)		TD (n=100)		t-Test	p
	Mean	SD	Mean	SD		
HC						
T0	-0.49	0.55	-0.35	0.59	-1.41	0.160
T1	-0.31	0.53	-0.28	0.79	-0.16	0.867
T2	0.12	0.69	-0.19	0.99	2.04	**0.042**
T3	0.74	0.83	0.37	1.06	2.14	**0.033**
Weight						
T0	-0.48	0.90	-0.34	1.04	-0.82	0.409
T1	-0.33	0.86	0.21	0.87	-3.62	**<0.001**
T2	0.06	0.68	0.39	0.88	-2.35	**0.020**
T3	0.02	0.76	0.34	1.00	-1.97	0.050
Height						
T0	0.18	0.82	0.29	0.72	-0.83	0.407
T1	0.06	0.75	0.05	1.04	0.03	0.970
T2	0.52	0.84	0.22	1.05	1.77	0.078
T3	0.58	0.87	0.62	1.36	-0.19	0.843

Figure 3.1. Changes of the z score for HC during the first 14 months of life (T0=birth) in ASD and TD groups, after controlling for weight and height.



HC, autistic symptoms and cognitive level in ASD group

HC was not different between AD and PDD-NOS diagnosis, at all time points. Moreover, no differences were found on HC growth, on the basis of regressive onset and on IQ level (< or ≥ 70) (see Table 3.3).

Table 3.3. ASD group: differences for gender, diagnosis, IQ level and type of onset, on HC at the four considered periods

	Subjects (50)	**T0 (birth)**	**T1 (1/2months)**	**T2 (3/5 months)**	**T3 (6/14 months)**	
Gender						p
Males	40	-0.53±0.73	-0.22±0.90	0.20±1.09	0.78±1.23	0.323
Females	10	-0.19±0.35	-0.005±0.85	0.15±1.01	0.79±0.81	
p		0.045	0.491	0.898	0.979	
Diagnosis						
AD	20	-0.56±0.68	-0.17±0.86	0.13±1.0	0.50±0.96	0.223
PDD-NOS	30	-0.40±0.70	-0.18±0.92	0.24±1.12	0.98±1.25	
p		0.435	0.967	0.725	0.156	
IQ Level						
IQ≥70	29	-0.42±0.64	0.02±0.66	0.18±0.86	0.86±0.96	0.745
IQ<70	21	-0.53±0.76	-0.46±1.08	0.21±1.32	0.68±1.40	
p		0.585	0.073	0.930	0.609	
Onset						
Early	39	-0.40±0.67	0.09±0.91	0.28±1.09	0.95±1.13	0.246
Regressive	11	-0.67±0.73	0.49±0.78	-0.12±0.97	0.21±1.08	
p		0.267	0.191	0.268	0.062	

3.4 Discussion

The purpose of the present study was to supervise the timing of HC development in the first year of life in Italian children with ASD. Our study confirms that the majority of children with autism display an increased acceleration of HC growth during the first year of life. Because some studies have suggested that an increase in the rate of head growth could be the result of an overall increase in general body growth of autistic children (Fukumoto et al., 2008; Dawson et al., 2007 van Daalen et al., 2007; Torrey et al., 2004), we have controlled HC for weight and height. After this correction, HC begins to growth overmuch and quickly from 1-2 months to 3-5 months reaching at 6-14 months the 75,8th percentile. This result is slightly

different compared to first Courchesne study (2003) where the abnormal growth reached the 84th percentile, HC at birth was significantly smaller in ASD group compared to subjects with typical development, and the acceleration last for all the first year of life. In contrast to Courchesne study (2003), but in agreement with several other reports (Lainhart et al., 1997; Dementieva et al., 2005; Torrey et al., 2004; Hultman et al., 2002), our ASD newborns show a HC measurement similar to typical control. Second, our findings indicate that 0-6 months represents the period at which the abnormal brain overgrowth (assuming that brain size is correlated to HC) (Bartholomeusz et al., 2002) has his peak; during the second semester of life the autistic brain continues to be significantly larger but without any other gain compared to TD. It seems that something causing an abnormal growth occurs during the first semester of life and not during the latter part of the first year as signalled by Elder (2008). Then, our research proposes that the 0-6 months period should be considered a specific sensible period for the starting of the disorder: not before when HC is not larger than in TD and not in the slope from 6 to 12 months when the rate of growth is similar to TD. Evidences for the first six months of life as a critical period for autistic onset come also from behavioural findings: retrospective home videotape analysis (Maestro et al., 2001) of infants later diagnosed as having autism reveals an incipient phase of developmental alteration which displays itself in early differences in social attention. Literature indicates that an early abnormal brain growth process precedes the full expression of the disorder and coincides with the first appearance of subtle behavioral abnormalities (Courchesne; 2004). In fact, prospective studies on children subsequently diagnosed as ASD agree that a clear expression of an altered social behavior is not likely to be found before 12 months of age (Zwaigenbaum et al., 2005; Bryson et al., 2007; Landa & Garrett-Mayer 2006).

We could suggest that combining measures of head circumference with behavioural (and/or instrumental) tests for early social and non social attention might improve our capacity of

screening autism at an earlier age. In our study, the HC overgrowth was present in the whole sample except 10 (20%) patients who did not present the growth acceleration regardless the value of HC at birth and regardless the presence of regression. Then, even if HC has the potential to be included in a check list for autism in infancy, we have to take in consideration that it is not able to recognise all subjects at risk for autism (Lainhart; 2006). For the subgroup of ASD without an early HC acceleration, we should imagine a different pathophysiologic pathway that remains to be elucidated in future studies. According to percentages reported in the recent literature (included between 14% and 34% of cases), we have found macrocephaly (HC > 97th percentile) in 18% of the ASD sample at 6-14 months. This condition is clinically, although no significantly, more present in ASD then in our control sample composed of children with TD. We could suggest that this is another special group of children with autism characterised by an accelerated growth of HC without reaching macrocephaly. Thus, we can outline the presence in the autism spectrum of three groups of children that differ as far as early abnormal HC growth and final macrocephaly are regarded: 1) abnormal early HC growth toward macrocephaly (18% of our cases); 2) abnormal HC growth without final macrocephaly (68%); 3) without abnormal HC growth; this latter group of children represents in our casistic only the 14% of children with autism. Further research is needed to establish whether these different groups could delineate subtypes of ASD useful for genetic and neurobiological studies.

We have also examined whether atypical great expansion in head size is associated with severity of autistic symptoms. Previous reports indicate contrasting results: Dementieva (2005) found a correlation between an increased rate of head growth and higher levels of adaptive functioning, while in the Courchesne's study (2003) HC at 6-14 months was significantly greater in Autistic Disorder than in PDD-NOS. In the current study, we have not found any significant difference between these two groups as far as HC at different point is

regarded. Furthermore, mean head circumference z scores were not significantly associated with IQ or regressive onset.

Because this is the first paper on Italian children, we can hypothesize that some of the differences between our and previous studies on HC could be related to this specific population. First, the weight was significantly smaller in ASD; this finding is opposed to other studies (Davidovitch et al., 1996; Fukumoto et al., 2008-2010; Mraz et al., 2007) reporting that body weight, as well as HC, was significantly bigger in ASD. Second, our study has considered males and females as a whole group because no difference was found between boys and girls in HC; differently, Fukumoto (2008) pointed out that body weight was significantly increased in boys with autism; but in his study Fukumoto (2008) considered boys and girls as two different groups. Third, unlike Dissanayake (2006) and Torrey (2004) who reported a general abnormal growth of the body sizes including the growth in stature, in our study mean length z scores did not differ significantly from controls at any age interval.

For these different reasons we propose that in future studies it will be considered appropriately HC together with body measures using similar ethnic group as we did.

In short, this study, while confirming the existence of an abnormal HC growth rate in the first year of life in children with ASD, points out the sudden and excessive increase in head size during the first six months of life. Second, it confirmed the association between ASD and macrocephaly only in a limited number of ASD children. Finally, it corroborates the importance to measure the HC in the first months of life of children because its abnormal rate of growth, in addition to other behavioral signs, could contribute to the process of early ASD identification.

Chapter 4

Structural magnetic resonance techniques

4.1 Voxel-based morphometry

The voxel-based morphometry (VBM) technique consists in a voxel-wise comparison of the local volume or concentration of grey/white matter between two groups of subjects (Ashburner & Friston, 2000). The procedure involves spatially normalizing high-resolution images from all subjects in the study into the same stereotactic space. This is followed by the segmentation of the grey/white matter from the spatially normalized images, and the smoothing of the grey/white-matter segments. Voxel-wise parametric statistical tests, which compare the smoothed grey/white-matter images from the two groups, are performed. Corrections for multiple comparisons are made using the theory of Gaussian random fields.

VBM is crucially dependent on registration performance. The recently introduced Diffeomorphic Anatomical Registration using Exponentiated Lie algebra (DARTEL) algorithm implements several methodological advances to address this limitation (Ashburner, 2007). A diffeomorphic warping is implemented to achieve an accurate inter-subject registration with an improved realignment of small inner structures. Several ASD structural imaging studies to date have used region-of-interest manual tracing methods that have the limitation of being operator dependent and thus invalidated by a low inter-laboratory reliability. On the other hand, automated VBM is more sensitive to subtle differences and can be standardized across laboratories. For example, in a recent study (McAlonan, 2002) MRI data of the same Asperger subjects were analyzed using both manual tracing and voxel-based analysis, revealing no differences in regional brain volumes when is applied the first method and significant alterations between groups in white matter as well as in grey matter when patients and controls were compared through VBM.

4.2 Diffusion tensor imaging

Diffusion tensor imaging (DTI) is a magnetic resonance imaging (MRI) technique that allows for the indirect assessment of the integrity of white matter tracts (Le Bihan, 2001) by means of measurement of the extent and direction of water diffusion within the brain. When unconstrained, water molecules diffuse randomly in all directions and thus exhibit isotropy. Within white matter tracts, the movement of water molecules is physically constrained along the direction of tracts by sheaths of myelin, a phenomenon referred to as anisotropy, which is represented as an ellipsoid in tensor form (Basser, 1994-1996).

A basic understanding of the influence of various structural components on anisotropic water diffusion is a prerequisite for interpreting alterations in diffusion and anisotropy as a result of various disease processes or abnormal development. DTI provides three valuable parameters: (1) the average extent of water diffusion (apparent diffusion constant—ADC) which provides information on restriction and boundaries (high packing density of cells); (2) the fractional anisotropy (FA) that is higher in dense and ordered structure; and (3) the orientation of the ordered structure (color coded DTI).

FA is a scalar value that ranges between 0 and 1. Increasing FA values indicate a higher tensor ellipsoid anisotropy. FA, with no other information, is a highly sensitive but fairly not specific biomarker of neuropathology and microstructural architecture. This combination produces challenges to the interpretation of DTI measurements for both diagnostic and therapeutic applications. However, most agree that FA is a marker of white matter integrity. In fact, reduced fractional anisotropy (FA), indicating more isotropic diffusion, is characteristic of damaged and/or disorganized white matter tracts (Beaulieu, 2002).

Another simple and clinically useful scalar invariant is the the average of the eigenvalues. This average is referred to as the mean diffusivity, or MD or Apparent Diffusion Coeffcient (ADC)

and it relates to the total amount of diffusion in a voxel, which is related to the amount of water in the extracellular space.

DTI may be visualized in a slice plane (a section through the data) or in three dimensions, depending on the subset of the data that is presented. Planar visualization methods are voxel-based, meaning an image is generated to display information from the tensor that is in each voxel in one slice plane. For example, images may be displayed of any anisotropy measure, or of the trace. Another type of image can represent the major eigenvector field using a mapping to colors. The color scheme commonly used to represent the orientation of the major eigenvector works as follows: blue is superior-inferior, red is left-right, and green is anterior-posterior (Figure 4.1).

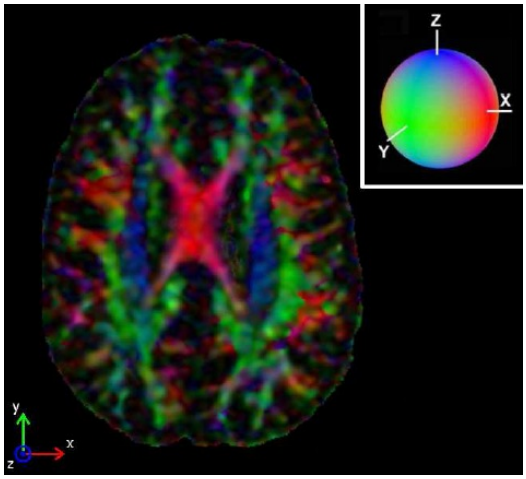


Figure 4.1. Colormap showing major eigenvector direction indicated by color (red: right-left; green: anterior-posterior; blue: superior-inferior).

The dominant method for three-dimensional visualization of DTI is tractography, a very commonly employed method which estimates the trajectories of major fiber tracts in the white matter (Figure 4.2). The central theme of tractography is tracing paths by following probable tract orientations, in order to reconstruct an estimate of the underlying white matter fiber structure. Many methods have been proposed in the literature for addressing this

problem, and most of them produce output, which corresponds well to known anatomy in regions where the data is not made ambiguous by crossing fibers.

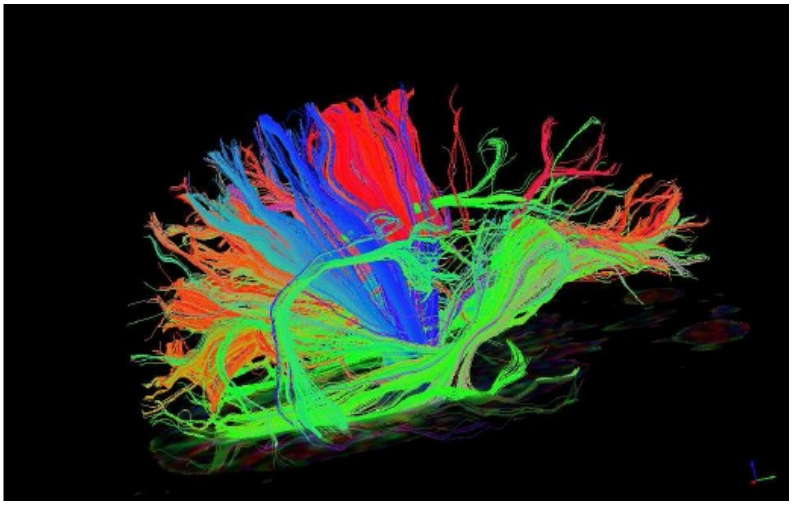


Figure 4.2. Three-dimensional DTI visualization in TrackVis.

Chapter 5

A diffusion tensor imaging study in Autism Spectrum Disorder

5.1 Introduction

There are some studies in which diffusion tensor imaging indices have been analyzed in Autism Spectrum Disorders (ASDs) subjects. Findings are conflicting: the age of subjects analyzed is a prominent variable that influences results. In fact, studies on toddlers and young children (Ben Bashat et al., 2007; Cheung et al., 2009; Kumar et al., 2009; Sundaram et al., 2008) found increased FA, while literature concerning children, adolescents and adults with ASD consistently reported reduced FA (Alexander et al., 2007; Barnea-Goraly et al., 2004; Brito et al., 2009; Catani et al., 2008; Ke et al., 2009; Keller et al., 2007; Lee et al., 2007; Pardini et al., 2009; Shukla et al., 2010; Thakkar et al., 2008). In particular, these studies have found lower FA in individuals with autism, compared with controls, in the corpus callosum (Alexander et al., 2007; Barnea-Goraly et al., 2004; Keller et al., 2007), the anterior cingulate, ventromedial and subgenual prefrontal areas, temporoparietal junction, and in the superior temporal gyrus (STG) white matter and temporal stem (Barnea-Goraly et al., 2004; Lee et al., 2007). Recent studies that combined DTI with VBM on ASD children reporting a mixture of increased and decreased white matter densities in different parts of the brain (Conturo et al., 2008; Ke et al., 2009; Mengotti et al., 2010).

Studies that have employed Diffusion Tensor Tractography (DTT) to examine the integrity of specific WM tracts in individuals with autism relative to typically developing individuals have focused primarily on intra-hemispheric tracts. For example, one study reported alterations in the structural integrity of long-range fibers in the frontal cortex in children within the autism spectrum (Sundaram et al., 2008), while another reported significant reductions in the micro-structural integrity of the right superior cerebellar peduncle and short intra-cerebellar fibers in adults with Asperger syndrome (Catani et al., 2008).

A more recent study revealed a significant increase in the number of streamlines (i.e. the lines that depict the fibers in a tract) in bilateral inferior longitudinal fasciculus (ILF) and the cingulum bundle, as well as a reduction in streamlines in the right uncinate fasciculus (UF) (Pugliese et al., 2009). Importantly, these tracts are associated with behavioral functions that are known to be impaired in autism. For example, the ILF and the inferior fronto-occipito fasciculus (IFOF) are critical for higher-level visual and emotion processing (Rudrauf et al., 2008; Thomas et al., 2010), domains atypical in individuals with autism (Behrmann et al., 2006; Bertone et al., 2005; Humphreys et al., 2008; Lee et al., 2007). In summary, these tractography studies reveal perturbations in intra-hemispheric WM tracts in individuals on the autism spectrum, which may account for some of their difficulties in information processing.

All these results are have been realized on late childhood subjects or on adults. More recent studies investigated children under 3 years old. In 2007 Ben Bashat and colleagues (2007) realized a DTI study on young children and found an FA increase in a lot of brain area (in particular in left hemisphere and in frontal lobe). These results are in agreement with the finding of abnormal brain and connections growth in the first years of life, but remains to clarify if FA reduction is due to increase in number, size of axons or myelination processes, or whether it is the result of reduced synaptic pruning early in development.

5.2 Materials and methods

Participants

Twenty-two children (age range 2–11) with autism spectrum disorder were recruited from our clinical autism research program at the IRCSS Stella Maris Institute and ten children (age range 2–11) without developmental delay (noDD) (Table 5.1). NoDD subjects underwent an MRI examination because of various reasons (including headache, head trauma, cataract, single -i.e. not recurrent in the next two years- unprovoked idiopathic seizure). Inclusion criteria were: 1) a standardized evaluation of cognitive abilities 2) clinical data records

providing sufficient information to ensure the lack of neurological, behavioural or developmental disorders. Some ASD patients and noDD participants did not have clear hand dominance; those who did were right-handed.

There were no significant between-group differences in age (control group mean age 5.25±2.46 years; ASD group mean age 5.54±2.03; p=0.73) while the non verbal IQ was found significantly different between the two groups (control group mean IQ 5.8±0.42; ASD group mean IQ 4.09±1.38; p=0.0006).

Table 5.1: Participants characteristics

	Control group (n=10)	**Autism Spectrum Disorder (n=22)**
Age, months: mean (s.d) range	5.25 (2.46) 2-11.22	5.54 (2.03) 2.88-11.33
IQ (non verbal): mean (s.d) range	5.8 (0.42) 5-6	4.09 (1.38) 2-6

Cognitive evaluation

Because different instruments were used (Leiter International Performance Scale, WISC-IV, Griffiths Mental Development Scales, Bayley Infant Scales of Developments) and in order to increase reliability of analyses, NVIQ (nonverbal intelligence quotient) scores were converted to the following categories: 1=below 26, 2=26-40, 3=41-55, 4=56-70, 5=71-90, 6=91-110, 7=above 110.

Image acquisition

Structural and diffusion tensor MRI of the brain were performed on a 1.5 T MR system (Signa Horizon LX, GE Medical System). A sagittal three-dimensional fast spoiled gradient (SPGR) dataset covering the whole head was acquired. The parameters were: TR=12.3 ms, TE=2.4 ms, voxel resolution 256 x 256, field of view 280 mm, 124 slices, 1.1 mm slice thickness. For the DTI analysis, a multislice echo-planar imaging (EPI) acquisition sequence, using 25 directions of diffusion gradients, was used. After an interpolation automatically applied by the MR

system the resolution is 0.7422mm x 0.7422mm x 3mm with a field of view 190mm x 190mm and coverage of the whole brain (TE= 107 ms, TR=11000 ms, b-value 1000 s/mm).

DTI processing

Maps reconstruction

Images were processed using the FSL (FMRIB Software Library, FMRIB, Oxford, UK) (Smith et al., 2004) software package. For each subject, all images including diffusion weighted and b0 images, were corrected for eddy current induced distortion and subject motion effect using FDT (FMRIBs Diffusion Toolbox) (Behrens et al., 2003). Brain mask was created from the first b0 image using BET (Brain extraction Tool) (Smith, 2002) and FDT was used to fit the tensor model and to compute the FA, MD, axial diffusivity and radial diffusivity maps.

TBSS analysis

Voxelwise analysis was performed using TBSS (Smith et al., 2006). First the most representative FA image was identified and all subjects' FA data were aligned to this target image using the nonlinear registration tool FNIRT (Andersson 2007a, 2007b), which uses a b-spline representation of the registration warp field (Rueckert 1999). Next, the mean FA image was created and thinned to create a mean FA skeleton, which represents the centers of all tracts common to the group. A threshold of FA > 0.25 was applied to the skeleton to include only major fiber bundles. Each subject's aligned FA data was then projected onto this skeleton and the resulting data fed into voxelwise cross-subject statistics.

Statistical analysis

Statistical analysis was performed voxel by voxel to detect regions of significant differences of FA among the two groups of subject. The correlation of FA with age and with IQ was also investigated introducing these parameters as covariate in the contrast matrix. Individual FA maps were included in a non-parametric permutation-based group model using “randomize” in FSL (Nichols and Holmes, 2002). The TFCE (Threshold-Free Cluster Enhancement) option

in randomize was used in order to avoid the need for the arbitrary initial cluster-forming threshold. Both contrasts were computed using 5000 permutations. Results are reported at corrected threshold $p < 0.05$.

Tractography

Tract reconstruction

Diffusion tensor vectors were computed using ExploreDTI software (http://www.exploredti.com/). After the application of motion and distortion correction, a deterministic tracking algorithm was applied. Tract data were then transformed in NifTi format using an homemade MATLAB program including also information on length, FA and MD of each tract. NifTi files were finally imported in TrackVis software (http://www.trackvis.org/) for the reconstruction of the tracks of interest. White matter areas that TBSS analysis showed to be significantly different in the groups were selected for the analysis: the cingulum and the arcuate fasciculus (Catani & Thiebaut de Schotten, 2008).

The cingulum is a medial associative bundle that runs within the cingulate gyrus all around the corpus callosum. It contains fibers of different length, the longest of which run from the anterior temporal gyrus to the orbitofrontal cortex. The short U-shaped fibers connect the medial frontal, parietal, occipital, and temporal lobes and different portions of the cingulate cortex. The cingulum was dissected using a one-ROI approach. A single region was defined on the top three slices. When the cingulum separated into two branches an anterior and posterior region were defined on each slice. Artifactual (callosal) fibers were removed using an exclusion ROI defined around the corpus callosum.

The arcuate fasciculus is a lateral associative bundle composed of long and short fibers connecting the perisylvian cortex of the frontal, parietal, and temporal lobes. A three ROIs approach was used to reconstruct the three segments of the arcuate fasciculus. The first ROI was defined on the Broca's area, in the frontal lobe selecting three coronal slices of brain. The

second ROI was identified on three axial slices catching the Wernicke's territory in the temporal lobe. The last ROIs was identified in the Geschwind area of the parietal lob selecting three appropriate slices in the sagittal view of the brain. Following this approach the three segments of the arcuate fasciculus were reconstructed: the long direct segment connecting Wernicke's area with Broca's area, the anterior indirect segment linking Broca's territory with the inferior parietal lobule and the posterior indirect segment linking the inferior parietal lobule with Wernicke's territory.

The reconstruction of the tracts was performed with an FA threshold of 0.2 to avoid false positive due to artifacts.

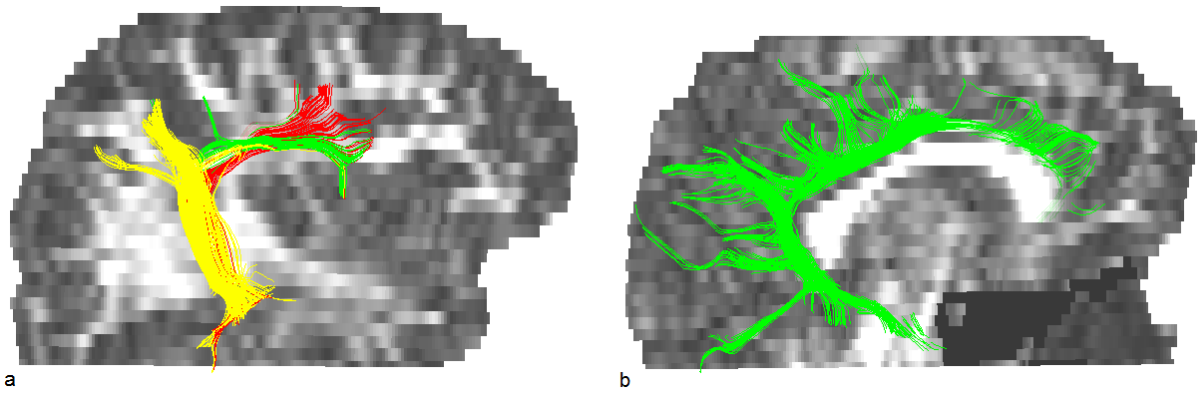


Figure 5.1. Selected tracts reconstruction: a) arcuate fasciculus and b) cingulum.

Tractography outcome measures

For each tract selected for the analysis we extract the following measures: number of streamlines, mean length of streamlines, volume of the tract, fractional anisotropy (FA), mean diffusivity (MD), parallel diffusivity and perpendicular diffusivity.

Statistical analysis

Statistical comparisons of the tractography outcome measures were performed using SPSS software (SPSS Inc, Chicago, Ill). General linear model (GLM) analysis for repeated measures was used with side (left and right hemisphere) and tracts (cingulum and the three segments of the arcuate fasciculus) as the within-subject factors and group as between-subjects factor.

Then, univariate ANOVA was performed on all the tractography measures. Where significant differences were detected, post hoc analysis was performed using independent student's t-test. The same analyses were repeated after co-varying for age.

5.3 Results

Group characteristics

There were no significant between-group differences in age (control group mean age 5.25±2.46 years; ASD group mean age 5.54±2.03; p=.73) while the non verbal IQ was found significantly different between the two groups (control group mean IQ 5.8±0.42; ASD group mean IQ 4.09±1.38; p=.0006).

FA differences between groups

Young children with autism spectrum disorder had a significant increase of FA in a lot of white matter areas. In particular, increase in corpus callosum, cingulum, external and internal capsula, arcuate fasciculus was found (p=.05) (Figure 5.2).

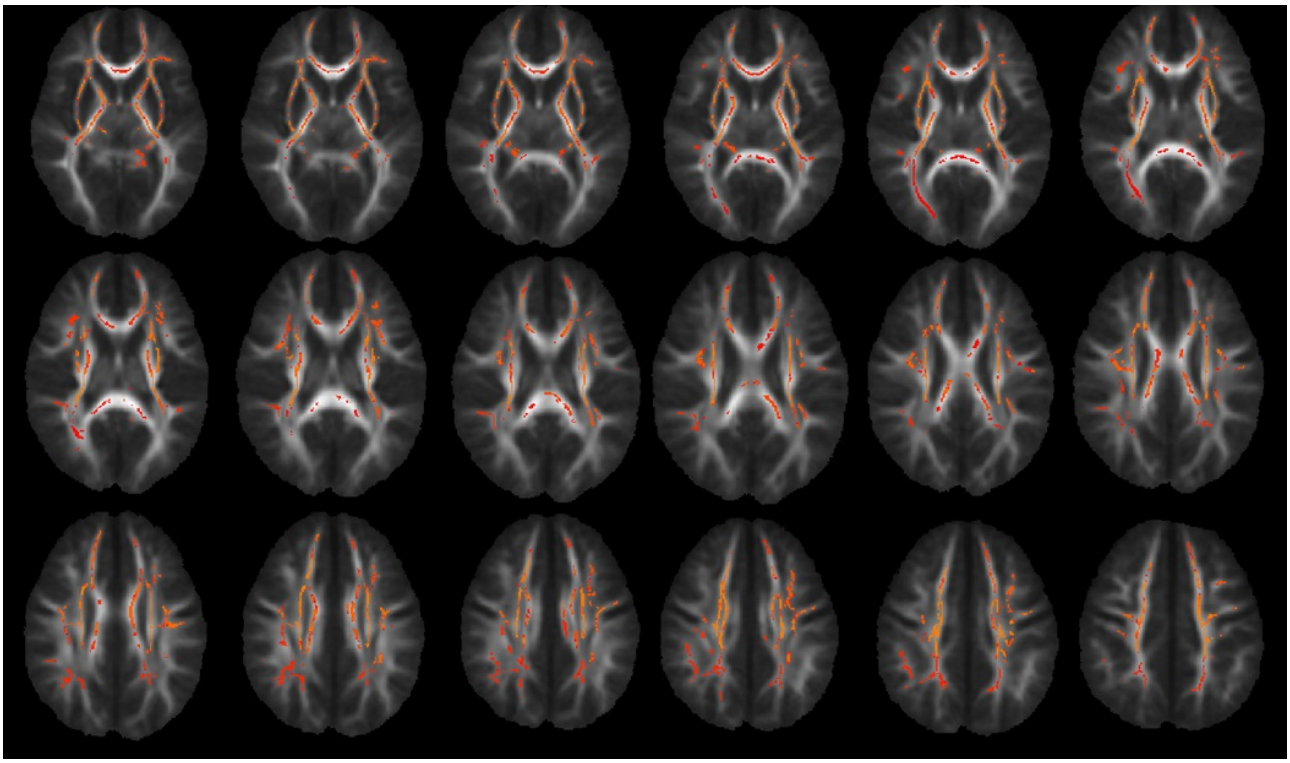


Figure 5.2. Regions of signi_cantly increased FA in ASD than in controls (in red), superimposed on the mean FA image (p<0.05, non parametric permutation test, corrected for

multiple comparisons). All images are in radiological convention, i.e. the right side of the subjects is on the left side of the images.

FA correlation with age

Positive correlation of FA with age was found for the control group and for the ASD groups (p=.05).

Tract-specific measurements

Number, length and volume of streamlines The GLM showed a significant group-by-side-by-tract interaction (p=.01) and tract-by-side-by-age (p=.03) interactions in the arcuate fasciculus. Comparison of the individual tracts revealed a significant increase in the length of streamlines bilaterally within the cingulum in ASD group (left cingulum: mean=88.7$\pm$14.2, right cingulum: mean=77.7$\pm$10.4) than in controls (left cingulum: mean =69.2$\pm$17.6; p=.002, right cingulum: mean=58.2$\pm$11.8; p=.0001). There was also a significant reduction in the number of streamlines within the posterior indirect segment of the left arcuate fasciculus in ASD group (mean=129.5$\pm$77.3) than in controls (mean=190.8$\pm$98.5; p=.06).

No significant difference in the volume of the analyzed tracts was found.

FA, MD, parallel and perpendicular diffusivity

FA was in ASD group was significantly increased bilaterally in the cingulum (left cingulum: p=.002, right cingulum: p=.003) and in the fornix (p=.004). There was also an increase of MD in ASD group in comparison to controls in the following areas: within the left cingulum (p=.05), within the right indirect posterior (p=.02) and the right indirect anterior (p=.04) segments of the fasciculus arcuate. Finally a significant increase in parallel diffusivity in the ASD group was found in the right cingulum (p=.02).

5.4 Discussion

This study was one of the first study on young ASD children. The TBSS analysis showed a significant increase of FA in ASDs with respects to control in corpus callosum, cingulum,

external and internal capsula, arcuate fasciculus. The increase of FA in young ASD children is in agreement with the study of Ben-Bashat et al. (2007) who found increased restricted di_usion in white matter in overall analysis as well as in selected ROIs in young children with confirmed diagnosis of autism. This increase of FA might be a confirmation of accelerated brain growth in autism in the years of life. The cingulum and the arcuate fasciculus were selected for the reconstruction and the tractographic analysis. Significant differences in these white matter tracts were found. The cingulum is the most prominent tract connecting limbic system and cerebral cortex and is involved in higher-level cognitive processes like error monitoring, attention, visuospatial and memory functions, abilities that are frequently compromised in ASD subjects. On the other hand, the arcuate fasciculus is a fiber bundle related to language, a function always impaired in ASD, at least from the qualitative point of view. In particular the most significative result was the increase in the length of streamlines of the cingulum in ASDs in comparison to controls. It is important to underline that the length measure is a mean of all the streamlines so that if the number of short streamlines is higher the mean is lower. This finding confirms the result found by Sundaram et al. (2008). These researchers found that the fiber length of the long association fibers was higher in the ASD group than in controls, although they said that it was unclear from their study which specific long association tracts were involved. From the findings of the present study it seems that the predominant long association fibers belong to the cingulum. The hypothesis of Sundaram and collegues was that one of the mechanisms related to increased long range fiber length could be altered serotonin. In fact, many previous studies have shown that serotonin acts as a neurotrophic factor involved in axonal outgrowth during development. In vitro measures of serotonin synthesis in animal models of autism could help to clarify the role of altered serotonin in autism in white matter structural changes. Recently, Hadjikhani (2010) has hypothesized that increased serotonemia during pregnancy due to frequent use of

antidepressants drugs such selective serotonin reuptake inhibitors (SSRI) could play a role in ASD pathogenesis

Moreover further studies are needed in order to correlate these findings with behavioral measures so that it would be possible to clarify the meaning and the clinical implications of these results.

Chapter 6

A view into the brain of female children with autism spectrum disorder. Morphometric regional alterations detected by structural MRI mass-univariate and pattern classification analyses.

6.1 Introduction

From an epidemiological perspective, autism spectrum disorders (ASDs) are a common disability, with a prevalence of 1:110 children in the U.S (Centers for Disease Control and Prevention –CDC-, 2009) and a strong male preponderance, varying according to IQ. In fact, the male to female ratio may range from 1.31:1 in patients with considerable intellectual disability (Tsai and Beisler, 1983), to 10.8:1 and 15:1 in AS and high-functioning autism, respectively (namely ASD individuals without mental retardation; Gillberg et al., 2006; Wing, 1981).

While some authors question the significance of male preponderance and ascribe it to a greater under-diagnosis or wrong diagnosis of ASD females [ASDf; (Faherty, 2006; Nydén 2000)], others trace back the biased sex ratio to a genetic and/or sex-related hormones pathogenesis. Genetic hypothesis declares that girls need a higher threshold of genetic vulnerability to result in an affected phenotype (Tsai et al., 1981) and brings forward both sex-linked (Skuse, 2000) and autosomal transmission (Stone 2004). On the other hand, hormonal influences could play a role in modulating genetic factors. A general hypothesis on ASD, the extreme male brain (EMB) theory of autism (Baron-Cohen, 2002), emphasises that ASD can be interpreted as a far end of the typical male pattern with impaired empathizing skills and enhanced systemizing abilities. The neurobiological mechanisms for this profile could rely on prenatal exposure to elevated levels of testosterone that may cause alterations in neural structure and function with the subsequent development, in its most pronounced

form, of ASD (Knickmeyer et al., 2005; Knickmeyer and Baron-Cohen, 2006; Auyeung et al., 2009). In particular, Auyeung (2010) indicates that a hyper-masculinized profile is present in ASD since infancy and is irrespective of sex.

Similarly, considering the neuroanatomical structures, the same authors (Baron-Cohen et al. 2005) argue that the brain of ASD patients represents an atypical extreme of typical male brain. The validity of this hypothesis has been proved inasmuch as the overall brain is concerned: studies have established that the mean total cerebral volume of typical young males is, on average, 10% larger than the typical female one (Caviness, 1996; Reiss, 1996; Lenroot, 2007; Giedd, 2009) and it is a well-replicated datum that ASD children have even larger brains than typical males (Piven, 1992; Piven, 1995; Courchesne 2001; Courchesne 2003; Aylward 2002; Sparks 2002; Hazlett 2005; Schumann 2010). As to the volume of specific brain subregions, results are less univocal (Amaral, 2008; Stanfield, 2008), also because of differences in age and clinical features of the study samples.

Up to date few neuroimaging studies have focused on ASD females. Literature predominantly concern samples of male subjects only (Piven 1995; Courchesne, 2001; Carper 2002; Herbert, 2003 Carper and Courchesne, 2005; Wassink, 2007; Hardan, 2009) or of males and females together, without separate gender analyses (Aylward, 2002; Hazlett, 2005; Mosconi, 2009; Zeegers 2009). This choice is explainable with the disproportionate rate between ASD males and females, which is not sufficient to reach the statistical power needed to detect differences. Since even in typical development there is an early sexual dimorphism of brain volumes (Geary, 1998; Durston, 2001, Gilmore, 2007), it could be crucial to obtain information related to gender effect. To our knowledge, only two studies (Bloss and Courchesne, 2007, Craig, 2007) are specifically addressed to the neuroanatomical structure in ASD females through structural MRI investigation. The preliminary study of Bloss and Courchesne (2007) is focused on 9 preschool ASDf and reveals statistically significant differences from typical girls,

consisting in the enlargement of whole brain, of frontal, temporal gray matter regions and of cerebellar white matter, and in the reduction of cerebellar gray matter volume. Craig's contribution compares 14 high-functioning adult ASD females to typically developed women, finding in the former's brains a reduction both of grey and the white matter of specific regions, and an enlargement of the white matter in certain cerebral structures and cerebellum. However, it is difficult to compare these two works with each other, as they do present substantial differences not only with regard to the sample age and clinical characteristics, but also with regard to the methods of anatomic parcellation (manual tracing and computer algorithms in Bloss study *versus* voxel-based morphometry analysis in Craig paper). Previous studies exploring sex effect on brain ASD volume found no differences between 9 ASDf and controls (Piven, 1996), and cerebral enlargement in 7 ASDf *versus* typical development girls (Sparks, 2002). In the first longitudinal MRI study of brain volume growth during early ASD childhood, Schumann (2010) accounted for the presence of a gender effect. This analysis reveals that ASDf present an abnormal growth of whole brain and specific anterior regions when compared to typical females and that this finding is more prominent with respect to ASD males versus typical males. To summarize, studies investigating volumetric brain differences between ASDf and controls suggest various and largely unreplicated findings. However, the small sample size of females, the different ages and IQ considered, as well as the variability of the analysis methodologies, prevent clear interpretation and generalization of the results.

The present study aims to extend the knowledge on variability in the neuroanatomical structures in ASDf children, using the voxel-based morphometry (VBM; Ashburner and Friston, 2000) and the support vector machine (SVM) analytic approach (Vapnik, 1995). While the VBM analysis is a well-established mass-univariate approach, the SVM multivariate technique has been rarely implemented to investigate brain MR data of ASD subjects (Ecker et

al., 2010a; Ecker et al., 2010b). Whereas a mass-univariate approach does require correction for multiple comparisons to avoid the occurrence of false positives, a multivariate technique has the advantage of intrinsically taking into account inter-regional brain correlations. Moreover, the SVM pattern recognition technique would allow investigations about the predictive value of structural MRI scan (Ecker et al., 2010a; Ecker et al., 2010b). However, both VBM and SVM approaches are implemented in the present study to validate the two methods in detecting structural brain abnormalities in ASDf subjects with respect to controls. In particular, the SVM classification capability to predict the class membership of undiagnosed subjects is just exploited as a preliminary step of our algorithm allowing identifying possible between-group structural differences.

6.2 Materials and methods

Subjects

The ASD sample included 38 female children (ASDf) and consists of 22 children with autistic disorder (AD) and 16 children with pervasive developmental disorder – not otherwise specified (PDD-NOS). These subjects were selected from a sample of 57 ASDf patients who underwent a brain MRI examination in absence of a specific clinical indication, as a completion of the diagnostic pathway with the aim of excluding brain abnormalities. The sample had to satisfy these requirements: a diagnosis of idiopathic ASD according to the DSM-IV-TR criteria coupled with clinical judgments and the absence of use of any psychotropic medication. Diagnosis was made by a multidisciplinary team including a senior child psychiatrist, an experienced clinically trained research child psychologist and a speech-language pathologist during 10 days of extensive evaluation and confirmed by the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord et al., 2000) in 33 of 38 patients. Exclusion criteria were: 1) anomalies detected by MRI; 2) a non verbal IQ, NVIQ< 40; 3) for children under 48 months, lack of follow-up after 48 months of chronological age confirming the clinical diagnosis of ASD; 4) infectious disorders; 5) neurological syndromes or focal

neurological signs; 6) dysmorphic features suggestive of a genetic syndrome; 7) significant sensory disabilities; 8) anthropometric parameters (weight, height and head circumference) lying outside two SD from the mean of normal subjects; 9) anamnesis of birth asphyxia, premature birth, head injury or epilepsy*;* 10) insufficient image quality.

Consequently, 19 patients were excluded from the study because they had: non verbal IQ < 40 (2), seizures (2), microcephaly (2), macrocephaly (1), findings on brain MRI [enlargement of subarachnoid spaces (1); arachnoid cyst (1); enlargement of the lateral ventricles (2), asymptomathic Arnold-Chiari malformation type I (1); white matter reduction in temporal lobe (1); hypoplasia of the corpus callosum (1)]; lack of follow-up after 48 months of age (3); low quality scans (2).

Control female subjects were retrospectively selected from our database of clinical structural MRI in order to match ASDf patients for age and NVIQ. This sample consists of 19 patients with idiopathic developmental delay (DD) in whom ASD had been ruled out and 19 children with NVIQ ≥ 70 (noDD).

As for ASD, the DD patients underwent structural MRI in absence of a specific clinical indication, as a completion of the diagnostic pathway with the aim of excluding brain alterations. The noDD subjects underwent an MRI examination because of various reasons (including headache, head trauma, cataract, single -i.e. not recurrent in the next two years- unprovoked idiopathic seizure). Inclusion criteria were: 1) a standardized evaluation of cognitive abilities 2) clinical data records providing sufficient information to ensure the lack of neurological, behavioural or developmental disorders.

The control group was selected so as to meet the same exclusionary criteria as the ASDf (except the third criterion specified above) with the further requirements of no family history of ASD and a score below 20 on the Childhood Autism Rating Scale (CARS; Schopler et al, 1988). CARS is a standardized clinical observation in which a trained observer rates child's

behavior on each of 15 clinical features and has good psychometric properties to screen for ASD (Magyar & Pandolfi, 2007).

ASD and DD patients performed also the recommended laboratory tests to rule-out medical causes of ASD/DD, including audiometry, thyroid hormone disorders, high-resolution karyotyping, DNA analysis of FRA-X and screening tests for inborn errors of metabolism (plasma and urine aminoacid analysis, urine organic acid measurement, urine mucopolysaccarides quantitation, plasma and urine creatine and guanidinoacetate analysis).

The diagnosis of idiopathic DD was performed after a 10 days multidisciplinary and thorough evaluation when the mental retardation (IQ<70) remains of unknown etiology after an exhaustive investigation (both clinical and laboratoristic) for underlying causes.

A number of well-standardized tests with valid and reliable psychometric properties were used to assess intellectual abilities due to differences in the age, verbal skills and functioning level of female children, as is common in clinical practice and research. These included: the *Leiter International Performance Scale - Revised* (Roid & Miller, 1997), the *Griffiths Mental Development Scale-Revised* (Griffiths, 1996), the Italian version of *Wechsler Preschool and Primary Scale of Intelligence* ((WPPSI, Wechsler, 1973) and *Wechsler Intelligence Scales for Children – Revised* (WISC-R, Wechsler, 1986). As some of these tests provide only a global cognitive score, separate verbal and nonverbal IQ scores were not available.

The characteristics of all samples (ASDf, DD and noDD) are reported in Tab. 6.1.

MRI data acquisition

MRI data were acquired using a GE 1.5 T Signa Neuro-optimized System (General Electric Medical Systems) fitted with 40 mT/m high-speed gradients. The standard MR protocol for children included FSE T2-wighted, FLAIR, DWI, SE T1-weighted sequences and a single voxel 1H MR spectrum. Moreover, a whole-brain fast spoiled gradient recalled acquisition in the steady-state T1-weighted series (fSPGR) were collected in the axial plane with repetition time

12.4 ms, echo time 2.4 ms, inversion time 700 ms, flip angle=10°, yielding 124 contiguous 1.1 mm axial slices of 256 x 192 voxels with an in-plane resolution of 1.1×1.1 mm^2.

All children were sedated with a general anaesthesia with a halogenated while spontaneously breathing. All MRIs were performed between October 2003 and March 2010 and the written informed consent from a parent or guardian of children participating in the study was obtained.

The research protocol was approved by the Institutional Review Board of the Clinical Research Institute for Child and Adolescent Neurology and Psychiatry.

VBM-DARTEL image preprocessing

We conducted a voxel-based morphometry (VBM) study (Ashburner and Friston, 2000) to investigate the differences in the volumes of grey matter (GM) and white matter (WM) between the autism and the control group. The T1-weighted volumetric images were analyzed with SPM8 package (Statistical Parametric Mapping, Wellcome Department of Imaging Neuroscience, London, UK, http://www.fil.ion.ucl.ac.uk/spm) using the VBM protocol with modulation. We implemented the Diffeomorphic Anatomical Registration using Exponentiated Lie algebra (DARTEL) algorithm (Ashburner, 2007), where a diffeomorphic warping is implemented to achieve an accurate inter-subject registration with an improved realignment of small inner structures (Yassa and Stark, 2009), and to generate a study-specific template.

The VBM preprocessing included the following steps: (1) checking for scanner artifacts and gross anatomical abnormalities for each subject; (2) setting the image origin to the anterior commissure; (3) SPM default segmenting of brain tissues; (4) importing the parameter files produced by the tissue segmentation in the DARTEL procedure to generate a study-specific template, and to obtain the brain tissues segmented according to it; (5) affine transforming of segmented brain tissues into the MNI space (Ashburner, 2007) according to the

normalize_DARTEL.m matlab script (McLaren et al., 2010; Canu et al., 2010); (5) checking for homogeneity across the sample and using standard smoothing (i.e. with 8-mm isotropic Gaussian kernel). After this preprocessing, we obtained smoothed modulated normalized data (in the MNI space) to be used for the statistical analysis. The aim of the modulation step was to render the final VBM statistics reflective of the "volume" differences rather than the "concentration" differences in GM (Ashburner and Friston, 2000).

VBM Statistical analysis

Whole-brain volume comparison

Group differences were evaluated for the volumes of gray matter (GM), white matter (WM), cerebrospinal fluid (CSF), and for total intracranial volume (TIV).

Estimates of the absolute GM, WM, and CSF volumes were obtained after the automatic brain segmentation had been carried out in the VBM-DARTEL preprocessing. The TIV was calculated as the sum of the volumes of GM, WM, and CSF. The analysis of variance (ANOVA) was performed to identify any significant difference in global tissue volumes between ASDf and control subjects.

Mass-univariate approach: VBM analysis

The regional GM and WM volumes were compared between the two groups using the VBM-DARTEL analysis. The normalized modulated and smoothed GM/WM image segments in each group were entered into a voxel-wise two-sample t-test analysis in SPM8. The conventional VBM–type analysis was employed using the stringent significance threshold $p<0.05$, family-wise error rate (FWE) corrected.

Pattern recognition approach: SVM classification

We followed the whole-GM classification approach with SVM proposed by Klöppel (2008) and Ecker (2010a). In contrast to the mass-univariate VBM analysis, the pattern recognition techniques, e.g. SVMs, are multivariate and thus take into account specific inter-regional dependencies, using that information to help categorize scans (Lao et al., 2004; Fan et al., 2005).

A SVM (Cortes and Vapnik, 1995) is a supervised classification method, i.e. it requires a training set, where to learn the differences between the two groups, and a validation set to evaluate the classification performance on previous unseen data. In our analysis, each image is treated as a point in a high dimensional space, where the space dimension is equal to the number of voxels in the SPM segmented GM. The input to the SVM are vectors of features/voxels extracted from the two categories of patients and controls, labeled with "1" and "-1", respectively. As the number of features/voxels is very high, whereas the amount of subjects considered in this study is rather limited, we considered only linear kernel SVM to avoid the risk of overfitting data. Training an SVM is a minimization problem where the largest margin hyperplane allowing for an optimal separation of the training examples is identified. The separating hyperplane is defined by a weight vector and an offset, $\underline{w} \bullet \underline{x} + b = 0$, where the weight vector $\underline{w}$ is a linear combination of the support vectors and it is normal to the hyperplane. The SVM is trained according to the leave-pair-out cross validation technique (LPO-CV), which is usually implemented to estimate the performance of a classifier when a rather limited dataset is available. The classification performance is evaluated according to the receiver operating characteristic (ROC) curve (Metz, 1986), where the sensitivity (true positive ratio, i.e. the percentage of ASD subjects correctly classified as ASD) is plotted against the false positive ratio (i.e. the percentage of misclassified control subjects). Different ROC curves are compared to each other in terms of the estimated areas under the curve (AUC). The

meaning of AUC has been proved to be the probability that a random pair of positive/diseased and negative/non-diseased individuals would be correctly identified by the diagnostic test (Green and Swets, 1966).

We used in this study the SVM-Light software package (Joachims, 1999).

Discrimination maps and recursive feature elimination

The implementation of a linear kernel SVM has a further advantage; it allows for a direct extraction of the weight vector $\underline{w}$ as an image, which is referred as discrimination map. The vector $\underline{w}$, which is normal to the separating hyperplane, indicates the direction along which the images of the two groups differ most. It can be used to generate a map of the most discriminating voxels in the images. As the intensity value reported in each voxel of the GM segment image is proportional to the amount of GM in that specific location (the modulation option has been selected in the SPM segmentation), a higher/lower value in the discrimination map indicates that patients have higher/lower GM volume in that specific location with respect to controls.

To identify the voxels with the highest discriminating power, we implemented the SVM recursive feature elimination (SVM-RFE) procedure (Guyon et al., 2002; De Martino et al., 2008). The SVM-RFE is a feature selection technique that iteratively eliminates features/voxels from the data set with the aim of removing as many non-informative features as possible, while retaining features that carry discriminative information. A new SVM classifier is trained at each iteration. The feature ranking criterion we implemented is the absolute value of each weight vector component $|w_i|$. The features/voxels are iteratively excluded from the dataset with the aim of removing as many non-informative voxels as possible (low $|w_i|$), while retaining those encoding the discriminative information (high $|w_i|$). We thus removed at each step j of the iterative procedure all voxels/features with $|w_i| < T_j$,

where the threshold value T_j is defined so that $T_j = min|w_i| + j\ (max|w_i| - min|w_i|)/N$, with $j=0, \ldots N$, and N defining how finely the AUC versus the number of retained voxels is sampled.

The SVM-RFE algorithm is implemented in this study with the main aim of estimating the SVM classification performance as a function of the number of GM voxels considered in input to the SVM. Once we estimated the AUC at each iteration, we can identify the minimum amount of voxels that have to be considered to achieve high classification accuracy. The discrimination map obtained at the corresponding threshold on $|w_i|$ encodes the anatomical information about the most discriminating voxels. Despite a statistical significance cannot be assigned to the regions of the brain where the SVM-RFE algorithm localized the voxel where the images of the two groups of subjects differ most, the discrimination map can at least visually be compared to the SPM map.

6.3 Results

Participant characteristics and volumetric analysis

The segmented brain regional absolute volumes generated by the VBM-DARTEL analysis were considered. Tables 6.1 and 6.2 show details of the entire dataset and the two subgroups of DD and noDD subjects, respectively. There are no significant differences between the groups on age and NVIQ. The average values and the standard deviation of age, IQ score, volumes of GM, WM, CSF, and TIV in the two groups and the between group statistics are reported.

Table 6.1. Sample characteristics and global volume group differences in the entire dataset

	Subject Group, Mean (SD)		ANOVA	
Variable	Autism (n=38)	Control (n=38)	F	p value
Age, months	53 (18)	53 (19)	0.0025	0.96
IQ	72 (20)	73 (25)	0.042	0.84
GM, ml	770 (57)	721 (72)	10.9	0.002
WM, ml	332 (42)	308 (54)	4.84	0.03
CSF, ml	181(53)	192(73)	0.52	0.47
TIV, ml	1283 (100)	1220 (140)	5.15	0.03

Abbreviations: GM, grey matter; WM, white matter; CSF, cerebrospinal fluid; TIV, total intracranial volume.

Table 6.2. Sample characteristics and global volume group differences in the subsets of ASDf and controls groups**.**

	Subject Group, Mean (SD)		ANOVA		Subject Group, Mean (SD)		ANOVA	
Variable	A-DD (n=19)	C-DD (n=19)	F	p value	A-noDD (n=19)	C-noDD (n=19)	F	p value
Age, months	47 (18)	51 (18)	0.52	0.47	59 (16)	56 (18)	0.43	0.52
IQ	55 (11)	51 (7)	1.9	0.18	89 (10)	95 (13)	2.75	0.11
GM, ml	764 (70)	712 (68)	5.31	0.03	776 (39)	729 (77)	5.49	0.03
WM, ml	327 (43)	300 (53)	2.94	0.10	338 (41)	316 (56)	1.92	0.17
CSF, ml	192 (48)	186 (67)	0.1	0.75	170 (57)	197 (80)	1.43	0.24
TIV, ml	1282 (120)	1200 (120)	4.45	0.04	1284 (76)	1242 (150)	1.14	0.29

Abbreviations: GM, grey matter; WM, white matter; CSF, cerebrospinal fluid; TIV, total intracranial volume; A-DD, ASD with developmental delay; C-DD, control with developmental delay; A-noDD, ASD without developmental delay; C-noDD, control without developmental delay.

The results of the ANOVA analysis for group differences, revealed the following results on $p < 0.05$: the GM volume was found to be significantly higher in ASD subjects as compared to control in all three datasets; the significant difference in WM volume found on the entire dataset is not preserved in the two subsets; the significant difference in TIV showed by the entire dataset and the DD subset has not been found in the noDD dataset.

The volumetric variables (GM, WM, CSF, TIV), the age and the IQ scores can be shown in a plot matrix, where each variable is plotted against each other, whereas its histogram is shown along the diagonal (Fig. 6.1).

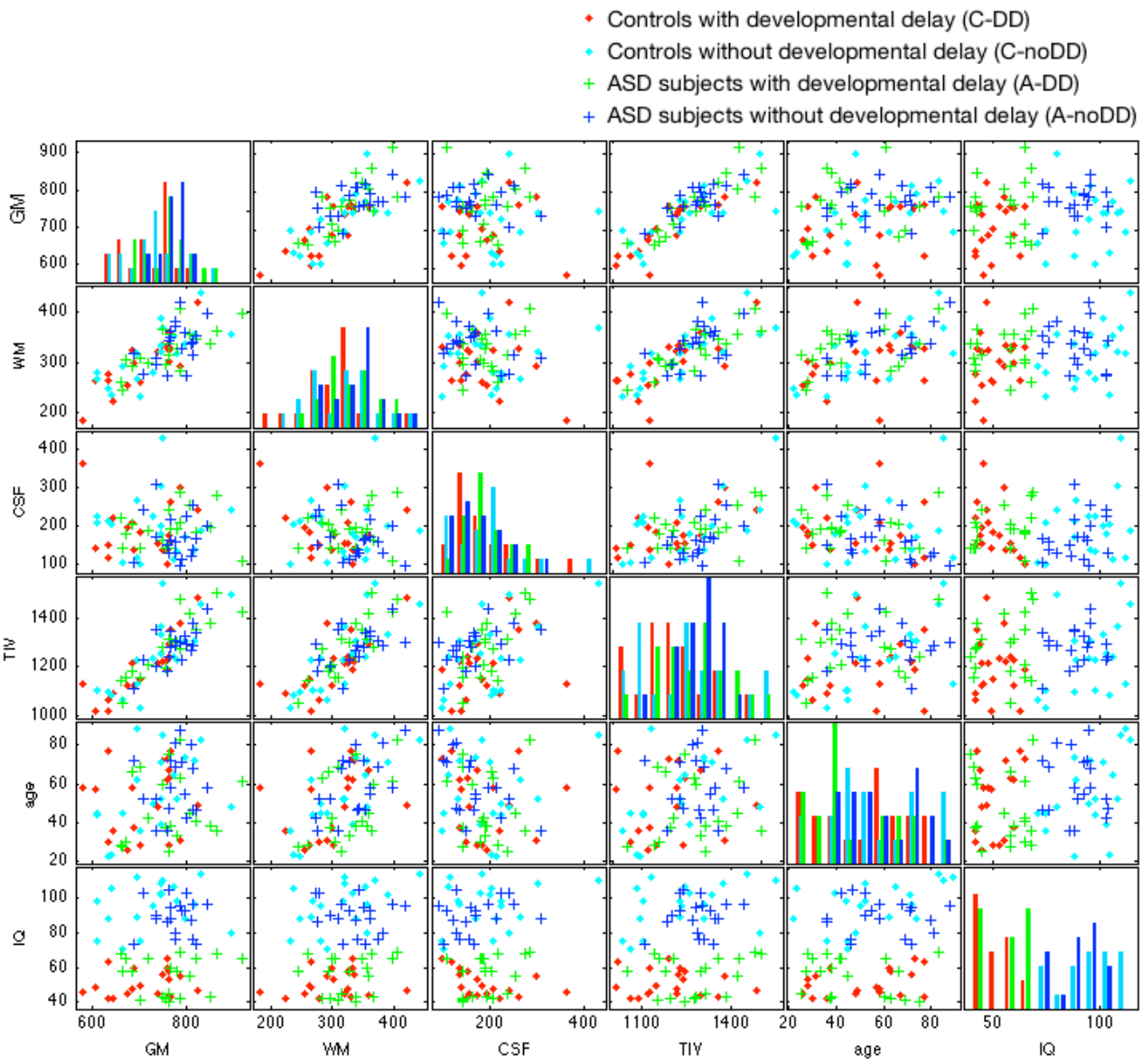


Figure 6.1. Single subject data on volumetric variables (GM, WM, CSF, TIV), age and IQ scores; the histogram of each variable is shown along the diagonal in arbitrary units.

Local GM/WM volume differences (VBM–DARTEL)

To detect possible regional between-group differences, we employed the conventional voxel-wise two-sample t-test VBM analysis on normalized modulated and smoothed (8-mm FWHM isotropic Gaussian kernel) GM and WM segments, using the stringent statistical threshold $p<0.05$, FWE corrected, with an extent threshold of 10 voxels. An absolute threshold mask of 0.1 on both GM and WM was used to avoid possible edge effects around the border between

GM and WM. Age and IQ were entered as covariates in the statistical analysis, thus ending up with 72 degrees of freedom.

Whereas no statistical significant difference has been detected in the WM volume, a significant volumetric between-group difference has been found in the GM. As shown in Fig. 6.2A, a significant increased GM volume is detected in the *left superior frontal gyrus.*

Support Vector Machine (SVM) classification of GM segments

The vectors of features given in input to the SVM are constituted by the sequence of the intensity values of the GM segments obtained in the VBM preprocessing; the vector entries represent the amount of GM in each voxel, as the modulation option has been selected in the SPM segmentation.

Linear kernel SVMs have been implemented to reduce the risk of overfitting the data as the number of features/voxels is very high (about 2.5×10^5), whereas the number of pattern in the dataset is limited to 76. The linear kernel SVM has only one parameter: the c value, that controls the trade off between having zero training errors and allowing for misclassifications. We used the default c values computed by the SVM-Light software through heuristics on the training dataset. As each patient in our dataset is matched to a control with respect to both age and IQ score, the SVMs have been trained according to the leave-pair-out cross validation (LPO-CV), thus excluding one couple of matched subjects from the training set at each iteration, and validating the trained SVM on it. The discrimination performance of SVM trained with all features/voxels of the GM segment is quite poor: AUC=0.62.

Discrimination maps and SVM recursive feature elimination (RFE)

The SVM-RFE algorithm has been implemented as follows: for each threshold T_j on the $|w_i|$ the LPO-CV is performed on the retained data to give an estimate of the classification performance of the SVMs obtained with the reduced set of features/voxels. The behavior of

AUC versus the number of retained voxels reported in Fig. 3 shows that the SVM-RFE procedure is very useful to optimize the classification performance leading to an improvement in the value of AUC from 0.62 to a maximum value AUC_{max}=0.80.

Despite an AUC value of 0.80 obtained in LPO-CV is itself a very interesting result from the point of view of the on-going debate about the predictive value of whole-brain structural MR scans in ASD (Ecker et. al 2010a), it is behond the aim of the present study to set-up a decision-making system on ASD data. We show here the behavior of AUC during the SVM-RFE iterations to infer that an objective criterion to set the threshold T_j on the weights $|w_i|$ to be shown on the discrimination maps could be defined for example as to choose T_j corresponding to AUC_{max}.

As shown in Fig. 3, the AUC shows a plateau region where the relative difference between AUC and AUC_{max} is less than 2%. The maximum value AUC_{max} is achieved by retaining about 200 voxels in the SVM classification (corresponding to less than 0.08% of the total amount of GM voxels), whereas the plateau region for AUC extends from a percentage of retained voxels of 0.01% to 1% of the total amount of GM voxels.

To build the discrimination maps we computed the weight vector $\underline{w}$ using all data to train the SVM. We reported in Fig. 2B the map obtained for the left extreme values of the plateau region of AUC (see Fig. 3), i.e. with the most exiguous number of discriminating voxels (0.01% of GM voxels with the highest $|w_i|$ values) retained in training the SVM, and leading to an AUC value (AUC=0.79) within the 2% of relative difference from AUC_{max}.

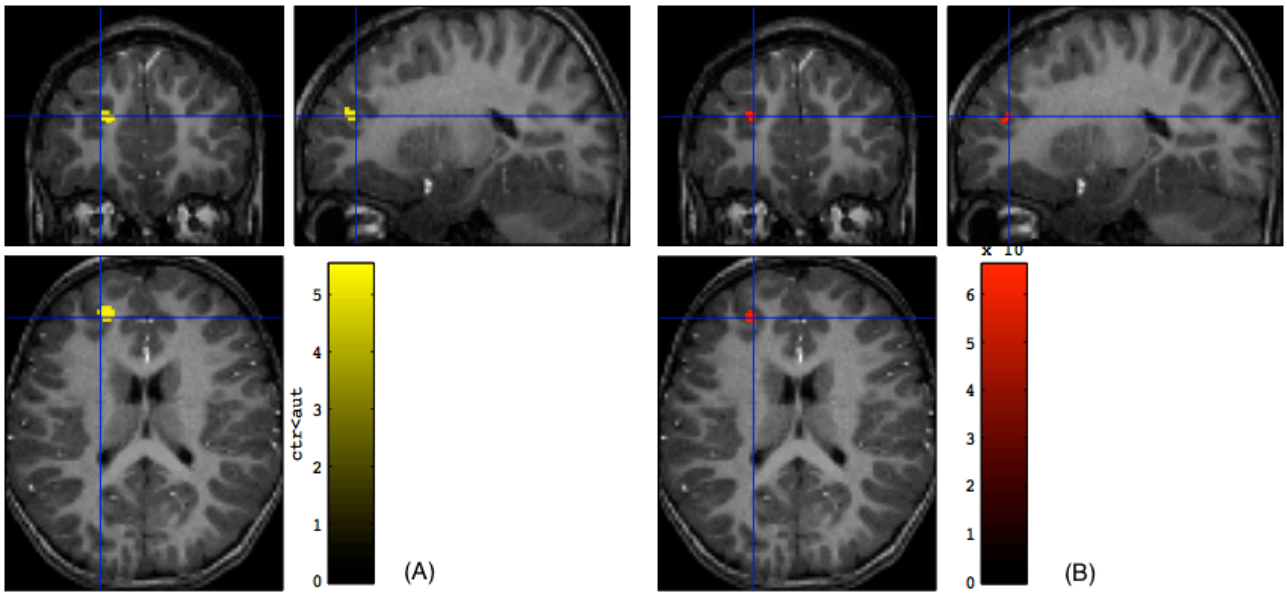


Figure 6.2. Brain region showing larger local GM volumes in the autism disorder group compared to control subjects (yellow/red areas) are overlaid to a representative structural MR image normalized to the MNI space. A): VBM result with p<0.05, FWE corrected, with an extent threshold of 10 voxels. B): SVM-RFE discrimination maps obtained with about the 0.01% of retained voxels (left extreme of the plateau where AUC=0.79, as shown in fig. 3). The VBM and SVM-RFE procedures reveal the same cortical volume alteration in the left superior frontal gyrus (MNI coordinates: -19 44 23).

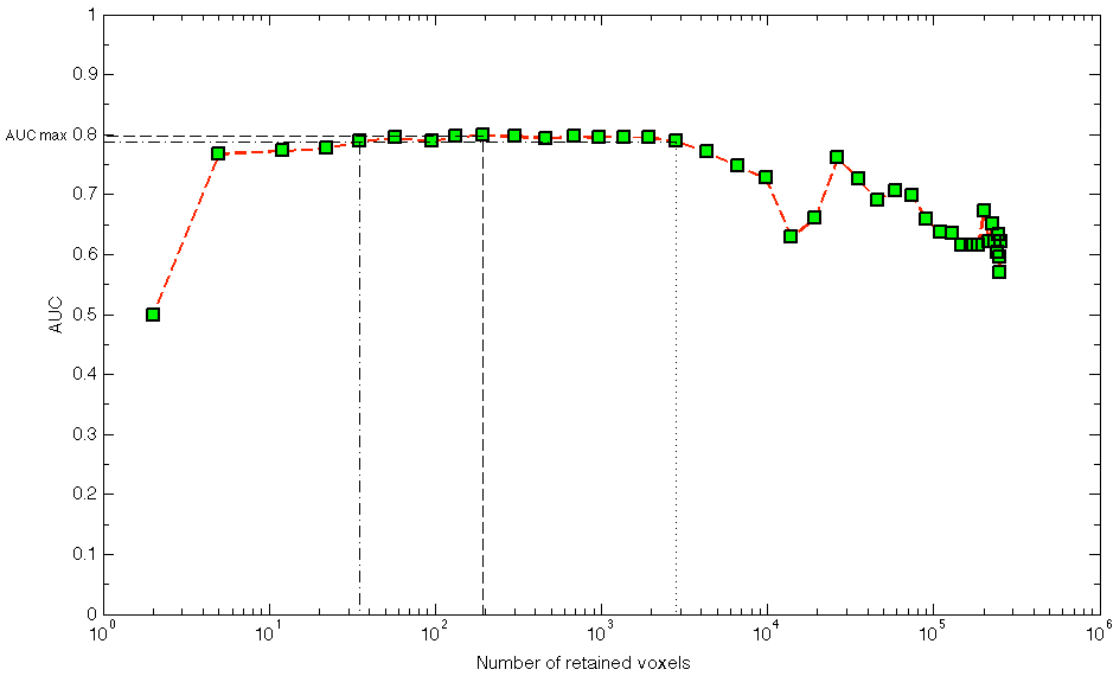


Figure 6.3. AUC versus the number of voxels with high $|w_i|$ values considered for the SVM classification of the GM segments. The maximum AUC value is obtained by considering about 200 voxels (0.08% of GM voxels) in the SVM training (AUC_{max}=0.80); a plateau region where AUC values are within 2% of relative difference from AUC_{max} is obtained for a percentage of retained GM voxels in the 0.01%–1% range.

The direct comparison between the SPM map obtained with the VBM analysis (Fig 6.2A) and the SVM-RFE discrimination map reported in Fig. 2B shows that the two procedures detected an increased amount of GM in ASD subjects with respect to controls in the same brain region, the *left superior frontal gyrus*.

As the w_i values are shown in the maps, it could be possible to distinguish the brain regions where GM is either greater or lower in the patient group with respect to the control group. However, as shown in the figures, only regions where GM is greater in ASD subjects with respect to controls are found for the corresponding threshold values on w_i.

We reported in Fig. 6.4 the map obtained by retaining only the small set of voxels (about the 0.08%) with the maximum discriminating power (AUC_{max}=0.80). It can be noticed that an increased GM volume in three brain regions is detected in ASD patient with respect to control subjects. The involved brain regions extended for more than 10 voxels are bilaterally the *superior frontal gyrus* and the *right temporo-parietal junction,* as reported in Table 6.3.

In order to make direct comparisons across studies, we translated MNI coordinates into Talairach coordinates using the mni2tal function (http://www.mrc.cbu.cam.ac.uk/Imaging/Common/mnispace.shtml).

Table 6.3. Results of the SVM analysis**.**

		Brodmann Area	Number of voxels	MNI Coordinates (x, y, z)	Talairach coordinates (x, y, z)
Superior frontal gyrus	L	9-10	116	(-26, 44, 20)	(-22, 39, 19)
	R	9-10	34	(26, 50, 10)	(22, 45, 11)
Temporo-parietal junction	R	39	24	(45, -55, 26)	(39, -57, 20)

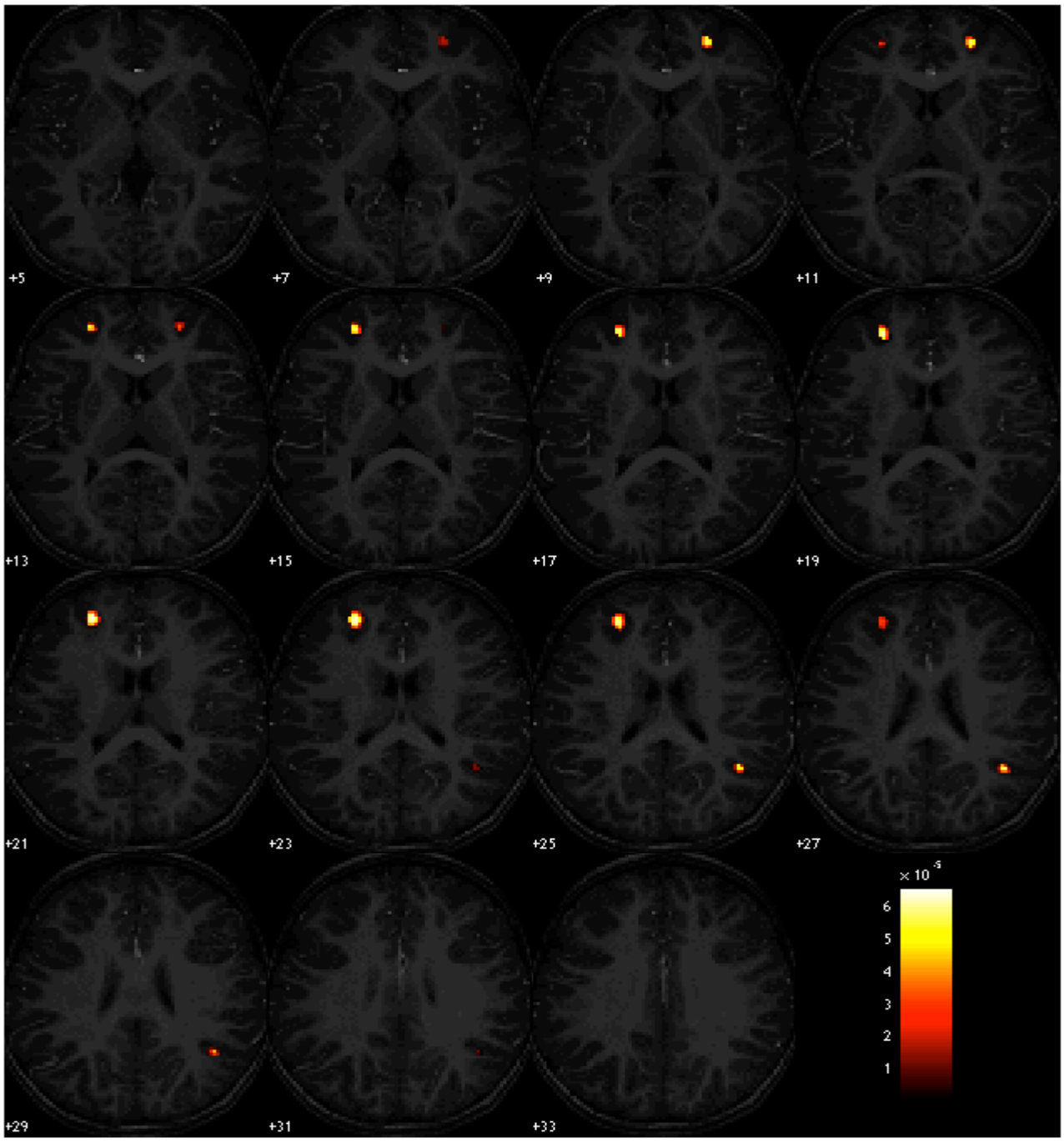


Figure 6.4. Discrimination maps overlaid to a representative structural MR image. The voxels (about 0.08% of the total amount of GM voxels) with the highest discrimination power (AUC_{max}=0.80) correspond to three areas of the brain where GM is greater in the ASDf group with respect to the control group: the bilateral superior frontal gyrus [number of voxels (MNI coordinates): 116 (-26 44 20); 34 (26 50 10)], and the right temporo-parietal junction (TPJ) [24 (45 -55 26)].

6.4 Discussion

Findings from several MRI-based morphometric studies on "brain's ASD children", actually refer to sample of "brain's ASD males children" (Piven 1995; Courchesne 2003; Carper 2002; Herbert 2003; Carper and Courchesne, 2005; Wassink, 2007; Hardan, 2009). When females take part to the sample, they are often not enough to perform a reliable separate analysis by sex (Courchesne, 2001, Aywlard, 2002; Kates 2004). Moreover, only high-functioning ASD patients usually participated to a MRI study (Abell, 1999; Aywlard, 2002; Herbert 2003; Mc Alonan, 2005, Salmond 2005) since they present a better compliance to complete the MRI exam without sedation. Therefore, the extent to which conclusions can be applied to the entire ASD population is limited.

This study attempts to fulfil part of this research lack by exploring brain volumes differences in a group of female children with ASD as compared with a control group that includes both female children with DD and without DD. The results provide three key findings.

First, the between-group whole-brain and brain-segment volume comparison revealed a total intracranial volume enlargement approximately of 5 % in female children with ASD with respect to age and IQ matched controls. The GM, WM and CSF segments were obtained during the VBM preprocessing based on the DARTEL algorithm, where a diffeomorphic warping is implemented to achieve an accurate inter-subject registration and a study specific template. The total intracranial volume has thus been estimated as the sum of GM, WM and CSF.

These findings are consistent with the well-replicated result of an accelerated postnatal brain growth in ASD samples of males or mixed subjects and with an attenuation of the difference between patients and controls with increasing age (Courchesne, 2007). Conversely, MRI data regarding early brain overgrowth in ASDf are inconclusive: Piven (1996) reports on an increased total brain volume in ASD males, but not in females, Sparks (2002) demonstrates

similar enlargement of cerebral volume across gender, while Bloss and Courchesne (2007) described an abnormal enlargement of whole brain, frontal cortex and temporal cortex greater in females than in males with ASD. Although causes of early brain overgrowth and its pathophysiology remains to be established (Amaral, 2008), possible consequences could be a reduction of connectivity (Ringo, 1991) and, from the clinical point of view, an interference on typical social behavior, as suggested by a murine model (Fatemi, 2002). Evidence for this early enlargement comes from retrospective longitudinal studies on disproportional head circumference size (Lainhart, 1997; Courchesne 2003; Torrey 2004), and are confirmed by an increased brain volume in ASD structural MRI investigations on toddlers and children (Hazlett, 2005; Courchesne 2001; Carper 2002; Sparks 2002). While some researchers argue that abnormal brain enlargement is mainly explained by excessive increases in WM (Herbert, 2003, Courchesne, 2001 Hazlett, 2005), others think that GM is involved, alone or in association to WM (Lotspeich, 2004; Palmen 2005; Schumann, 2010). In the present study, the greater GM ($p=0.0015$) primarily accounted for overall brain volume enlargement in children ASDf ($p=0.0262$) relative to control children; however, an atypical and excessive development is also present in WM ($p=0.0310$) and CSF ($p=0.4744$).

Second, the conventional VBM analysis we implemented (smoothing with 8-mm FWHM isotropic Gaussian kernel, voxel-wise two-sample t-test, stringent statistical threshold $p<0.05$, family-wise error corrected, extent threshold of 10 voxels) highlighted an increased GM volume in a specific region of the left superior frontal gyrus (lSFG; BA9/10) of ASDf. A regional cortical volume alteration has been revealed by the SVM-RFE analysis obtained with the most exiguous set of voxels (0.01% of the total GM voxels) and the results are extremely consistent with the lSFG region identified by the VBM analysis.

According to an anteroposterior gradient of early brain enlargement in ASD, the frontal lobes are the site of the peak of overgrowth and abnormalities (Carper 2002) as indicated also by an

excessive cortical folding, measured by the gyrification index (Hardan, 2004, Jou, 2010). Our results are in agreement with an advanced analysis of frontal area which restricts the enlargement to dorsal (which contains the SFG area) and mesial regions (Carper & Courchesne, 2005). Indirect information regarding structural abnormalities in the lSFG derives also from mapping of cortical sulcal patterns (Levitt 2003) revealing an anterior and superior shifting of superior frontal sulcus in ASD children and adolescents compared to controls. At the microscopic level, histologic studies provide evidence for disturbances in the brain maturation process in this area, as indicated by alteration of cell microcolumn morphology in BA 9 (Casanova, 2002, Buxhoeveden, 2006).

According to Amodio and Frith (2006), the SFG belongs to the anterior rostral region of the medial frontal cortex (arMFC), an area activated by a wide range of tasks including executive functions (Duncan & Owen, 2000), as well as evaluative judgment (Zysset, 2003), self-knowledge, monitoring of one's own emotional state, person perception, mentalizing (see Amodio & Frith for a review). In particular, "mentalizing" (Frith et al., 1991) or "theory of mind" (ToM; Premack and Woodruff, 1978) is the human ability to attribute mental states to self and others and represents the ASD deficit that mainly explains the social and communication impairment of these patients (Frith, 2001). While the typical anterior core area in mentalizing system corresponds to the medial prefrontal cortex (Van Overwalle, 2011), our GM increased area is localized in a more lateral region and is implicated in at least some aspects of ToM. For instance, the joint attention, one of the first manifestations, at about 18 months, of the mentalizing capacity is sustained by a neural network involving an area of increased volume in lSFG (Waiter, 2004; Williams, 2005).

Ramnani and Owen (2004) argue that BA 10 is activated in high-level tasks requiring the coordination and integrations of multiple related cognitive operations. A deficit in abilities that need integration of information is also the characteristic neuropsychological profile of

ASD children, regardless of IQ (Williams, 2006). There is a large body of research demonstrating that ASD patients have an altered activation in tasks requiring SFG contribution, such inhibition of inappropriate responses/cognitive control (Minshew, 1999; Solomon, 2009), self-referential processing (Lombardo, 2010) and reward achievement (Schmitz; 2008).

Recent investigations on the macaque monkey (Petrides & Pandya, 2007) indicates that the rostral prefrontal cortex, which contains the analogous of our increased GM density area in SFG, projects to the multisensory processing region of the superior temporal sulcus. This area is involved in an higher order level elaboration process, consisting in integration of information coming from multiple sensory sources to reach an abstract interpretation and elaboration of the experiences (Petrides & Pandya, 2007). Atypical responses to typical sensory stimulation have been described as a common feature of ASD patients (Wing, 1969; Baranek, 1997) and a possible explanation focuses on impairments in the cortical integration of perceptual input (Gomot 2002). In addition, difficult to integrate sensory information in a gestaltic manner lies at the bottom of the weak central coherence hypothesis of ASD (Frith 1989; Happé and Frith 2006). Therefore, it could be hypothesized that the structural alteration in SFG interferes with the connections to superior tempotal sulcus determining, in its turn, an altered activity of this area. The rostral prefrontal cortex is also connected with amygdala, orbitofrontal and cingulate cortex, areas all involved in ToM and empathy tasks processing (Völlm, 2006).

Third, the implementation of the SVM analysis on the GM segments obtained in the VBM-DARTEL preprocessing revealed a more complex circuitry that discriminates ASDf from control subjects. In fact, the GM volume excess previously limited to the lSFG, is now flanked by a volume increase in the homologous area (MNI coordinates = 26 50 10) in the right hemisphere, and, furthermore an increase of right temporo-parietal junction (rTPJ; MNI

coordinates = 45 -55 26). Abnormalities in GM temporal lobe of ASD subjects are reported by several structural MRI investigations (Carper, 2002; Boddaert 2004; Waiter, 2004; Hazlett, 2005; Bonilha, 2008; Schumann, 2010) and are in agreement with neuropathologic findings (Bauman & Kemper, 1985; Bailey, 1998; Casanova, 2002). Moreover, these subtle, but significant GM alterations correspond closely to areas implicated in social cognitive function in other studies. In particular TPJ is a fundamental neural substrate for ToM abilities (Saxe & Kanwisher, 2003) and for attributions of representational mental states (e.g. beliefs) mainly (Zaitchik, 2010), but it is also implicated in reasoning, human-like shape motion, goal-directed action and moral judgments tasks (Van Overwalle, 2009). Neuroimaging studies in adult ASD have contradictory reported weaker activation of ToM circuit involving TPJ (Castelli, 2002), enhanced activations of these areas (Mason, 2008) or no differences between patients and controls (Happé, 1996). On the other hand, a recent investigation on ToM network (Kana, 2009) correlates the TPJ alteration in ASD patients with a lower functional connectivity between this region and frontal areas.

Approaches based on SVM and SVM-RFE classification have been implemented in the last few years both on structural data of subjects with different pathologies, e.g schizophrenia (Fan et al., 2005), Alzheimer's disease (Kloppel et al., 2008) and also autism (Ecker et al., 2010a; Ecker et al., 2010b), and to functional data to classify for example brain states (Mourão-Miranda et al., 2005).

Some of those studies are voxel based, i.e. the SVM classifier works in an N dimensional space, where N can be either the size of the whole image or of the ROI considered for the analysis (Mourão-Miranda et al., 2005; Kloppel et al., 2008; Ecker et al., 2010a). The other studies consist in feature-based analyses, where morphological (volumetric, geometrical) features are first extracted from the data, and then classified by the SVM (Fan et al., 2005; Ecker et al., 2010b).

The procedure we adopted in this study belongs to the voxel-based category, and can be directly compared with the paper by Ecker et al. (2010b), which is focused on autism data as well. A comparative discussion about the results is not possible as the sample characteristics are totally different in the two studies: males, adults and high-functioning ASD in the Ecker's paper versus females, children, and high- and also low-functioning individuals in the current analysis. Ecker et al. found that SVM succeded to identify a spatially distributed networks able to discriminate between adult ASD males and controls. These included the limbic, frontal-striatal, fronto-temporal, fronto-parietal and cerebellar systems. However they didn't found any consistency between SVM and VBM results. In particular, the VBM analysis they performed did not revealed any significant region with enlarged GM in the autism group with respect to the control group at the conventional stringent levels of statistical significance ($p<0.05$, FWE corrected). As the authors suggest, this can be been explained in term of the availability of a not enough populated dataset, thus not allowing achieving a sufficient statistical power for VBM not implemented with the DARTEL algorithm.

By contrast, results from VBM-DARTEL and SVM-RFE analyses are extremely consistent in the present study. The fact that SVM-RFE allowed us to considered two additional interesting brain regions with respect to the VBM output can be explained as follows. The VBM analysis implemented with the stringent settings for correction for multiple comparisons (FWE correction, $p<0.05$) is a safe procedure allowing only 5% false-positive voxels in the SPM output; however, it is unfortunately prone to miss a lot of true positives. SVM, which is a multivariate approach, intrinsically takes into account inter-voxel correlation, and it does not need to the multiple comparison correction procedure. Through the RFE algorithm, SVM allows to identify and localize even very subtle differences in the brain anatomy between two groups of subjects.

From the methodological point of view, whereas in Ecker's study the SVM potential in discriminating patients from healthy controls and the predictive value of whole-brain structural MR scans have been investigated, the implementation of SVM in the present study is finalized to the generation of the discrimination maps. In other words, The SVM approach has not been implemented in this study with the main aim to partitioning subjects into the patient and control categories, but as an effective alternative approach to localize the brain regions possibly involved in or affected by the disease. In particular, the SVM-RFE procedure has been used not to optimize the predictive accuracy of the SVM classifier, but to remove non-informative features from the weight vector to represent only the most informative voxels in the discrimination maps.

We showed the behavior of AUC during the SVM-RFE iterations to infer that an objective criterion can be implemented to choose the most appropriate amount of voxels to be displayed in the discrimination maps. Despite an AUC value of 0.80 obtained in LPO-CV is itself a very interesting result from the point of view of the on-going debate about the predictive value of whole-brain structural MR scans started some time ago (Lao et al, 2004; Davatzikos, 2004), and more recently focused on autism data (Ecker et. al 2010a), it is beyond the aim of the present study to set-up a decision-making system on autism data.

The systematic evaluation of the predictive power of structural MR scans, the comparison with and the possible combination to the information extracted from DTI data, deserve to be addressed in a future dedicated study.

The present study has several methodological advantages as well as important limitations that should be carefully considered. The strengths of this study included the large number of carefully selected, medication-naive ASDf subjects and the availability of a large database, which allowed us to examine both a well-matched noDD comparison group and a group of idiopathic DD patients. However, the retrospective nature of the study implies an incomplete

data assessment (lack of ADOS in 5 of 38 ASDf patients) and a non-homogeneous evaluation of intellectual abilities. The focalization of this study on brain of children ASD females prevents us to extend conclusions to males. Future research on a specific comparison of ASD male and female children, matched for age and IQ, could address the issue if a sex difference in regions of brain enlargement exists, and investigate its possible correlation with ASD phenotype.

6.5 Conclusions

This study suggests that brain enlargement is a hallmark of early ASD, independent of sex and that GM could represent a fundamental component of the altered developmental process. The VBM-DARTEL analysis localized a particular increase of the volume of the left superior frontal gyrus. By integrating the VBM-DARTEL analysis with the SVM classification approach we can identify a broader pattern that differentiate ASDf from control subjects. This altered circuit, which includes the bilateral frontal gyrus and the right temporo-parietal junction, could have a seminal role in ASD core dysfunction.

Current findings can shed light on the specific neuroanatomy of ASD females, including also lower-functioning subjects often excluded from research projects on structural MRI in ASD. This study could represent an initial step toward an ASD diagnosis that includes brain endophenotype in addition to standard approaches currently used, exclusively based on behavioral criteria.

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