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Gastrointestinal condition, nutritional aspects and gut microbiota in Autism Spectrum Disorders: a new perspective for research and intervention

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ABSTRACT

In the last two decades several studies have been trying to explore a possible role for gut microbiota in Autism Spectrum Disorders (ASD), supported by the high incidence of gastrointestinal disorders among ASD children and by the now well recognized existence of the brain-gut-microbiota axis (the complex system of bidirectional interactions between central nervous system, gastrointestinal tract and microorganisms inhabiting the gut).

Nevertheless, results about alterations in gut microbiota composition and/or activity in ASD are to date strongly contrasting.

A possible explanation could be that these studies tend to treat ASD as a unique pathology, whereas it includes different cognitive-behavioural phenotypes. Moreover, they do not consider factors which are important for children's gut flora development, such as type of delivery, nutritional history (e.g. formula milk during lactation) and medical history (e.g. antibiotics intake) as well as factors that may affect the present composition of microbiota, such as the current diet (e.g. the strong food selectivity that often occurs in ASD children) and the presence of gastrointestinal disorders.

In this study, I developed an interview to parents to assess whether there are differences related to the above mentioned aspects between ASD children and typically developing children and among ASD themselves, considering differences in cognitive level and severity of autistic traits.

I also explored the use of special diets such as gluten-, lactose and casein free diets, the reasons for their adoption and the possible benefits for the child.

Moreover, I decided to include in this interview also a section dedicated to parental difficulties in managing mealtime in order to collect information useful to plan future interventions.

I found differences between ASD- and typical children in the incidence of gastrointestinal disorders and food selectivity. Especially, some children initially eat everything and then switch to a more and more restricted diet. This could be considered an early symptom of the pathology. I also found an association between gastrointestinal disorders and severity of autistic traits.

Furthermore, I collected faecal samples from ASD families (two parents, an ASD child and a typically developing sibling) and analysed them with metaproteomics and bioinformatics techniques in order to assess microbiota activity and evaluate it in light of ASD phenotype, nutritional habits, gastrointestinal disorders and genetic proximity.

Demonstrating the existence of a different microbiota composition in ASD or at least in a subgroup could allow to identify a biomarker of a possible development of ASD and to design preventive interventions, even through probiotics intake. Moreover, it could help to better understand the molecular mechanism underlying this pathology.

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CHAPTER 1: INTRODUCTION

1.1 Autism Spectrum Disorders: what are they?

Autism spectrum disorders (ASD) are pervasive early- onset neurodevelopmental disorders, that appear with the notable incidence of 1% - 2%, according to different studies conducted in Asia, Europe and North America. This occurrence seems influenced by gender, with a prevalence 4.5 times higher among males than females, and can be found in all racial, ethnic and socio-economic groups (for a more detailed discussion see Christensen et al., 2016).

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5 - American Psychiatric Association, 2013), these disorders are characterized by persistent deficits in communication and social interaction, which result in an abnormal approach or lack of initiative towards the others, failure in normal conversation and / or reduced tendency of sharing interests. A deficit in understanding and implementation of nonverbal communicative behaviors (e.g. abnormalities in eye contact, lack of facial expression and gestures) is also present. All this results in difficulties in establishing and maintaining social relationships.

These difficulties are associated with restricted and repetitive interests, activities and behaviors that may occur in the context of language (e.g. echolalia, idiosyncratic phrases), movement (motor stereotypies) and repetitive and stereotyped use of objects. An excessive adherence to routines, a resistance to change and a fixation in highly restricted interests with abnormal intensity or focus can also be present, so that an external request to change activity can even elicit tantrums or behaviors that are inappropriate to the context.

All this can be accompanied by hypo- and/or hyperreactivity to sensory stimuli (e.g. apparent insensitivity to temperature, nuisance to loud noises) and unusual interests with respect to certain aspects of the environment (e.g. bright objects) (American Psychiatric Association, 2013).

The symptoms, although present from early childhood, cannot become completely recognizable as long as the social demand is not higher than the child's ability to cope. This is the reason why the diagnosis is generally possible starting from 2-3 years of age. However, it is not uncommon that these disorders are diagnosed at school age or even in adulthood, especially for milder forms (Davidovitch et al., 2015).

In a certain percentage of cases, moreover, parents report normal development of the child up to 18-24 months and a subsequent loss of acquired skills: this is the case of the so-called “autism spectrum disorder with regression” (Stefanatos, 2008).

However, the set of symptoms has mostly a chronic development (Aman, 2005) and often prejudices dramatically the person's functioning in the everyday life (American Psychiatric Association, 2013). Nevertheless, the characteristics and severity of symptoms can significantly vary from person to person along a continuum: this is the reason behind the use of the word "spectrum" in naming these disorders.

In addition, the symptoms pattern described above can be associated with delayed development or even absence of language, resulting in a further impairment of communicative aspects (Kjelgaard and Tager-Flusberg, 2006).

Moreover, consistent differences on cognitive level can be found among subjects on the spectrum. In particular, it comes to high-functioning autism in the presence of IQ equal to or higher than 70 and low-functioning autism for IQ below 70.

To conclude, the frequent comorbidity with other disorders, such as epilepsy, ADHD, schizophrenia, anxiety disorders, and gastrointestinal disorders, contributes to make the phenotypic framework even more heterogeneous and complex (Kohane et al., 2012; Lai et al., 2013; Mannion et al., 2013). Moreover, in the more severely impaired end of the spectrum, subjects can also show aggressive or self-injurious behavior (Karst and Van Hecke, 2012).

Taken together all the above mentioned difficulties, it becomes clear that many individuals need a lifelong support, usually provided by family members, and that this may have negative consequences also on the quality of life of these relatives, in addition to economic costs and need for private and public services (Karst and Van Hecke, 2012).

In this complex scenario it is easy to understand the high efforts that have been made to understand the etiology of these disorders, in order to find ways to prevent them, if possible, or at least to allow early diagnosis and intervention with better outcomes for the child.

Thus, after a long time in which it has been believed that Autism Spectrum Disorders were caused by the poor responsiveness of inefficient mothers in their relationship with their child, nowadays they are considered the result of pre- and/or postnatally impaired neurodevelopmental processes. In fact, neuroimaging studies showed alterations in the frontal and prefrontal areas, their linked structures and their connections to the temporo-parietal areas. (Minshew et al., 2006). Such abnormalities in the neurological development seem to underlie the child impaired ability to put him-/herself in relationship with others in the early years of life, causing in this way cognitive and behavioral effects (Trevarthen and Aitken 1998; Venuti, 2012; Vicari et al., 2012).

Interestingly, post-mortem analysis of brain tissues from individuals with ASD supports a role for chronic neuroinflammatory processes that could potentially alter synaptic connections and change brain connectivity (Rodriguez and Kern, 2011). In fact, different studies have highlighted innate and adaptive immune dysfunction in ASD, not only related to the brain but also at systemic level, with an

interesting correlation with worse behavioral measures (Li et al., 2009; Ashwood et al., 2011; Ashwood et al., 2011; Depino, 2013; Ricci et al., 2013; Masi et al., 2014)

It seems also crucial to establish if these immune abnormalities are an epiphenomenon in ASD or if they are cause or consequence of the neurodevelopmental impairments (Matelski and Van de Water, 2016).

Also a genetic basis for ASD has been hypothesized, even if studying it is challenging due to the lack of large samples and the tendency of ASD individuals not to reproduce (Rish et al., 2014).

Although the first studies indicated a high genetic heritability (Folstein and Rutter, 1977; Steffenburg et al., 1989; Bailey et al. 1995; Dowson et al., 2002; Rutter, 2005), more recent findings put these results on debate (Hallmayer et al., 2011): in fact, even if several tens of genes and genomic regions have been identified as directly related to ASD etiology or at least to a susceptibility, only about 10% of ASD cases seem to be syndromic, whereas the others 90% seem to be idiopathic (Betancur, 2011; Iossifov et al., 2012).

In addition, the high presence of heterogeneous symptoms patterns among ASD must be taken into account.

All these aspects suggest to consider ASD as a multifactorial disease resulting from the interaction between genetic and environmental factors (Herbert, 2010).

Therefore, a growing interest has been directed to epigenetics. In fact, it may be possible that a genetically susceptible pattern becomes target of an environmental insult that causes dysregulating changes in a specific window of neurodevelopmental vulnerability (Stamou et al., 2013; Kim and Leventhal, 2015; Mazina et al., 2015).

Unfortunately, identifying these complex interactions is challenging because of the high number of environmental factors that theoretically could play a role (Matelski and Van de Water, 2016).

Nevertheless, a possible way to address these difficulties is to consider not only the typical symptoms of this pathology but also other aspects that are often related to ASD, such as for instance the presence of gastrointestinal disorders.

1.2 Gastrointestinal disorders: an interesting comorbidity

In fact, many individuals with ASD are subjected to gastrointestinal problems (Parracho et al., 2005) with a prevalence of constipation and diarrhea, followed by abdominal pain, vomiting and gastroesophageal reflux (de Magistris et al., 2010; Kang et al., 2014). According to a recent population-based prospective study in Norway, these symptoms seem to be more common in children

with ASD than in typically developing children or in those with developmental delay (Bresnahan et al., 2015).

Nevertheless, the actual incidence of gastrointestinal disorders among ASD patients is under debate. In fact, it has been estimated to range between 9% and 90%, depending on the study (Buie et al., 2010). This discrepancy can be accounted as the result of methodological issues related to the characteristics of sample and data collection (interviews with parents, diagnostic database). However, another reason is the difficulty of diagnosis of gastrointestinal problems in these subjects: many, in fact, cannot express pain or discomfort through verbal and/or nonverbal channels (Buie et al., 2010). In recent years there has been a growing awareness of these diagnostic difficulties. This has led to the development of guidelines for the assessment and treatment of gastrointestinal disorders in individuals with ASD, within which numerous behaviors are listed that, apparently disconnected, could instead be indirect symptoms of gastrointestinal disorders: e.g. lethargy, pushing objects towards the belly, grinding teeth, biting clothes, often throat clearing, swallowing, sobbing for no apparent reason, repeating words/phrases related to pain, seemingly inexplicable increase in repetitive behaviors / stereos or oppositional behavior, restlessness, screaming, self-injurious behaviors, sleep difficulties, etc. (Horvath and Perman, 2002; McAtee et al., 2004; Carr and Owen-Deschryver, 2007). Furthermore, Chaidez et al. found that children with ASD presenting frequent abdominal pain, gaseousness, diarrhea, constipation or pain on stooling scored worse on irritability, social withdrawal, stereotypy, and hyperactivity compared with children with ASD having no frequent GI symptoms (Chaidez et al., 2014).

In this scenario, a proper diagnosis and treatment of gastrointestinal disorders could lead to a reduction or even the disappearance of some problem behaviors listed above.

In addition, these results are in line with what has also been highlighted by Adams et al., namely that there is an association between high severity of autistic symptoms, in particular with regard to the communication area, and the presence of gastrointestinal disorders (Adams et al., 2011). It could be speculated that more communicative impairment may induce higher levels of stress, resulting in somatization.

On the other hand, however, it could also be assumed that gastrointestinal discomfort is a further phenotypic manifestation of an organic condition implicated in the etiology of autism spectrum disorders, or at least in a subgroup.

This would be in line with the above explained considerations about possible gene-environment interactions. A candidate for this role could be the gut microbiota, which means the microorganisms inhabiting the intestinal tract.

1.3 Microbiota: a key role in the microbiota-gut-brain axis

There is, in fact, a complex system of bidirectional connections that links the central nervous system, the intestinal tract and the microbiota, known as microbiota - gut - brain axis, of fundamental importance for the maintenance of the homeostasis (Cryan and O' Mahony, 2011) and in which the intestinal microorganisms seem to play an important role (Rhee et al., 2009).

The human gastrointestinal tract is a complex system that includes more than 10^{14} bacteria, whose genome is 100 times larger than the human genome (Del Chierico et al., 2012) and that adapted to co-exist in a commensal or symbiotic relationship with the host (Ley et al., 2008). In fact, these microorganisms play a very important role for the host's health, preventing the invasion of pathogenic microorganisms dynamically, exerting essential metabolic functions (e.g. fermentation of non-digestible fiber, energy recovery from short-chain acids and vitamin K production) and stimulating the proper development of the immune system (Hooper and Gordon, 2001; Forsythe et al., 2010). Moreover, these microorganisms promote the orderly development of the gastrointestinal mucosal barrier function. Interestingly, many individuals with ASD show alterations in gut permeability (D'Eufemia et al., 1996; de Magistris et al., 2010) and innate and adaptive immune dysfunctions, as discussed above.

Moreover, gut microbiota develops in a timespan also sensitive for neurodevelopment. In fact, in the maternal womb the fetus has not microbiota yet, but he/she is exposed to the mother's microbiota metabolism products through the placental circulation. During and immediately after birth, the newborn is colonized by strains of the maternal intestinal and vaginal flora, as well as by microorganisms from breast milk, environment and later from food (Stanghellini et al., 2010).

The colonization process completes between the first and second year of life with regard to the amount of bacteria, while the composition of the microbiota is influenced by the genetics of each subject and by environmental factors, such as diet and living conditions: therefore, it can vary during lifespan (Parracho et al., 2005; Chaste et al., 2012; David et al., 2014; Goodrich et al, 2014).

Regarding possible pathways by which microbiota might influence the nervous system, several mechanisms have been highlighted (Cryan and Dinan, 2012 – Fig.1.1), such as vagus nerve activation (Wang et al., 2002; Wang et al, 2003; Goehler et al., 2008; de Lartigue et al., 2011), immune activation (Sternberg et al., 2006; Dantzer et al., 2008) and production of metabolites with neuroactive properties, like short chain fatty acids (Gundersen and Blendy, 2009; MacFabe et al., 2011; Thomas et al., 2012). In addition, bacteria synthetize many neurotransmitters and neuromodulators that are also active in the nervous system, such as GABA, noradrenalin, serotonin, dopamine and acetylcholine (Lyte, 2011; Barrett et al., 2012).

Therefore, it could be hypothesized that an altered composition or activity of intestinal microbiota could interfere with the proper neurological development, directly or through epigenetic processes after birth and/or in the early years of life.

1.4 Animal models for microbiota-gut-brain interaction in Autism Spectrum Disorders

Animal models have been used to collect information about physiological pathways that could actually be involved in a possible microbiota-gut-brain interaction in ASD.

In fact, mice kept from birth without the intestinal bacterial flora show increased motor activity and a reduced state of anxiety associated with an alteration in the expression of genes involved in intercellular communication processes and long-term synaptic potentiation in specific brain regions also involved in ASD (frontal cortex, striatum, amygdala, and hippocampus). Interestingly, the exposure of these mice to microorganisms of the intestinal microbiota within a certain time window immediately after birth leads to behavior manifestation that are similar to those of the control organisms (Sudo et al., 2004; Heijtz et al., 2011).

Furthermore, the intraventricular administration of short chain fatty acids (one of the products of the intestinal bacterial metabolism) in mice is likely to affect cognition and social behavior: two of the most compromised areas in ASD (Shultz et al., 2008; MacFabe et al., 2011).

In addition, in mouse models known to show characteristics related to ASD (MIA-mice) the administration of *Bacteroides fragilis*, a commensal bacteria of the gut flora, has allowed the restoration of a proper intestinal permeability, the change in microbial composition and the improvement of communication deficits and stereotypic behavior shown by this type of mice (Hsiao et al., 2013).

It has also been demonstrated that the chronic administration of the probiotic *Lactobacillus rhamnosus* in mice leads to a modification in the expression of the receptors of GABA, a neurotransmitter with inhibitory function in certain brain areas also involved in autism spectrum disorders (Bravo et al., 2011).

These studies endorse the hypothesis of a possible role for the microbiota in the neurodevelopment and seem to open new scenarios for the development of therapies based on the administration of probiotics to prevent the onset of ASD.

1.5 Previous studies on microbiota in ASD: many critical aspects

Following these promising results showed by animal models, some research based on the analysis of fecal samples of ASD subjects have been conducted. These studies seem to indicate a microbiota composition partially different in individuals with ASD compared to typically developing controls. Unfortunately, these findings are not only highly contrasting but also affected by several methodological problems regarding the characteristics of the participants and the lack of consideration for aspects that can influence microbiota composition (Mayer et al., 2014).

In fact, Parracho at al. found an increased presence of *Clostridia* species in an ASD group (N=58) compared to siblings (N=12) and to typically developing controls (N=10). Moreover, siblings showed a bacterial flora composition intermediate compared to those in healthy and ASD subjects, indicating that environmental factors, such as diet and living conditions, and the genetics of each subject can have an impact on the intestinal bacterial population (Parracho et al, 2005). Nevertheless, this study presents some critical aspects, such as the small number of siblings and controls and the participants heterogeneity in terms of age, sex, gastrointestinal problems, diet and antibiotic/probiotic intake.

In another study, also Finegold et al. compared three groups, but with even less participants: 33 ASD children, 8 siblings and 7 controls. In this case, increased *Bacteroides* and decreased *Firmicutes* were highlighted. Additionally, *Desulfovibrio* species and *Bacteroides vulgatus* were present in higher number in ASD. Again, this study presents different male:female ratio in the groups, some participants were on special diets or were taking antifungal agents, and there is no regard to the presence of gastrointestinal symptoms (Finegold et al., 2010).

Similarly, De Angelis et al. found lower *Firmicutes* and higher *Bacteroides* in an ASD group (N=10) compared to 10 controls. Also in this case, the sample size is small and there is a different male:female ratio in the groups. In addition, ASD subjects with gastrointestinal symptoms had been excluded from the study (De Angelis et al., 2013).

Williams et al., instead, compared a group of 15 male ASD children with gastrointestinal problems and 7 male controls also with gastrointestinal problems and showed, differently from the previous studies, lower levels of *Bacteroides* and higher ratio of *Clostridia* to *Bacteroides* and *Firmicutes* to *Bacteroides* in ASD. Despite the attempt to control some variables such as sex and gastrointestinal problems, the number of participants is still exiguous (Williams et al., 2011).

Furthermore, Wang et al. found an increased presence of *Sutterella* species and a decreased relative abundance of *Bifidobacterium* in an ASD group (N=23), differently from siblings (N=22) and controls (N=9). Again, there is a different male:female ratio in the groups and sample size between ASD/siblings and controls. Moreover, even if information about gastrointestinal problems has been

collected, no distinction has been made between children taking probiotics/antibiotics or according to different dietary habits (Wang et al.,2011; Wang et al. 2013).

Adams et al. compared 58 children with ASD and 39 typically developing children of similar ages. The ASD group showed lower levels of *Bifidobacter* species and higher levels of *Lactobacillus* species, whereas other bacteria had similar levels. Although the number of subjects involved in this study is higher and a particular attention for gastrointestinal problems has been payed, there is a wide age range, some children took probiotics and no information about diet is provided (Adams et al., 2011).

In contrast, Kang et al. highlighted a less diversity in gut microbiota in ASD (N=20) vs. a control group (N=20). Also in this case, the participants are heterogeneous in terms of sex, age, gastrointestinal symptoms, diet, and probiotics/prebiotics intake (Kang et al., 2013).

To conclude, Gondalia at al. compared 28 ASD with gastrointestinal problems, 23 ASD without gastrointestinal problems and 53 sibling control. They did not find any meaningful differences between groups. Nevertheless, it must be considered the differences among the participants in terms of age, sex, probiotic use and the absence of formal dietary assessment.

Furthermore, also the techniques used to analyze gut microbiota in these studies are worthy of a consideration: all of them focus on bacterial genome in order to provide information on microbiota composition but are far from describing functional activity, which could play a key role, instead. For this purpose, metaproteomics techniques could open a more intriguing scenario (Xiong W. et al., 2015; Zahng et al., 2016).

In addition, the results of these studies are not put in association with the microbiota of the family members, with their eating habits and/or gastrointestinal disorders in order to better assess genetic and environmental influences.

In the studies conducted so far, however, there is also another critical aspect: Autism Spectrum Disorders are treated as a single disease, whereas they are a complex phenomenon that includes highly different cognitive levels and different severity of autistic symptoms.. This could mean that the association between ASD and microbiota may be valid for certain subgroups of individuals and not for others. Therefore, this could be an additional possible explanation for the mixed results achieved so far.

It seems clear that there is the need for further studies that try to better control the so many variables implicated in the possible relationship between microbiota and ASD and that allow to collect enough information in order to identify different subgroups among ASD. This could also lead to a better understanding of the molecular mechanism underlying this complex pathology.

1.6 Hypothesis and aims

Considering all the aspects discussed above, the central hypothesis that has guided this research project is that gut microbiota could play a role in Autism Spectrum Disorders.
In order to address this hypothesis the aims of this work have been:

1. to develop an interview to collect information about factors that influence gut microbiota development and its present composition;
2. to compare ASD children and typically developing children for these factors;
3. to compare ASD children themselves for these factors, taken into consideration at the same time also level of functioning as well as severity of autistic traits, in order to identify possible subgroups and also to follow the onset, if present, of both gastrointestinal disorders and food selectivity in relation to the onset of autistic symptoms;
4. to typify microbiota activity of ASD families (two biological parents, an ASD child and a typically developing sibling) with regard to the above mentioned factors, as well as to shared environment and genetics, in order to find out a possible biomarker for the development of ASD, or at least for a subgroup.

Another more clinical aim of this work has been to explore parents' difficulties in managing mealtime with their ASD children to collect information useful for planning future support interventions.

CHAPTER 2: A NEW INTERVIEW to PARENTS

2.1 Introduction

According to the literature, numerous different factors can theoretically exert an influence on gut microbiota development. Nevertheless, previous studies on gut microbiota in ASD focused on typifying its composition without controlling these possible confounding factors sufficiently: this could be a possible explanation for the contrasting results provided so far (Mayer et al., 2014).
Thus, first step of my work has been the development of an interview to parents that could allow to collect easily this kind of information.
In this chapter, the main factors that can have an influence on gut microbiota will be described together with the development of the dedicated sections of the interview. Also, all questions will be presented in details.

2.2 Method

2.2.1 Interview development

General information about parents and prenatal factors

Different studies report the association between ASD and some risk factors, such as the age of parents, especially fathers, and aspects related to the prenatal period. In fact, advanced parental age seems to contribute to altered methylation in gametes due to increased oxidative stress causing DNA damage and fragmentation (Menezo et al., 2015).
Others factors associated with autism risk are maternal bleeding, gestational diabetes and being first born vs. third or later. Instead, factors with evidence against a role in autism risk include previous fetal loss and maternal hypertension, preeclampsia, and swelling (Gardener et al., 2009).
Also medicine intake during pregnancy seems to be involved in ASD: valproic acid, thalidomide, and antidepressants (specifically selective serotonin reuptake inhibitors), especially during the first trimester of pregnancy, has been associated with an increased risk of ASD in the child (Croen et al., 2011; Christensen et al., 2013). However, these results should be carefully interpreted because of the difficulty to isolate medication-related effects from those of the mother's underlying condition that may also influence autism risk (Lyall et al., 2014).

Therefore, the effect of smoke and alcohol during pregnancy on the onset of ASD is still under debate (Tang et al., 2015).

Thus, questions about these topics were inserted in this interview, even if they seem not to be directly connected to the microbiota of the child. In fact, they could be important anyway in order to identify subgroups with similar risk factors among ASD children.

Moreover, some of these factors could have affected the mother's microbiota during pregnancy (e.g. antibiotics intake, particular food choices, prebiotics/probiotics intake etc...) and possibly its metabolites, exerting in this way an effect on the fetus through the placental circulation.

Also a question about parents' present occupation was included because it can be related to their educational level (a higher parents' educational level has been associated with an increased diagnosis rate of ASD in the child – Van Meter et al., 2010) and also with the perceived stress level in managing mealtime with the ASD child, which is explored in another section of this interview.

In this section there is also a question about where the child has been living after birth. Since environment has a role in microbiota development, it may be assumed that a child who experienced often moving in the first years of life could have been exposed to different environmental microbes and this could have played a role in shaping his/her microbiota.

GENERAL INFORMATION

- parents' age;
- parents'occupation;
- parents' place of birth;
- child's place of birth and where he/she has lived;
- child's date of birth;
- other children and their dates of birth.

PREGNANCY**:**

- general information about pregnancy course:
    - problems (e.g. gestational diabetes?);
    - mother's diseases/infections;
- use of medicines (which and for how long);
- special dietary attention;
- positivity to toxoplasmosis;
- use of backing soda to wash vegetables and fruits;
- supplements intake;
- food intolerances not present previously;

- consumption of alcohol;
- smoke;
- previous miscarriages (causes).

Delivery

Microbiota starts to develop during delivery, when the baby is colonized by strains of the maternal intestinal and vaginal flora (Dominguez-Bello et al., 2010).
To date literature indicates that there are meaningful differences in gut microbiota composition in babies born through C-section compared to vaginal delivery (Mueller et al., 2015) and that babies born through C-section and swabbed soon after birth with maternal vaginal microbes show a partially restoration of the microbiome they had missed (Dominguez-Bello et al., 2016).
Regarding ASD, the role of C-section is still under debate. In fact, although some works showed an association, a new study has found that this association did not persist when using typically developing sibling controls, implying that familial genetic and/or environmental factors could play a role in the incidence of C-section, independently of the subsequent onset of ASD or not (Curran et al., 2015).
Thus, not only a questions about the type of delivery was put in the interview but also about the reasons for a C-section, if any, and also about possible difficulties happened during vaginal delivery. Also, other perinatal factors, such as being preterm, small for gestational age and low Apgar index have been highlighted as possible autism risk factors (Schieve et al., 2014).
Also a question about medicine intake (e.g. antibiotics) in the perinatal period was included, hypothesizing a possible effect on the microbiota development of the baby and possibly on the composition of mother's milk.
Furthermore, also neonatal jaundice has been associated with Autism Spectrum Disorders (Amin et al., 2011).

BIRTH:

- mode of delivery:
    - vaginal: problems (e.g. water breaking, breech birth, baby stuck between subsequent contractions, umbilical cord around the neck,...)
    - caesarean section: reasons (convenience or due to particular problems)
- preterm birth:
    - at how many weeks?

    - incubator for how long?
    - medicine administered?
- child's weight and length;
- Apgar index;
- jaundice;
- infections (for both the mother and the child) and treatments.

Lactation, weaning and introduction of solid foods

The development of a healthy microbiota strongly depends on the neonatal and early childhood period (Koshaleva et al., 2016). For instance, the gut microbiota of formula-fed children differs from that of breast-fed infants (Bezirtzoglou et al., 2011).
Later, significant changes in the gut microbiota occurred after weaning (Koening et al., 2011; Bergstrom et al., 2014) and between the second and third year of life an adult-like microbiota is established (Fallani et al., 2010). Nutritional habits play an important role in this process: for instance, a diet rich in polysaccharides promotes the presence of bacteria able to fermenting them (De Filippo et al., 2010). Nevertheless, a healthy microbiota establishment is crucial because about 60% to 70% of microbiota bacteria remain stable for the whole life (Faith et al., 2013; Raijlic-Stojanovic et al., 2013).
Moreover, the risk of ASD was found to be increased by the late initiation of breast-feeding, a non-intake of colostrum, prelacteal feeding, and bottle-feeding and to be decreased by longer periods of exclusive breast-feeding and continued breast-feeding (Al-Farsi et al., 2012).
Thus, this interview section was structured to be able to collect this information.
Also a question about the brand of formula milk, if any, was included: that could allow to control its actual composition.
For all 3 parts of baby nutrition development reported below, a question about gastrointestinal disorders was added, in order to study the onset and development of these disorders, if present, and also to possibly associate them with particular food, revealing in this way possible intolerances. Furthermore, in these sections there is also a question about supplementations because of the possible role of prebiotics/probiotics on microbiota composition.
Also characteristics of sleep and crying of the babies are addressed: both these aspects could be affected by problems in digesting some foods or by gastrointestinal problems, sometimes even not expressed in other ways.

In the section “Lactation”, questions about mother‘s disease and medicine intake during lactation are present because of their possible consequences on breast milk composition (directly and even through an influence on mother’s microbiota).
Moreover, also the topic “smoke” is addressed. In fact, a study has shown that mothers who smoked 15 or more cigarettes a day were twice as likely to have babies with colic (Reijneveld et al., 2005) and smoke seems to be also associated with baby’s sleep disturbance (Mennella et al., 2007).
In the sections “Weaning” and “Introduction of solid foods” there is a question about how the child accepted the new tastes and consistencies. It is well know that children with ASD have restricted interests and food selectivity (as described later in this chapter), thus this question allows to explore when this aspects, if present, started to affect the child nutrition.
In the section “Weaning” also a question about the entry to the nursery was included because it represents a big change in the child’s everyday life.

LACTATION

- mode:
    - breast feeding:
        - since when? Colostrum intake?
        - for how long exclusive breast milk?
        - for how long breast milk in total (even after having started weaning)?;
    - formula (brand):
        - reasons for introduction;
        - integration: proportion, since when and for how long;
        - only formula: since when and for how long;
- growth trend;
- supplements intake (e.g. vitamins, fluorine);
- gastrointestinal problems (reflux, regurgitation, colic, ...): for how long?
- cry characteristics before, during and after feeding;
- sleep characteristics;
- mother’s diseases and medicines intake;
- smoke.

WEANING

- age;

- duration;
- difficulty in accepting new tastes/textures?;
- growth trend;
- supplements intake;
- food intolerances/allergies;
- gastrointestinal problems;
- behavioral problems, especially after eating certain foods / after the meal;
- cry characteristics before, during and after the meal;
- sleep characteristics (since when slept all night long?);
- nursery (specify age)

INTRODUCTION of SOLID FOODS

- age;
- duration (since when started eating like the parents?);
- difficulty in accepting new tastes/textures?
- growth trend;
- supplements intake;
- food allergies/intollerances;
- gastrointestinal problems;
- behavioral problems, especially after eating certain foods / after the meal;
- cry characteristics before, during and after the meal;
- sleep characteristics;

Current diet

30% to 40% of adults' gut microbiota can be modified during the lifetime, and diet is one of the most powerful factors (Kashtanova et al., 2016).
In this scenario it is important to consider the remarkable incidence of food selectivity among autistic children (Bandini et al., 2010), because of its possible effect on microbiota and also on gastrointestinal disorders, although indirectly.
To date, it has been hypothesized that food selectivity could be the end effect of an altered sensory sensitivity or a form of restricted and repetitive patterns of behavior (Suarez et al., 2014).

On the other hand, food selectivity could result from gastrointestinal discomfort experienced in the past. This aversion could also be generalized to a broader food category in the years (Buie et al., 2010).

Since a children food frequency questionnaire validated in Italy is not yet available, a new one was created specifically for this research.

Thus, common foods in children's diet were selected and grouped on the basis of their composition affinity, with special attention to fibers content, given the well-known role of the microbiota in the fibers metabolism and the role as prebiotics of some types of fibers. Regarding meat, fish and potatoes, a special category was dedicated to the fried preparation, because of the especially high content of fat and its possible influence on microbiota.

For each food item, also a standard portion was provided to facilitate the quantification by the parents. ("Reference assumption levels of nutrients and energy for the Italian population-IV Revision "- "Livelli di Assunzione di Riferimento di Nutrienti ed energia per la popolazione italiana- IV Revisione", 2014).

However, also a question was included about other foods eaten by the child but not present in the questionnaire: this should allow not to miss possible strange food choices, which are not likely to be excluded, considering the tendency of ASD children to have sometimes strange interests (American Psychiatric Association, 2013).

In order to better assess food selectivity, for each item that parents categorized as not eaten by the child, it is asked if it is actually eaten by the rest of the family or if it is a kind of food the child could have not came in contact with because of parents' food choices, for example, but that he/she would eat outside the family.

Parents are also asked about the tendency of the child to try easily new foods and to selects foods on the basis of some characteristics like shape, color, consistency, presentation according to some previous studies (Bandini et al., 2010; Postorino et al., 2015).

In addition, there is also a question about pica, since this practice is sometimes associated with ASD (Kinell, 1985): depending on what is eaten, it can have an influence on gastrointestinal conditions and gut microbiota.

In this section also the highly debated topic of special diets is explored.

In fact, diets gluten-/lactose- and / or casein-free have been associated with reduced gastrointestinal disorders and with improvements of autistic symptoms (Knivsberg et al., 2002).

But according to the experts, such diets should be followed only in presence of a confirmed intolerance / allergy, like for typically developing children (Buie et al., 2010).

Even the Linea Guida "Il trattamento dei disturbi dello spettro autistico nei bambini e negli adolescenti" (Guideline "The treatment of autism spectrum disorders in children and adolescents", Istituto Superiore di Sanità, 2012) agrees with this approach.
Moreover, the wrong adoption of such diets could only add discomfort to the child. Indeed, it must be considered for example the difficulties of a parent who deprives her child of a very welcomed food like pasta and who should manage the oppositional reaction of the child, with whom the father already has difficulties to communicate. Another problematic topic is nutrition at school: here the child with ASD, already considered different, is also forced to follow a different diet compared to his/her schoolmates.
In addition, it is also asked if the child eats in a hasty way and if he can regulate his-/herself on quantities. Both these aspects may have an effect on the subsequent digestion of food.
At the end of this section, again information about possible notable child's behavior after particular foods and about the quality of sleep is requested.

CURRENT DIET

- special diet (ovo-lacto-vegetarian, vegan, lactose free, casein free, gluten free or a combination of them)
    - If YES:
        a. since when?
        b. for how long?
        c. why (if on medical advice, specify the type of doctor; if as a result of clinical examinations, specify the type of exam)
        d. changes in the child's behavior
        e. reasons for having stopped the diet

- Weight and height of the child at the moment
- Impressions on the amount of eaten food (too much/a proper amount/ few)
- Hasty swallowing of food
- Ability to self-regulate the amount of eaten food

- Food items (portion and frequency)
  (for each item not eaten by the child verify if the reason is a child's food choice or if the parents usually do not present that food to the child):

Food item	Reference serving	N° servings /week
Pasta and rice (not whole-grain)	1medium serving (80g) ½ serving if in soup	
Pasta and rice (whole-grain), spelt, barley	1medium serving (80g) ½ serving if in soup	
Pizza	1 piece (200 g)	
Bread (not whole-grain)	1 little “rosetta” (little Italian bread) 1 medium slice (50 g)	
Bread (whole-grain)	1 little “rosetta” (little Italian bread) 1 medium slice (50 g)	
Crackers, breadsticks, rusks	1 crackers serving, 2,5 rusks	
Meat (hamburger included)	1 little slice (70g)	
“Cotoletta” (Italian breaded meat)/Cordon bleu	1 little slice/1 piece	
Ham	3-4 medium slices (50g)	
Sausages	5 slices salami, 2 slices “bologna”, ½ wurstel (50 g)	
Fish	1 little slice (100g)	
Breaded fish	1 little slice (100g)	
Cheese	1 medium serving (fresh 100 g, hard 50g)	
Uova	1 egg	
Soy	1 medium serving (80-120g)	
Soy products (tofu, tempeh, seitan, vegetarian hamburger....)	1 medium serving (70g)	
Other legumes (peas, beans, chickpeas, lentils ...)	1 medium serving (80-120g, cooked)	

French fries and chips	2 small potatoes (200g) or 50 g chips	
Potatoes (other preparation, including gnocchi)	2 small potatoes (200g)	
Total vegetables (excluding potatoes)	----------	
Cabbage (cauliflower, cabbage, broccoli, Brussels sprouts …)	1 medium serving (125 g)	
Salads (all types) and spinach/chard	1 medium serving salad (50g) or spinach/chard (200g, cooked)	
Carrot	1 medium carrot (75 g)	
Peppers, eggplants, zucchini, cucumbers	1 medium seving (150 g)	
Tomatoes	1 medium serving (150 g)	
Vegetable soup	1 plate	
Garlic and onion	2 cloves of garlic, 1 medium onion	
Totale fruits	----------	
Apple and pear	1 medium fruit (150 g)	
Kiwi	2 pieces (150 g)	
Banana	1 medium fruit (150 g)	
Citrus (including citrus juices)	1 medium fruit (150 g)	
Summer friuts (strawberries, cherries, melon ...)	1 medium serving (150 g)	
Nuts (walnuts, hazelnuts, peanuts ...)	3 pieces	
Milk	1 glass (125 ml)	
Soy milk	1 glass (125 ml)	
Yogurt (specify type and brand _____)	1 cup (125g)	
Cakes, cookies, sweet snacks	2-4 coockies / 1 snack	

Cereals	1 serving (30 g)	
Jam, Nutella	1 tea spoon (3g)	
Ice cream, pudding	2 scoops of ice cream (100g) o 1 pudding	
Candies	1 piece	
Chocolate	2 pieces/1 Kinder bar (12,5 g)	
Fruit juices	1 small bottle (125 ml)	
Sweet beverages (Coca-Cola, thè)	1 glass (125 ml)	
Tea and herb teas	1 glass (125 ml)	
Sugar	1 tea spoon (3g)	
Honey	1 tea spoon (3g)	
Oil	1 little spoon (10 g)	
Butter	1 serving (10g)	

- Food items not listed above but usually eaten by the child (serving and frequency)
- Different food choices at school
- Tendency to try new foods
- Food selectivity (shape, colors, texture, presentation, brand)
- Pica (type e frequency)
- Daily amount of drunk water
- Probiotics/ prebiotics/ vitamin supplements intake (type and frequency)
- Problems behavior after having eaten some foods
- Sleep quality

Gastrointestinal disorders

Many individuals with ASD are also subjected to gastrointestinal problems (Parracho et al., 2005).

Moreover, it has been highlighted a possible difficulty of diagnosis: many, in fact, cannot express pain or discomfort through verbal and/or nonverbal channels. Nevertheless, numerous behaviors, apparently disconnected, could instead be indirect symptoms of gastrointestinal disorders (Buie et al., 2010).

Considering the possible association between gastrointestinal problems, microbiota, food selectivity and maybe different cognitive-behavioral ASD phenotypes, numerous questions were included to assess presence, development and characteristics of gastrointestinal problems. In particular, one question is about gastrointestinal problems in conjunction with stress events, in order to better distinguish the etiology of the disorders.

For gastrointestinal disorders, reference was made to the Rome's III criteria (Drossman D.A. and Dumitrascu D.L., 2006)

GASTROINTESTINAL DISORDERS

- sphincter control;
- gastrointestinal disorders:
  - dysphagia (liquid? solid?);
  - gastroesophageal reflux or regurgitation;
  - gastritis;
  - early satiety;
  - vomiting;
  - recurrent abdominal pain;
  - flatulence or bloating;
  - diarrhea (> 3 times daily) (ask for frequency if diarrhea attributed to intestinal viruses at kindergarten);
  - constipation (< 3 times per week);
  - mixed disorder (diarrhea and constipation together);
  - stools with foul smell;
  - infections.

- if gastrointestinal disorders:
  - time of onset;
  - evolution;
  - frequency;
  - appearance after having eaten specific foods;

    - appearance in case of specific events.

- previous gastroenterological assessments:
    - when;
    - medical exams undertaken;
    - diagnosis;
    - therapy.

- specific behaviors, possible sign of gastrointestinal discomfort (especially in no speaking children):
    - lethargy;
    - pressing hands/objects into abdomen;
    - special sensitivity when touched on the abdomen;
    - grimaces;
    - teeth grinding;
    - biting clothes;
    - often clearing throat;
    - frequent swallowing;
    - tics;
    - sobbing for no apparent reason;
    - echolalia related to pain;
    - seemingly inexplicable increase in repetitive / stereotypic behaviors;
    - restlessness, motor agitation;
    - screams;
    - aggressive/self-injurious behaviors.

Vaccinations

There is a hot debate about a possible role for vaccinations in Autism Spectrum Disorders. Nevertheless, the official position of the scientific community is against this hypothesis.
This interview section not only collects information about possible adverse reactions of the child against the vaccines, but also tries to assess if these vaccination procedures could have had effects on microbiota and its development, showed by the child possibly through a change in gastrointestinal habits or through other reactions, dermatitis for example.

VACCINATIONS

- which and at how many months;
- discomfort;
- gastrointestinal problems;
- behavioral changes.

Diseases and medicine intake

Microbiota can also be influenced by medicines intake. In particular, ASD subjects seem to be exposed to antibiotic therapies more frequently than typically developing children due to several health problems (especially bronchitis and otitis). This fact has been put in relation to a reduction of the intestinal commensal flora and to the proliferation of potentially pathogenic microorganisms, able to produce neurotoxins, which reach in mice models the CNS via vagus nerve (Bolte, 1998). In this context, the administration of vancomycin, an antibiotic effective against this type of bacteria, seems to register an improvement of autistic symptoms, suggesting that the intestinal microflora may have a role in autism (Sandler et al., 2000).
Therefore, this interview section allows to collect information about diseases and hospitalization of the child and related medicines intake (especially antibiotics), with special attention for the first 3 years of life, given the importance of this timespan for microbiota development, as already discussed before. Possible changes in gastrointestinal habits and behavioral symptoms as consequences of these health problems are also assessed.

DISEASES

- diseases and medicines intake:
    - typical children diseases (e.g. varicella): when?
    - infections (otitis, bronchitis, flu): occurrence, especially in the first 3 years of life
    - intestinal viruses: events (vomiting, diarrhea ..) and recurrence

- hospitalizations: when, for how long, treatments / therapies
- changes in gastrointestinal disorders related to diseases and hospitalizations;
- changes in the behavioral phenotype in relation to diseases and hospitalizations;
- dermatitis;

- allergies;
- current intake of medicines.

Other information

There are some other information that can be useful to better evaluate microbiota composition.
In fact, emerging literature show how living with pets can shape its composition (Fujimura et al., 2013). Thus, also a question about this topic is present, with particular attention to animals with whom the child could have spent time in the first period of life.
Therefore, individuals that live together tend to have a more similar microbiota. It must be considered, in fact, that there are multiple ways in which bacteria can pass form a person to another one, e.g. saliva, skinn contact etc. (Kashtanova et al., 2016). With the aim of studying similarities in microbiota composition among families, it could be helpful to know the time rate the child usually spends with each parent.
Finally, a question about genetics tests already conducted was included, since literature evidences indicate how individual genetics shape microbiota (Goodrich et al., 2014). This kind of information is also useful to identify subgroups among ASD children.

OTHER INFORMATION

- genetics tests (when and why);
- pets at home (in particular since the first period of life);
- mutual contamination in the family between parents and children (time spent with each parent).

Other children

In order to better study the microbiota composition of ASD subjects it can be useful to extend the analysis also to siblings. In this way it is possible to better evaluate the effect of genetic proximity and shared environment.
Therefore, a section is especially dedicated to explore the presence of gastrointestinal problems and also food selectivity in siblings.

OTHER CHILDREN

Meaningful elements related to eating habits and possible gastrointestinal disorders in siblings of the ASD child (if interesting aspects, go in depth following the same questions already used for the ASD child).

Information about parents and their families

Since it seem probable that alterations in the immune system are related to ASD (Matelski and Van de Watere, 2016), some questions are included about the incidence of some autoimmune diseases in the family (not only parents but also grandparents or even brothers and sisters), about allergies or intolerances and about the presence of gastrointestinal disorders and their origin.
The idea is that an alteration in immune system function can have been acquired by the child from the parents. Thus, having allergic parents could represent a risk factor for ASD.
Moreover, these information are also important again for better evaluate microbiota composition if the analysis is extended also to parents.

INFORMATION ABOUT PARENTS/THEIR FAMILIES:

- diseases:
    - celiac disease;
    - Type I diabetes;
    - rheumatoid arthritis
    - Hashimoto's thyroiditis (autoimmune hypothyroidism)
    - Systemic Lupus Erythematosus (LES)
    - Multiple Sclerosis
    - Fibromyalgia
- allergies (food, seasonal, medicines, other substances...)
- persistent gastrointestinal disorders (diagnosis and treatment, mother in particular);
- sporadic gastrointestinal disorders (e.g. in stressful occasions).

2.2.2 Participants

The interview was administrated to a first group of parents of 10 ASD children and to parents of 10 typically developing children. All 10 ASD children were patients of the Laboratory of Observation Diagnosis and Education, University of Trento.

The parents of typically developing children were recruited among acquaintances whose children matched with the ASD children for gender and age range (Tab. 2.1).

Group	Size	Age (average + SD)	Age (minimum)	Age (maximum)	Male	Female
ASD	10	7.98 ± 3.33 years	3 years	10.08 years	7	3
TD	10	7.78 ± 3.23 years	3.5 years	11.67 years	8	2

Tab.2.1: Participants' age and gender.

2.2.3 Interview procedure

The first contact with the parents of ASD children took place in person. On this occasion the purpose of this study was explained to the parents and it was agreed on when to meet to carry out the interview. All interviews were conducted at the Laboratory while the child was attending an intervention section. Instead, contact to parents of typically developing children was took by phone and interviews took place at parents' home, in order to make it more convenient for them.
All interviews were recorded after having obtained the permit from the parents.
During the interviews, questions were proofed for their understandability to the parents. It was also payed attention to the questions order, so that it allowed to conduct the interview like a fluent talk and that the parents could feel comfortable.

2.3 Results

Talking with the parents made clear that some factors had to be addressed with more precise and in depth questions in order to really acquire the information needed. For example, in a couple of interviews parents mentioned that their child had started to reduce the number of eaten foods after a first period in which he was used to eating a large variety of different foods. Therefore, a special question about this topic was added at the end of the section "Introduction of solid foods" ("reduction in the number of foods after initial varied diet"). Moreover, with regard to intestinal viruses, after the first interviews it became clear the need to explore this topic with particular attention in order to distinguish between pure infections and recurrent gastrointestinal problems. In fact, some parents have the tendency to attribute recurrent episodes of diarrhea to common viruses, whereas it seemed

more probable that they were actual gastrointestinal problems, considering the high frequency of these episodes.

In addition, some parents of ASD children told us about their difficulties in managing the food choices of their ASD children and the mealtime in general. In fact, food selectivity is part of the everyday management of feedings problems in ASD and is a challenge for the families (Dominick et al., 2007), since problematic mealtimes and a negative impact on dietary habits of other members of the family have been reported (Curtin et al., 2015). Moreover, parents of children with ASD and food selectivity tend to show higher levels of parenting stress (Postorino et al., 2015) and even spousal stress (Curtin et al., 2015).

Thus, a section dedicated to this topic was inserted at the end of the interview with the idea to use this information in the future to plan possible support interventions.

In this way it is also possible to collect information about strategies parents use with their children, for example to get them sited or to bring them to eat some food they do not like. These practices could be shared with other parents in the future.

Also a question about the nutrition at school was included, considering that it could be potentially a source of concerns for the parents, especially in the case of children with high food selectivity.

PSYCHOLOGICAL EXPERINCE RELATED TO THE MEALTIME WITH THE ASD CHILD

- meal as a stressful event;
- concerns related to the child's diet / to the mealtime;
- agreement between the parents about food choices for the child or conflicts;
- parents' diet conformed to that of the child, and related feelings;
- rituals;
- eating at school: organization and related concerns.

To conlcude, this interview presents the minimum set of questions that has to be asked. In fact, given the extreme variety of answers that parents gave to some of these questions during the first 20 interviews (such as diseases the child had or stress they felt in managing the mealtime), it is obviously necessary to improvise time by time new questions during the interview in order to follow parents' speech and obtain richer data.

This is one of the reasons why the use of an interview seems more appropriate than a questionnaire.

And it is also the reason why the required time for the interview can vary: it generally ranges between 45 minutes and 2 hours. As expected, interviews with parents of ASD children tended to be longer, especially for the cases with gastrointestinal problems and/or food selectivity.
Another important point is to ask parents to bring also the “libretto pediatrico” (pediatric notebook), because of the difficulties to remember some information in detail.

2.4 Conclusion

I strongly believe that it is essential to establish an alliance with the family in order to explore such complex and intimate topics, where facts and personal representations of what is right, normal, and healthy interweave. This covenant, offered by an interviewer interested not only to the mere data but also open to listen to the doubts of the parents, their effort and discomfort, allows on one hand to get richer data for studies on microbiota and on the other hand to gather information about parents' needs, useful to design support interventions. This interview wishes to be a valid tool for these purposes.

CHAPTER 3: LOOKING FOR DIFFERENCES

3.1 Introduction

In the last two decades an increasing number of studies on Autism Spectrum Disorders has been focusing on a possible role for gut microbiota in this pathology. In fact, it seems plausible in light of the high incidence of gastrointestinal disorders among ASD children (Buie et al., 2010) and considering the lack of a well-recognized genetic basis (Betancur et al., 2011): all this suggests to take into consideration also environmental factors that might act as a trigger for ASD in susceptible individuals.

Unfortunately, different attempts towards a definitive typing of gut microbiota in ASD children did not help to make progress in solving the puzzle: they provided, instead, highly contrasting results (Mayer et al., 2014).

This could mean that microbiota is not involved in Autism Spectrum Disorders, and that the gastrointestinal disorders, which many people suffer by, are the result of psychological distress caused by the difficulties in communication and social interaction that are typical for these disorders.

But on the other hand it is now well recognized that there is a system of bidirectional connections between gut and brain, where the microbiota seems to play an important role: thus, an alteration in microbiota composition or activity could indeed affect the central nervous system, as it has been demonstrating in the case of depression (Cryan et al, 2012).

It could therefore be assumed that the mixed results achieved so far are rather attributable to some methodological problems, such as not properly considering factors that may affect the microbiota such as medical and nutritional history of the subject. These factors have been described in depth previously in Chapter 2 of this thesis.

In addition, the studies conducted so far tend to consider ASD as a unitary disease, while they include different cognitive levels and autistic symptoms severity. Thus, it is possible that microbiota may play a role not for all subjects with ASD but only for a subgroup.

Following these considerations, it becomes clear the need to conduct this kind of studies on microbiota and ASD involving only well characterized subjects both cognitively and with respect to the severity of autistic symptoms, as well as collecting at the same time information on the various factors that can influence the microbiota: all this in order to better control the different variables in play.

Therefore, the aim of this second part of my work has been to assess possible differences in factors that can affect microbiota in ASD children compared to typically developing children and among

ASD children themselves, considering their differences in cognitive level and severity of autistic traits.

3.2 Method

3.2.1 Participants

Parents of 24 children with ASD and parents of 18 typically developing children were involved in this study.

19 of these ASD children were patients of the Laboratory of Observation, Diagnosis and Education of the University of Trento, Italy. The majority of them was followed not only for the diagnostic process but also for the subsequent intervention that sometimes lasts over several years. The others of them, instead, have been recruited among those who came to the Laboratory only to receive a diagnosis or a revaluation of previous diagnosis, made by the Laboratory itself or by other facilities within the Italian territory. The families' satisfaction for the activity of the Laboratory has allowed to have a good adhesion to the present study and to establish immediately a good relationship with the researcher based on the trust of the parents.

In all these cases, children were subjected to an assessment of autistic symptoms by psychologists well experienced in the use of the elective instrument for this purpose, the Autism Diagnostic Observation Schedule - Second Edition (ADOS, Lord et al., 2001).

This tool also allows to assess the gravity of autistic symptoms by matching the ADOS scores with four levels of increasing severity according to the following scale (Tab. 3.1):

Gravity	Minimum-no evidence		Low		Moderate			High		
ADOS score	1	2	3	4	5	6	7	8	9	10

Tab. 3.1: Correspondence between ADOS score and gravity level of autistic symptoms.

Furthermore, all children were subjected to an assessment of their cognitive profile through intelligence scales, such as The Leiter International Performance Scale, Third Edition (Leiter-3,

Leiter, 1940) and The Wechsler Intelligence Scale for Children (WISC, Wechsler, 2003), and / or through The Griffith Mental Development Scales (GMDS, Griffith, 2006) that assesses the quotient of development of the child, especially useful in the case of children with deficits in verbal communication, that for this reason cannot be assessed through the other intelligence scales. Regardless of the tool used for the assessment, it comes to high-functioning ASD in presence of IQ equal to or higher than 70 and to low-functioning ASD for IQ below 70.

Finally, 1 family were recruited thanks to its participation in a summer camp for children with ASD organized by the Laboratory of Observation, Diagnosis and Education, whose name is "Terapia in vacanza" (Therapy on holiday). In this case, reference was made to the evaluation documents submitted by the parents at the time of enrolling for the summer camp.

The following table summarizes cognitive level and symptoms gravity of the ASD participants (Tab.3.2):

Cognitive level		**Symptoms gravity**		
High	Low	Low	Medium	High
11	13	10	10	4
Tot. subjects = 24		Tot. subjects = 24		

Tab. 3.2: Participants' cognitive level and gravity of autistic symptoms.

With regard to the group of parents of typically developing children, friends, acquaintances and acquaintances of acquaintances who had children similar to the group of ASD children by age and gender were asked to take part into this study.

The following table displays the general characteristics of the ASD group and of the typical developing (TD) group (Tab.3.3):

Group	**Size**	**Age (average + SD)**	**Age (minimum)**	**Age (maximum)**	**Male**	**Female**
ASD	24	8.04 ± 3.32 years	2.92 years	13.92 years	20	4
TD	18	8.18± 3.36 years	2.92 years	13.67 years	15	3

Tab. 3.3: Age and gender of ASD- and TD group.

3.2.2 Interview

Especially for this study, an interview was set up in order to collect information about different factors that can affect microbiota development and its actual composition.
The creation process of this tool has already been described in Chapter 2 of this thesis.
The first contact between the researcher and the families took place in person in the lab or at the summer camp "Terapia in Vacanza" or by phone. On this occasion it was explained to the parents the purpose of this study and it was agreed on when to meet to carry out the interview.
Regarding the parents of children with ASD, interviews were conducted in the Laboratory or at the facility that housed the summer camp.
Instead, interviews with parents of typically developing children took place at their home, in order to make it more convenient for them.
All interviews were recorded after having obtained the permit from the parents.

3.2.3 Data analysis

According to the contents of the interview and the information about ASD children's IQ and autistic symptoms gravity, several variables were analyzed. Following table summarize the main features of these variables, presenting categorical and numerical variables separately (Tab. 3.4 and 3.5)

Categorical variables	
Name	***Levels***
Type of delivery	0 = vaginal 1 = caesarian
Lactation	0= maternal only 1= maternal+formula 2= formula only
Shift to a restricted diet between 2-2,5 years of age	0 = no 1 = yes
Special diets	0 = no 1 = yes
Pica	0 = no 1 = yes
No self-regulation of amount of eaten food	0 = no 1 = yes
Hasty swallowing of food	0 = no 1 = yes
Gastrointestinal disorders	0 = no 1 = yes

Parents' persistent gastrointestinal disorders	0 = no	1 = yes
Grandparents' persistent gastrointestinal disorders	0 = no	1 = yes
Child's food intolerances/allergies	0 = no	1 = yes
Child's other intolerances/allergies	0 = no	1 = yes
Parent' food intolerances/allergies	0 = no	1 = yes
Parents' milk intolerance/allergy	0 = no	1 = yes
Child's other intolerances/allergies	0 = no	1 = yes
Grandparents' food intolerances/allergies	0 = no	1 = yes
Grandparents' other intolerances/allergies	0 = no	1 = yes
Autoimmune disease in the family	0 = no	1 = yes
Celiac disease in the family	0 = no	1 = yes
Mealtime as a stressful experience	0 = no	1 = yes
Level of cognitive function	0 = high	1 = low

Tab. 3.4: Categorical variables (name and levels)

Numerical variables	
Name	***Unit of measure***
Maternal age at child birth	years
Paternal age at child birth	years
Duration of exclusively breast feeding	months
Total duration of breast feeding (even after weaning)	months
Food selectivity	percentage of refused foods
ADOS score	integer (range: 1 to 10)

Tab. 3.5: Numerical variables (with unit of measure).

Regarding IQ values, cognitive functioning levels (high or low) were preferred instead of IQ scores, since children had been assessed using different tools. In fact, the correspondence between high functioning level for IQ scores equal or above 70 and low functioning for IQ scores below 70 is valid anyway.

Regarding statistical analysis, a model was designed to identify variables that might predispose to the development of ASD. Since having this pathology or not is a categorical variable on two levels, the model was tested through a logistic regression analysis and a stepwise backward procedure with the R software.

The same was performed with respect to the variables that might discriminate between ASD- and typically developing children but without having a predisposing effect.

Moreover, the incidence of parents' perceived stress related to mealtime was tested for statistical significance using Fisher's exact test.

Subsequently, only the group of children with ASD was considered and further analysis were conducted in order to find differences between children with high and low cognitive level. Again, one model for predisposing variables and one model for discriminating variables were tested with a logistic regression analysis and a stepwise backward procedure using R software.

Finally, the relationship between ADOS scores as indicator of symptoms severity and possible predisposing/discriminating variables was assessed. In this case, since ADOS score are a numerical variable, a multiple regression analysis and subsequent stepwise backward procedure were performed.

3.3 Results: ASD vs TD

In this section, a possible relationship between interview's variables and being part of the ASD- or the TD group is assessed.

First, the results of the interviews are presented. Then, logistic regression analysis on possible explanation models are described. Finally, the incidence of stress at mealtime perceived by parents is reported.

3.3.1 Description of interviews results

Parental age at child's birth

Considering that advanced parental age, especially of the father, has been highlighted as possible risk factor for ASD through methylation pathways (Menezo et al., 2015), mothers' and fathers' age at child's birth were assessed separately between ASD- and TD group (Tab. 3.6)

Group	Father age (average + SD)	Mother age (average + SD)
ASD	34.29 ± 4.62 years	31.79 ± 4.39 years
TD	34.61 ± 4.27 years	31.94 ± 3.89 years

Tab. 3.5: Mothers' and fathers' age at child's birth

Delivery

Since microbiota starts to develop during delivery, differences in the incidence of C-section and vaginal section between ASD children and typically developing children were hypothesized.
A higher incidence of C-section among ASD children was found.

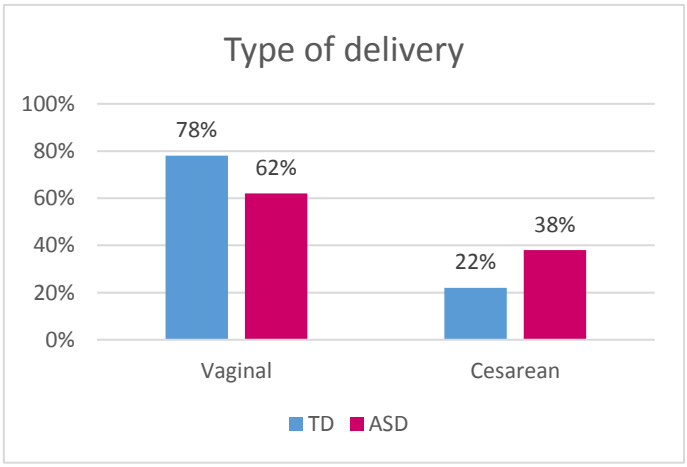


Fig. 3.1: Type of delivery

Breast feeding

So far, there are some evidences about increased ASD risk and suboptimal breast-feeding practices (Al-Farsi et al., 2012). Therefore, ASD- and TD group were compared for different type of lactation (Fig. 3.2). A higher incidence of formula milk combined with breast milk was found among ASD children. Furthermore, only 1 ASD child received formula milk exclusively.

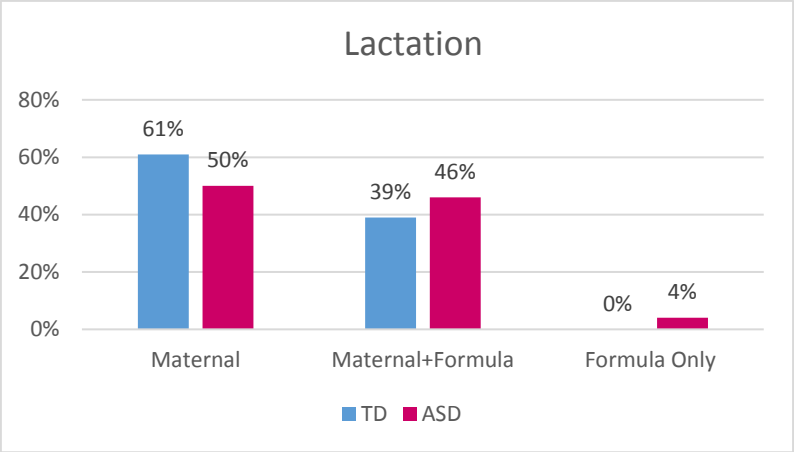


Fig. 3.2: Type of lactation

In addition given the importance of breast feeding for microbiota development, also possible differences between ASD- and TD children regarding the duration of exclusive breast feeding and the total duration of breast feeding, eventually also together with formula or during weaning, were assessed. Comparing the two groups, average duration of breast feeding (both exclusive and total) seems almost similar (Fig 3.3).

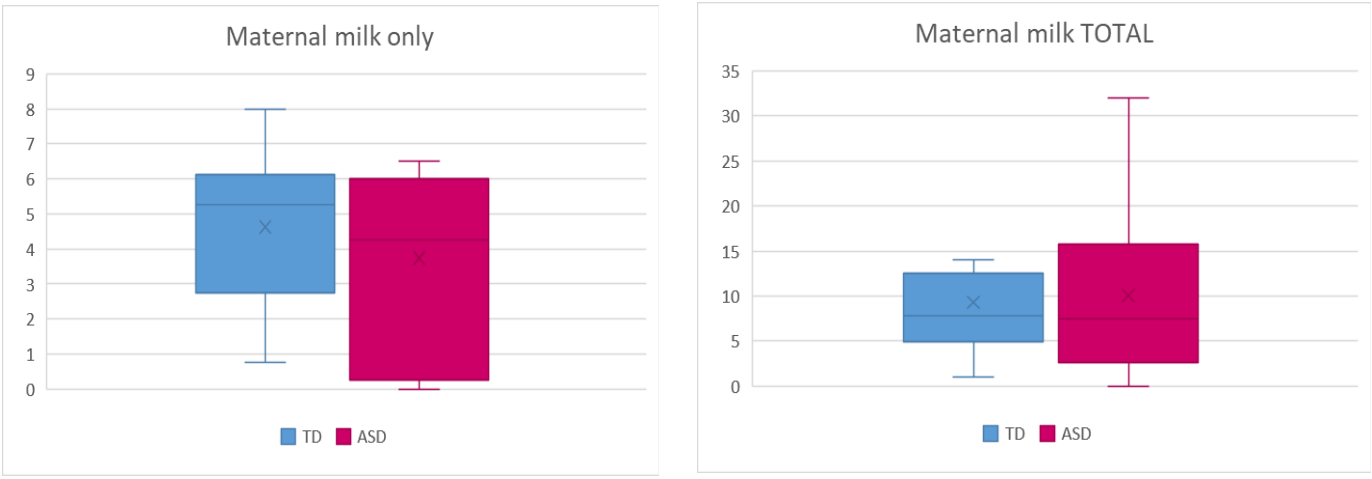


Fig. 3.3: Duration of exclusive breast feeding and of total breast feeding (in months).

Food selectivity

Food selectivity was measured as percentage of refused foods out of the list provided in the interview.

ASD children showed a higher selectivity: this result is in line with the current literature as discussed previously in this thesis. Moreover, some parents reported that their child was less selective at school compared to at home, in an almost similar percentage in both groups (Fig. 3.4)

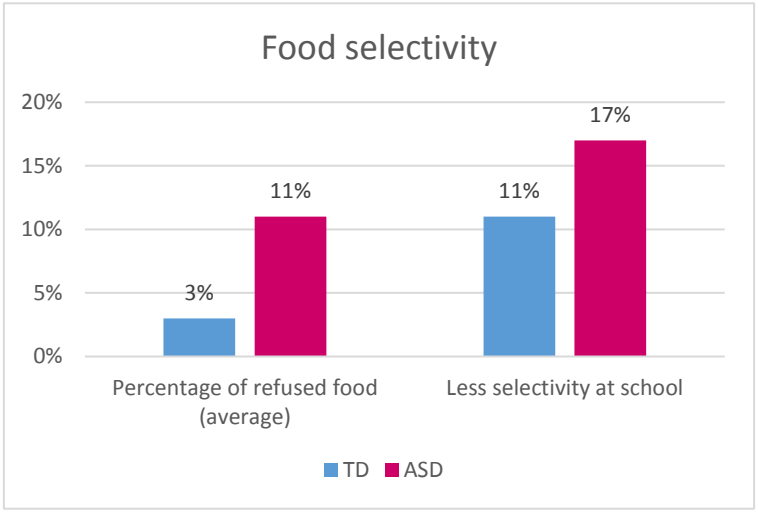


Fig. 3.4: Percentage of refused foods at home and incidence of being less selective at school.

Therefore parents of 6 ASD children reported a new interesting observation: their children had started to eat everything and then they had switched to a more and more restricted diet. This happened between 2 and 2,5 years of age. None of the parents of typically developing children referred a similar experience (Fig. 3.5).

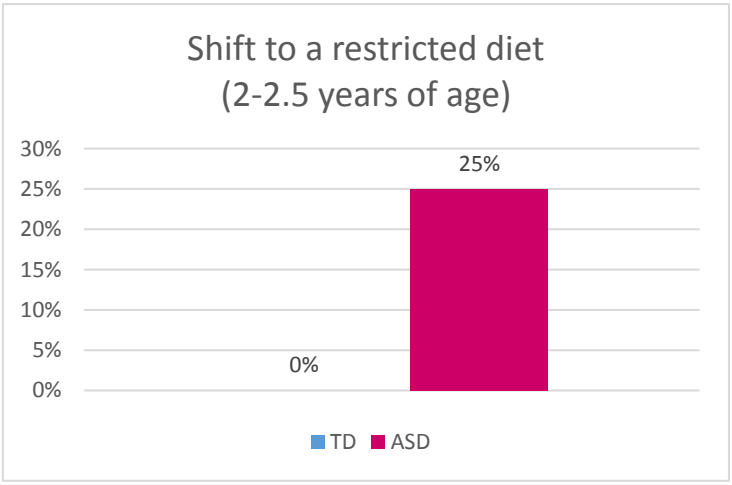


Fig. 3.5: Shift to a restricted diet between 2 and 2,5 years of age

Self-regulation of the amount of eaten food

According to the interviews, ASD children were more often reported as not able to regulate themselves in the amount of eaten food compared to the typically developing children. This aspect might be related to gastrointestinal disorders (Fig. 3.6).

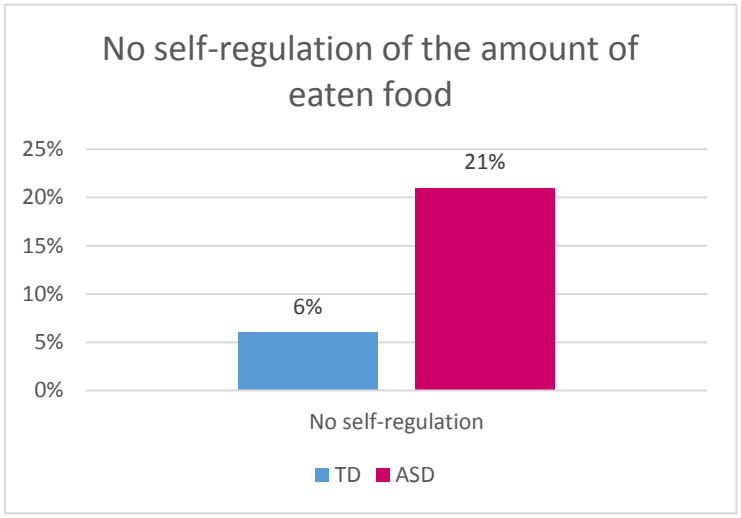


Fig. 3.6: No self-regulation of the amount of eaten food.

Hasty swallowing of food

Also the tendency to swallow food hastily might be involved in gastrointestinal disorders. Again, the incidence was higher in the ASD group (3.7):

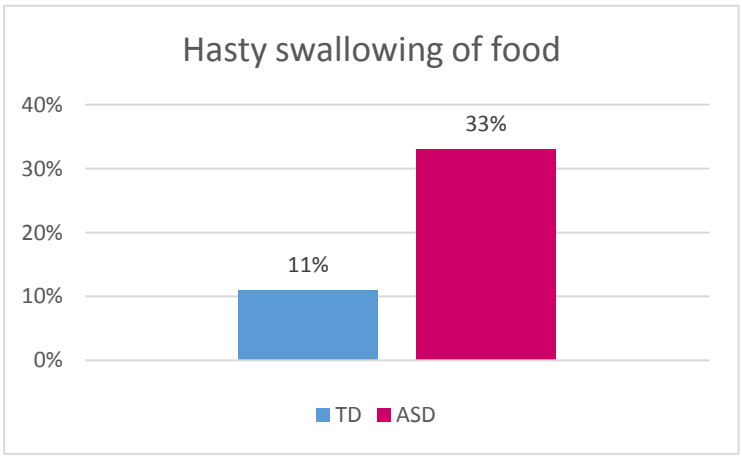


Fig. 3.7: Hasty swallowing of food.

Special diets

4 ASD children were on a gluten-, casein free diet and 3 are on a diet without milk (30% all together). All these children suffered from gastrointestinal problems and the parents reported an improvement in gastrointestinal symptoms, but only 2 of them reported also an improvement in behavioral aspects that could be related to ASD.

Moreover, 3 other families tried a gluten-, casein free diet in the past but stopped it soon after because the child had lost too much weight, or because this kind of diet was too complicated to follow or because they had not seen any results on behavioral level.

None of TD children was on a special diet (Fig. 3.8)

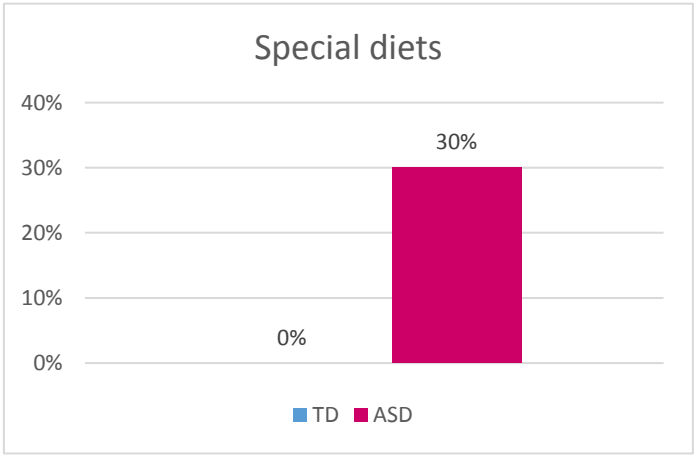


Fig. 3.8.: Special diets

Pica

An incidence of 8 % of pica among the ASD group (2 children) was reported, whereas none of the TD children showed this kind of behavior (Fig. 3.9).

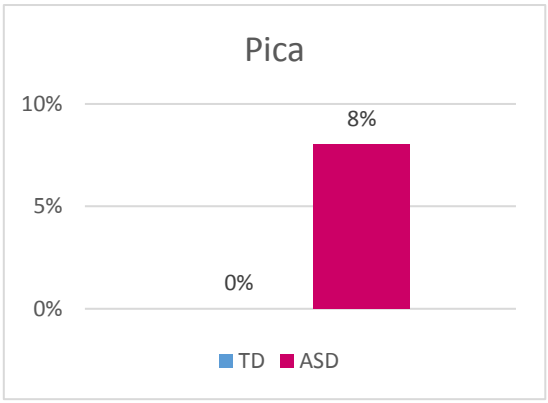


Fig. 3.9: Pica

Gastrointestinal disorders

A considerable incidence of gastrointestinal problems among ASD children was found, as expected according to the literature. Again, none of the TD children suffered from gastrointestinal disorders (Fig. 3.10).

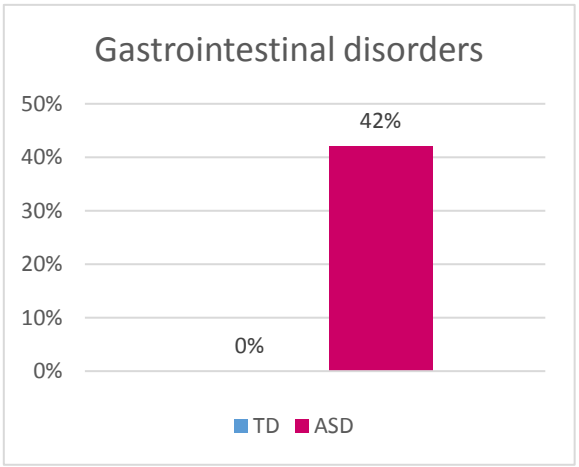


Fig. 3.10: Gastrointestinal disorders in ASD and TD children

Moreover, also differences in persistent gastrointestinal disorders among parents and grandparents of ASD children and TD children were assessed, in order to find a possible genetic predisposition.
A higher incidence was found in the ASD group, especially with regard to the grandparents (Fig. 3.11).

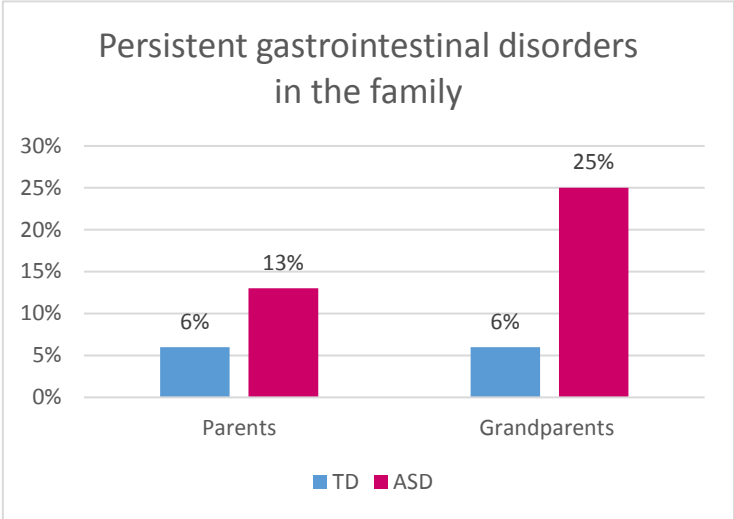


Fig. 3.11: Gastrointestinal disorders in the family.

Immunological aspects

Considering the well-known role of gut microbiota in the immune system development and their continuous mutual interaction, and the reported abnormalities at immune level in ASD (as discussed in Chapter 1), ASD- and TD group were compared for some aspects that could be related to the immune system.

In particular, a higher incidence of food intolerances/allergies was found in the ASD group, whereas the opposite for other types of intolerances/allergies (Fig. 3.12).

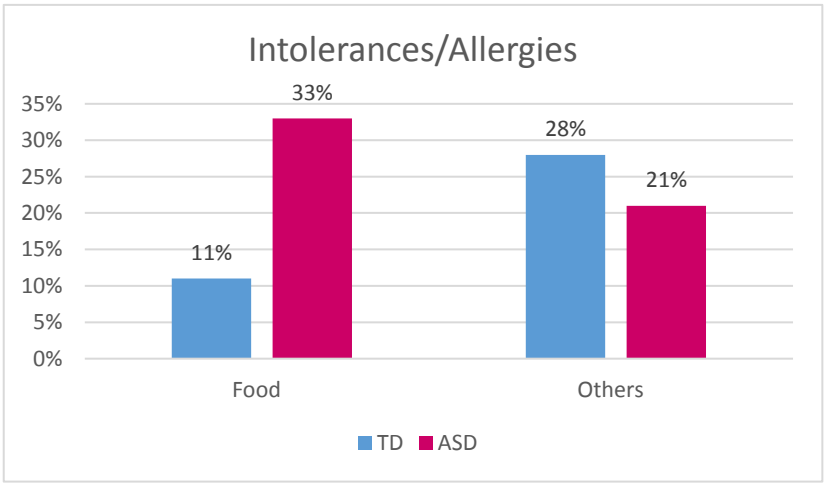


Fig. 3.12: Intolerances/allergies.

Moreover, the incidence of dermatitis was slightly higher in ASD children (Fig. 3.13).

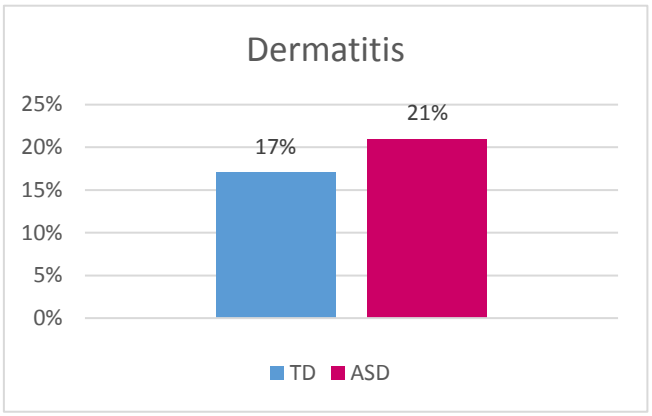


Fig. 3.13: Incidence of dermatitis.

In addition, also parents and grandparents were compared for the incidence of allergies, looking for possible genetic predisposition.

Interestingly, parents of ASD showed a higher incidence of food intolerances/allergies, especially for milk, whereas parents of TD children had a higher incidence of other types of allergies not related to food (Fig. 3.14).

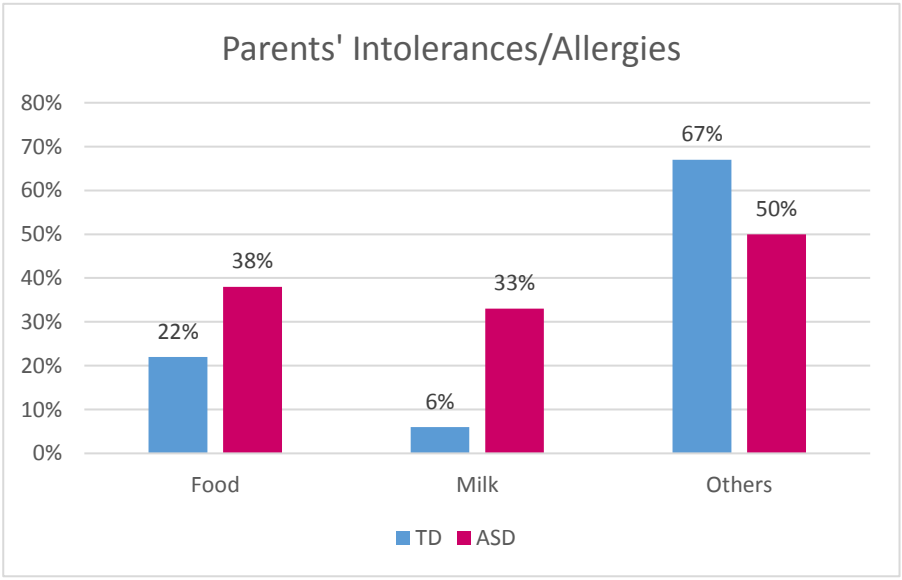


Fig. 3.14: Parents' intolerances/allergies.

Opposite results were obtained for grandparents (Fig. 3.15)

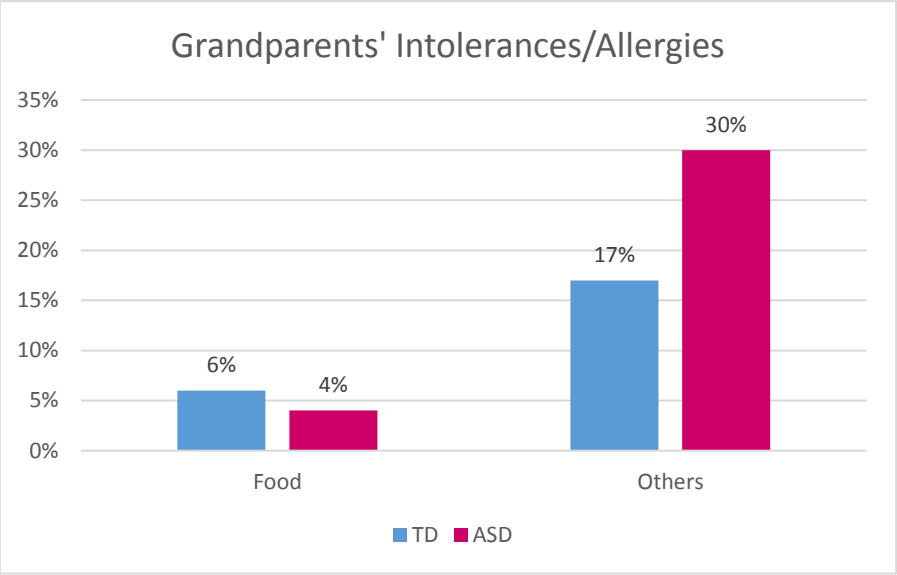


Fig. 3.15: Grandparents' intolerances/allergies.

Finally, a higher incidence of some autoimmune diseases in the family (celiac disease, type I diabetes, rheumatoid arthritis, Hashimoto's thyroiditis, systemic lupus erythematosus, multiple sclerosis, fibromyalgia) was found in the TD group.
Interestingly, even the incidence of celiac disease was higher in the TD group compared to the ASD group (Fig. 3.16).

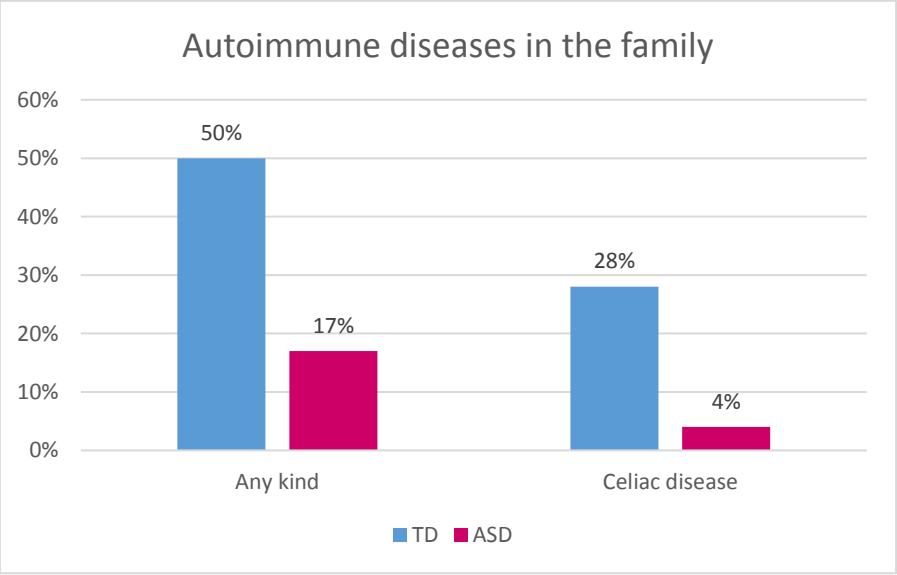


Fig. 3.16: Autoimmune diseases in the family.

3.3.2 Logistic regression with predisposing variables

Some of the above described variables have been reported as possible predisposing factors for ASD, as previously discussed in this thesis. Moreover, some are known also for having an effect on microbiota development. In addition, some aspects related to the family of ASD children (parents and grandparents) could perhaps represent a further risk factor, being sign of alterations in gastrointestinal and/or immune system. In this case, the reason for these alterations could be related to the genetics of the subjects but also to microbiota, as discussed before.

Since having ASD or not is a categorical variable on two levels, a logistic regression analysis on a model made of these supposed predisposing variables was performed.

Considering the features of this kind of analysis, variables having levels with only 1 or 0 occurrences were not included in the model.

Following table shows the list of predisposing variables and specifies which ones were not included in the model (Tab. 3.6)

Predisposing variables	
Maternal age at child's birth	
Paternal age at child's birth	
Type of delivery	
Lactation	Not included
Duration of exclusive breast feeding	
Total duration of breast feeding	
Parents' persistent gastrointestinal disorders	Not included
Grandparents' persistent gastrointestinal disorders	Not included
Parents' food intolerances/allergies	
Parents' milk intolerance/allergy	Not included
Grandparents' food intolerances/allergies	Not included
Parents' others allergies	
Grandparents other allergies	
Autoimmune diseases in the family	
Celiac disease	Not included

Tab. 3.6: Possible predisposing variables for ASD vs TD.

Using R software, following logistic regression model output was obtained (Tab. 3.7):

	Estimate	**Std. Error**	**z value**	**p-value**
(Intercept)	8.15749	4.76402	1.712	0.0868
Maternal age at child's birth	0.07183	0.14670	-0.490	0.6244
Paternal age at child birth	-0.16381	0.15026	-1.090	0.2756
Delivery 1	2.26341	1.35033	1.676	0.0937
Exclusive breast feeding	-0.23072	0.18108	-1.274	0.2026
Total breast feeding	0.11070	0.07197	1.538	0.1240
Parents' food allergies 1	2.13772	1.22418	1.746.	0.0808
Parents' other allergies	-1.22800	1.00764	-1.219	0.2230
Grandparents other allergies	0.83889	1.02279	0.820	0.4121
Autoimmune diseases1	-3.10546	1.33106	-2.333	0.0196
Null deviance: 57.364	Residual deviance: 39.071		AIC: 59.071	

Tab. 3.7: Logistic regression on a model with predisposing variables.

A stepwise backward procedure on this model were performed with the R software, resulting in a reduced model with 2 variables as follows (Tab. 3.8)

	Deviance	**AIC**
Parents' food intolerances/allergies 1	51.972	55.972
Autoimmune diseases in the family 1	56.216	60.216
Null Deviance: 57.36	Residual Deviance: 46.94	AIC: 52.94

Tab. 3.8: Stepwise backward procedure on logistic regression model.

Hence, a logistic regression analysis conducted on the reduced model provided the following output (Tab. 3.9):

	Estimate	Std.Error	z value	p-value
(Intercept)	0.5149	0.4233	1.216	0.2239
Parents' food allergies 1	2.1325	1.1465	1.860	0.0629
Autoimmune diseases1	-2.7064	1.1190	-2.419	0.0156
Null deviance: 57.364	Residual deviance: 46.945		AIC: 52.945	

Tab. 3.9: Logistic regression on reduced model.

According to these results, the only variables that seem to have a possible predisposing effect are parents' food intolerances/allergies and autoimmune diseases in the family.
In particular, parents' food intolerances/allergies seem more related to having an ASD child, even though the p-value is slightly above the statistical significance (p-value= 0.06).
Instead, a higher incidence of autoimmune diseases in the family seems to favor the opposite condition (p-value=0.015).

3.3.3 Logistic regression with discriminating variables

A similar analysis was performed with respect to variables that might discriminate between ASD- and TD children but without having a predisposing effect.
Also in this case, variables having levels with only 1 or 0 occurrences were not included in the model.
Following table shows the group of the discriminating variables and specifies which ones were not included (Tab. 3.10)

Discriminating variables	
Food selectivity	
Less selectivity at school	
Shift to a restricted diet at 2-2,5 years of age	Not included
No-self regulation of amount of eaten food	Not included
Hasty swallowing of food	
Special diets	Not included
Pica	Not included
Child's gastrointestinal disorders	Not included

Child’s food intolerances/allergies	
Child’s other allergies	
Child’s dermatitis	

Tab. 3.10: Possible discriminating variables for ASD vs TD.

A logistic regression analysis was conducted using R software, resulting as follows(Tab. 3.11):

	Estimate	**Std. Error**	**z value**	**p-value**
(Intercept)	- 0.47219	0.52604	- 0.898	0.369
Food selectivity	0.11382	0.06392	1.781	0.075
Less selectivity at school	- 0.46411	1.29509	- 0.358	0.720
Hasty swallowing of food	0.98504	0.94908	1.038	0.299
Child’s food intolerances/allergies	1.72738	1.05707	1.634	0.102
Child’s other allergies	-1.17665	0.97862	- 1.202	0.229
Child’s dermatitis	- 0.44237	0.98938	- 0.447	0.655
Null deviance: 57.364	Residual deviance: 45.442		AIC: 59.442	

Tab. 3.11: Logistic regression model with discriminating variables for ASD vs TD

Again, a stepwise backward procedure on this model was performed with the R software, resulting in a reduced model with 2 variables as follows (Tab. 3.12)

	Deviance	**AIC**
Child’s food intolerances/allergies 1	51.686	55.686
Food selectivity	54.369	58.369
Null Deviance: 57.36	Residual Deviance: 48.39	AIC: 54.39

Tab. 3.12: Stepwise backward procedure on logistic regression model.

Finally, a logistic regression analysis on the reduced model was performed with this output (Tab. 3.13):

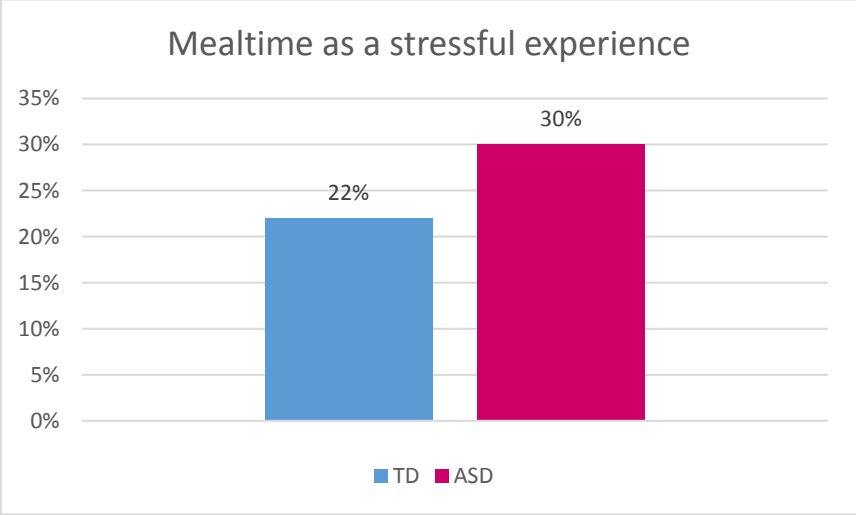
	Estimate	Std.Error	z value	p-value
(Intercept)	0.56257	0.44577	-1.262	0.2069
Child's food intolerances/allergies 1	1.51829	0.89963	1.688	0.0915
Food selectivity	0.09709	0.05342	1.817	0.0692
Null deviance: 57.364	Residual deviance: 48.391		AIC: 54.391	

Tab. 3.13: Logistic regression on reduced model.

According to these results, among the hypothesized discriminating variables only food selectivity seems to be related to the ASD condition, even though its p-value is slightly above the statistical significance (p-value= 0.06). In fact, ASD participants seem to be more selective than TD children. This result is in line with the literature on this topic, previously discussed in Chapter 2.

3.3.4 Mealtime as a stressful experience

Surprisingly, no statistical difference in the incidence of parents' perceived stress related to mealtime was found (Fisher's exact test, odds ratio=1.42, p-value = 0.73, Fig. 3.17).



Tab. 3.17: Incidence of mealtime perceived as a stressful experience by parents.

3.4. Results: comparison between different cognitive level among ASD

In this section, a possible effect of interview's variables on different levels of cognition among ASD children is explored. High functioning level corresponds to IQ equal or above 70, whereas low functioning level to IQ less than 70.

3.4.1. Description of interviews results

Parental age at child's birth

Following charts show the distributions of maternal and paternal age at child's birth, which seem similar in average between high and low cognitive functioning ASD children (Fig. 3.18).

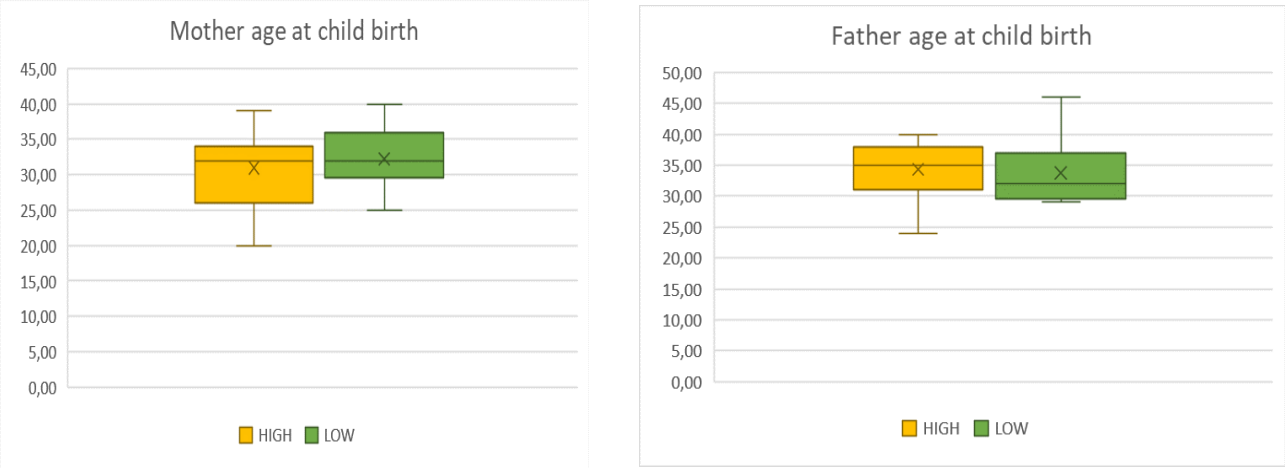


Fig. 3.18: Maternal and paternal age at child's birth.

Type of delivery and lactation

Although according to previous described results, type of delivery and lactation seem not significantly different between ASD- and TD children, it could be hypothesized that they might play an indirect role among ASD subjects in influencing, perhaps through the gut microbiota, some aspects such as cognitive level.

Among the participants, low cognitive level children showed a higher incidence of C-section (Fig. 3.19), whereas a less use of formula milk (Fig.3.20).

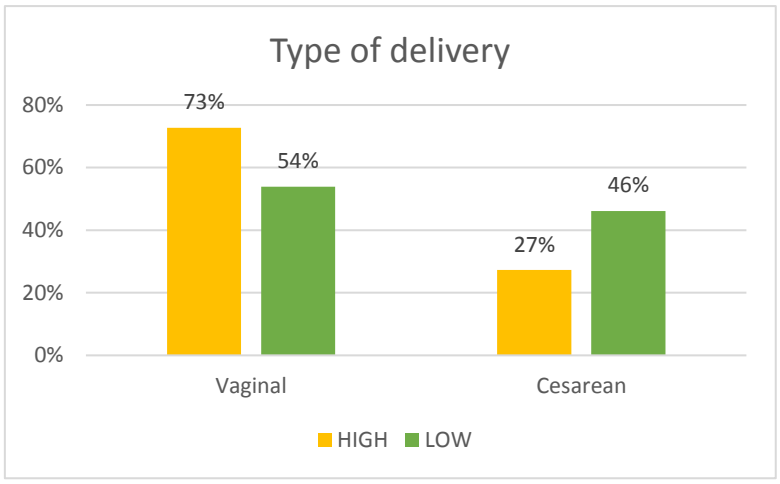


Fig. 3.19: Type of delivery

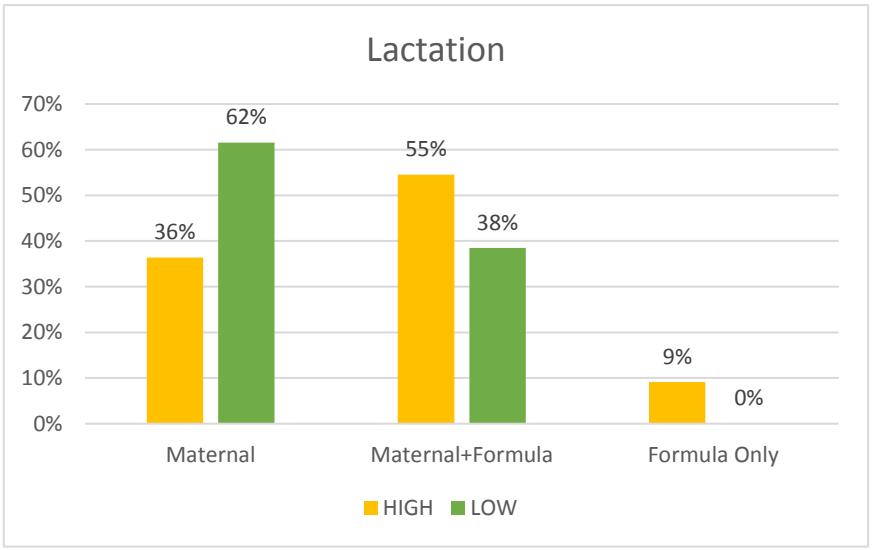


Fig. 3.20: Lactation

Also regarding the actual months of breast feeding, low functioning children received maternal milk for a longer period, both exclusive as well as total (Fig. 3.21).

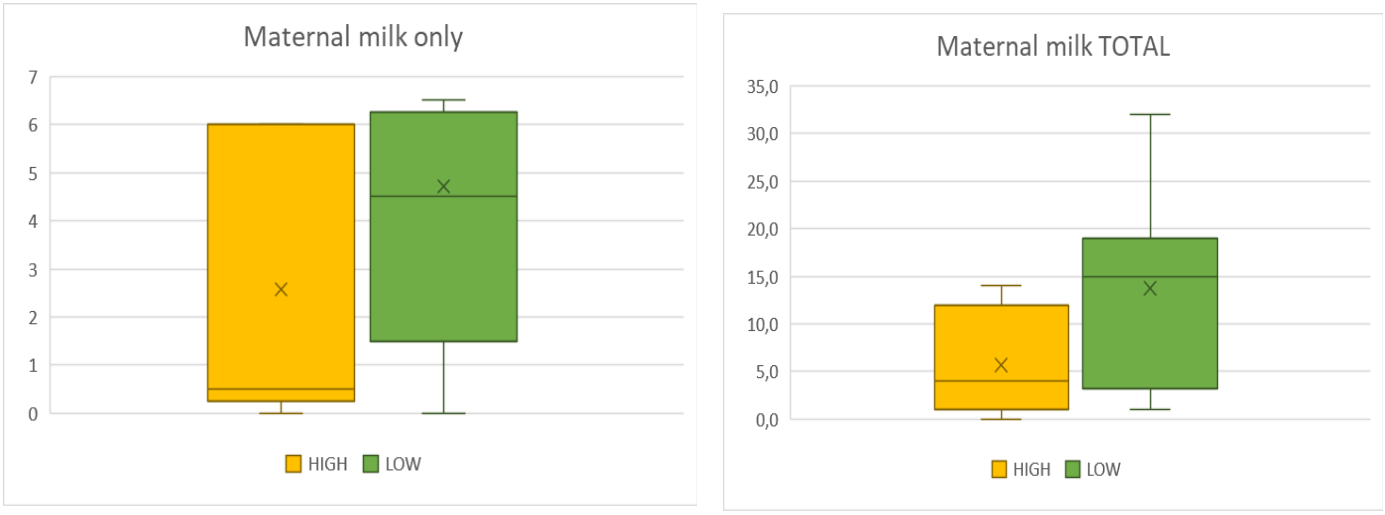


Fig. 3.21: Duration of exclusive breast feeding and duration of total breast feeding (in months).

Food selectivity and other aspects related to nutrition

High functioning children were more selective in their food choices (Fig. 3.22), and none showed pica behavior. Instead, low functioning children had more often the tendency to swallow food hastily (Fig. 3.23).

Regarding less selectivity at school (Fig. 3.22), shift to a restricted diet at 2-2,5 years of age and the difficulty to self- regulate in the amount of eaten food (Fig. 3.24), and the use of special diets (Fig. 3.25), high and low functioning children seemed to be almost similar.

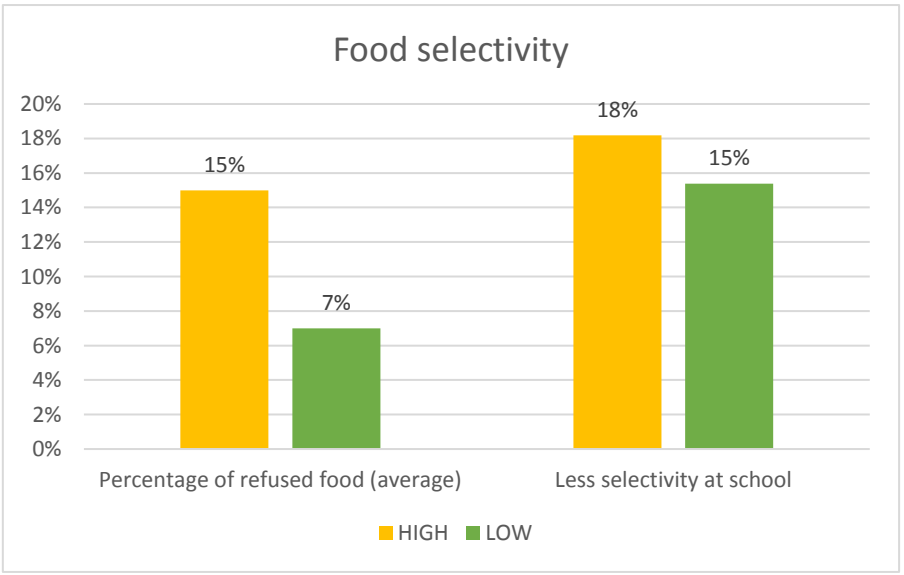


Fig. 3.22: Food selectivity.

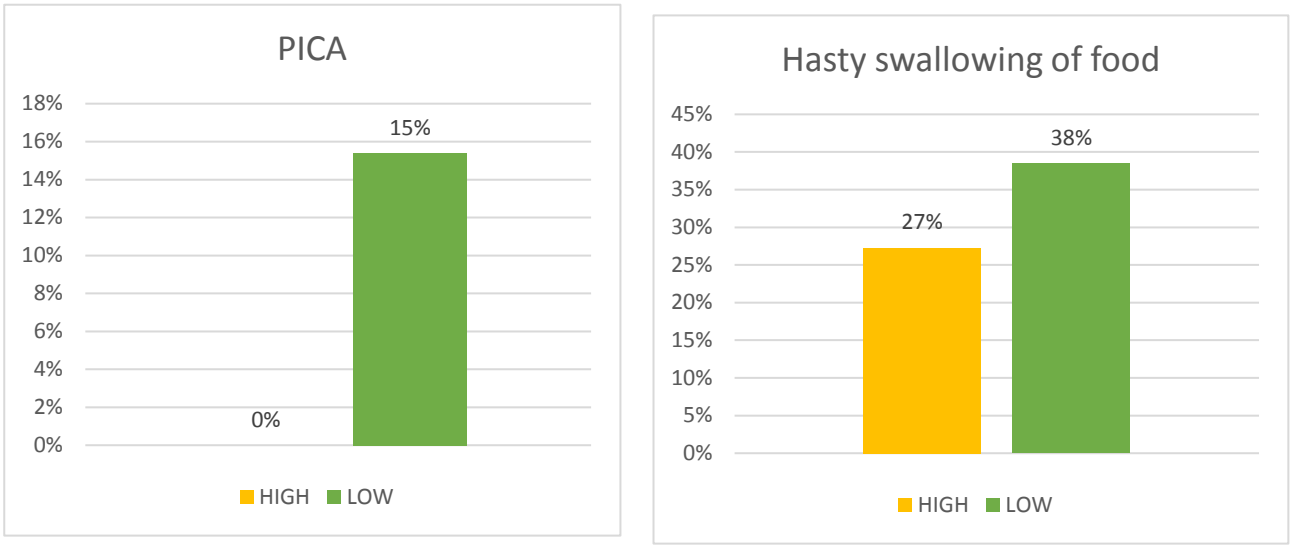


Fig. 3.23: Pica and the tendency of swallowing food in a hasty way.

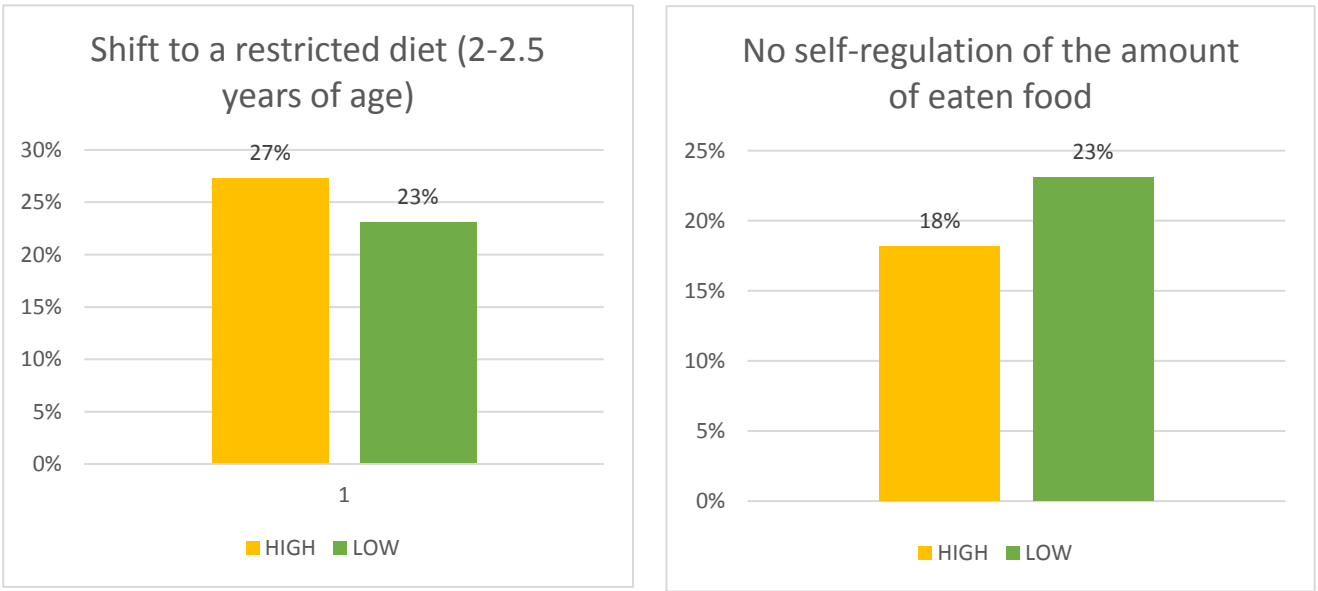


Fig. 3.24: Shift to a restricted diet between 2-2,5 years of age and no self-regulation of the amount of eaten food.

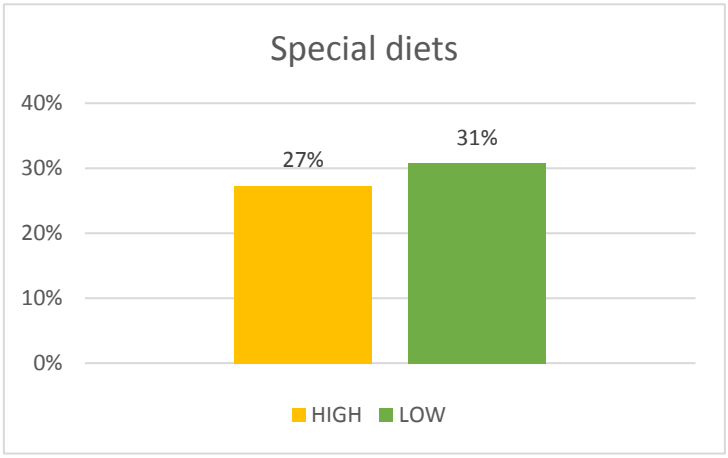


Fig. 3.25: Use of special diets

Gastrointestinal disorders

A higher incidence of gastrointestinal disorders was found in low functioning children and a slightly higher one in their parents. Instead, grandparents of high functioning children were reported to have more often gastrointestinal problems (Fig. 3.26).

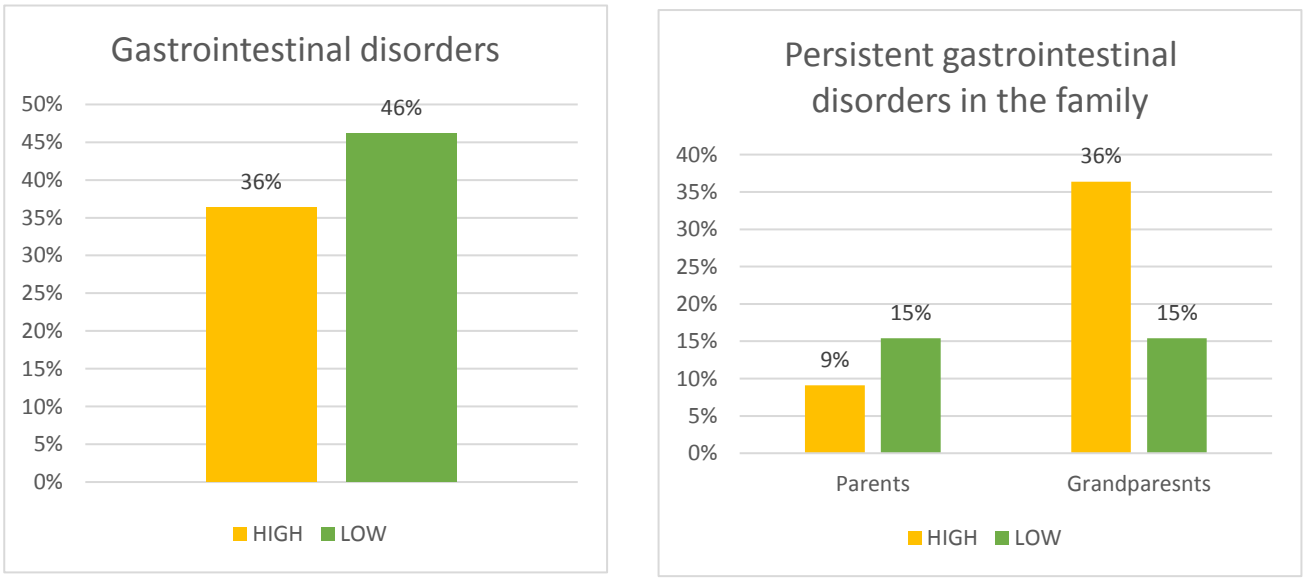


Fig. 3.26: Gastrointestinal disorders in the children (left) and in the family (right).

Immunological aspects

A higher incidence of food intolerances/allergies and dermatitis occurred among high functioning children, whereas other type of allergies are almost similar in the two groups (Fig. 3.27).

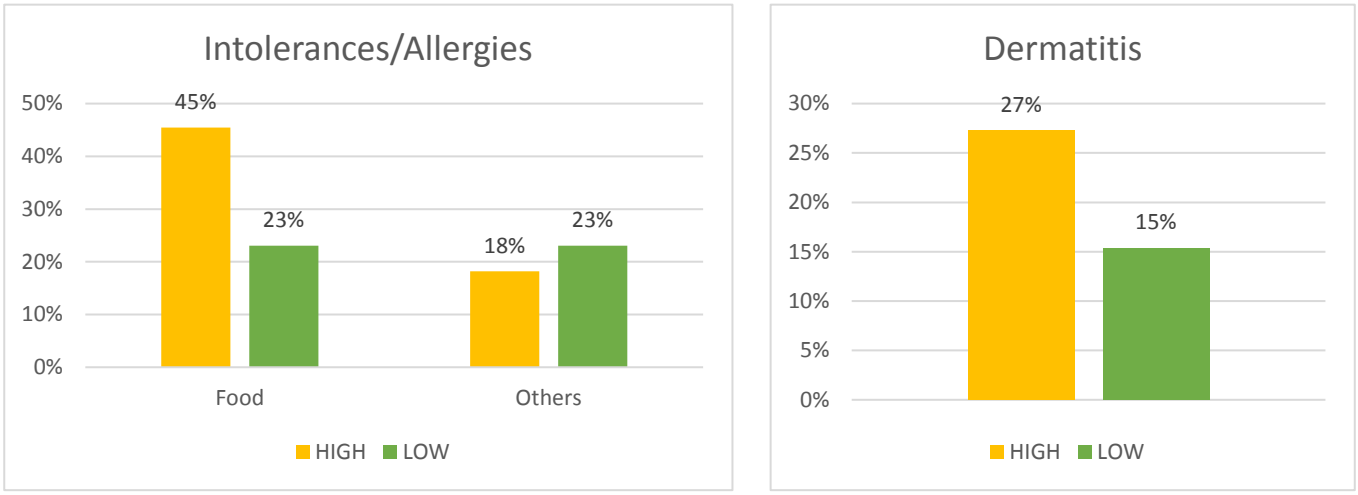


Fig. 3.27: Child's intolerances/allergies (left) and dermatitis (right).

As far as immunological aspects in the family are concerned, parents of low functioning children had more likely milk or other types of allergies not related to food (Fig. 3.28)

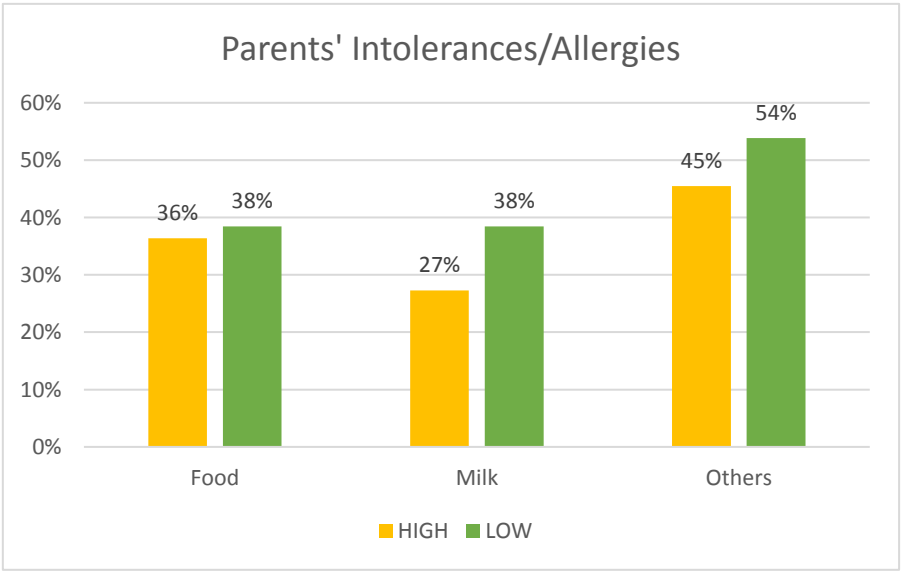


Fig. 3.28: Parents' intolerances/allergies.

Instead, grandparents of high functioning children had more often other types of allergies, whereas none had food allergies (Fig. 3.29).

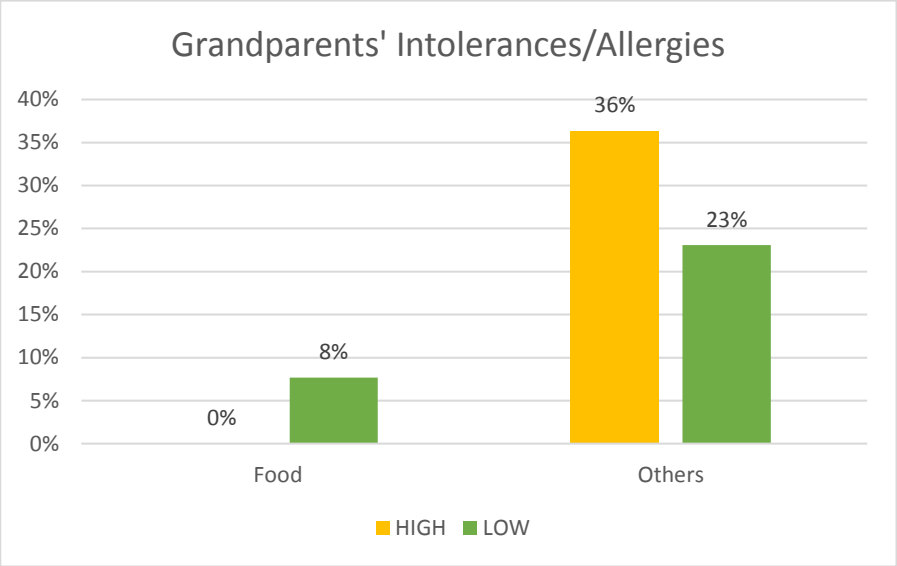


Fig. 3.29: Grandparents' intolerances/allergies.

To conclude, the incidence of autoimmune diseases seems to be similar in both groups (Fig. 3.30).

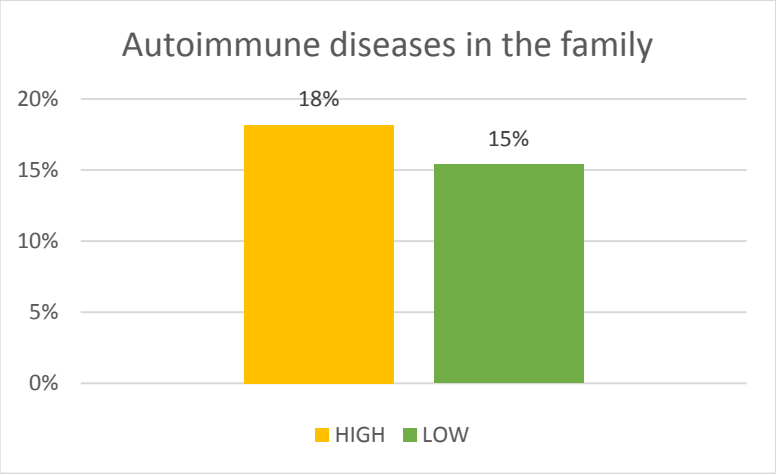


Fig. 3.30: Autoimmune diseases in the family

3.4.2 Logistic regression with predisposing variables

The same predisposing variables, already considered for the comparison between ASD and TD children, were used to assess a possible effect on cognitive level among the ASD group.
In fact, it could be hypothesized that these variables do not predispose themselves to the ASD but that they can influence some aspects of this pathology in a subject already genetically predisposed to develop ASD.
Since cognitive level is a dichotomous variable (levels: 0 = high, 1 = low), a logistic regression analysis was performed.
Again, considering the features of this kind of analysis, variables having levels with only 1 or 0 occurrences were not included in the model (Tab. 3.14).

Predisposing variables	
Maternal age at child's birth	
Paternal age at child's birth	
Type of delivery	
Lactation	Not included
Duration of exclusive breast feeding	
Total duration of breast feeding	
Parents' persistent gastrointestinal disorders	Not included
Grandparents' persistent gastrointestinal disorders	
Parents' food intolerances/allergies	
Parents' milk intolerance/allergy	
Parents' others allergies	
Grandparents' food intolerances/allergies	Not included
Grandparents other allergies	
Autoimmune diseases in the family	

Tab. 3.14: Possible predisposing variables for high vs low cognitive level.

Using R software, following output was obtained (Tab. 3.15):

	Estimate	**Std. Error**	**z value**	**p-value**
(Intercept)	10.9606	11.8184	0.927	0.354
Maternal age at child's birth	0.5265	0.4863	1.082	0.279
Paternal age at child birth	-0.9791	0.6793-	-1.442	0.149
Type of delivery 1	6.2333	6.4753	0.963	0.336
Exclusive breast feeding	-0.7709	0.7716	-0.999	0.318
Total breast feeding	0.7828	0.5672	1.380	0.168
Grandparents' persistent gastrointestinal disorders 1	0.3491	1.8957	0.184	0.854
Parents' food intolerances/allergies 1	-13.8326	3956.1973	-0.003	0.997
Parents' milk intolerance/allergy	18.5448	3956.1971	0.005	0.996
Parents' other allergies	-2.7325	2.6933	-1.015	0.310
Grandparents other allergies	3.0690	3.6753	0.835	0.404
Autoimmune diseases1	-7.7397	12.0529	- 0.642	0.521
Null deviance: 33.104	Residual deviance: 16.171		AIC: 40.171	

Tab. 3.15: Logistic regression model with predisposing variables.

A stepwise backward procedure on this model was performed with the R software, resulting in a reduced model with 1 variable as follows (Tab. 3.16)

	Deviance	**AIC**
Total breast feeding	33.104	35.104
Null Deviance: 33.1	Residual Deviance: 27.28	AIC: 31.28

Tab. 3.16: Stepwise backward procedure on logistic regression model.

Hence, a logistic regression analysis conducted on the reduced model provided the following output (Tab. 3.17):

	Estimate	Std.Error	z value	p-value
(Intercept)	-1.11082	0.73029	-1.521	0.128
Total breast feeding	0.14106	0.07002	2.015	0.044
Null deviance: 33.104 Residual deviance: 27.280 AIC: 31.28				

Tab. 3.17: Logistic regression on the reduced model.

According to this analysis, the total duration of breast feeding, even together with formula or during weaning, is positively related with a low level of cognitive functioning (p-value=0.044). The other variables seem not to play any role in shaping the cognitive level of ASD children.

3.4.3 Logistic regression with discriminating variables

A similar analysis was performed with variables that might discriminate between ASD children with high and low cognitive level but without having a predisposing effect.
Also in this case, variables having levels with only 1 or 0 occurrences were not included in the model (Tab. 3.18).

Discriminating variables	
Food selectivity	
Less selectivity at school	
Shift to a restricted diet at 2-2,5 years of age	
No-self regulation of amount of eaten food	
Hasty swallowing of food	
Special diets	
Pica	Not included
Child's gastrointestinal disorders	
Child's food intolerances/allergies	
Child's other allergies	
Child's dermatitis	

Tab. 3.18: Possible discriminating variables between high and low cognitive level.

A logistic regression analysis was conducted using R software, resulting as follows (Tab. 3.19):

	Estimate	**Std. Error**	**z value**	**p-value**
(Intercept)	0.7376	0.8762	0.842	0.3999
Food selectivity	-0.1391	0.0687	-2.024	0.0430
Less selectivity at school	2.7709	2.3316	1.188	0.2347
Shift to a restricted diet at 2-2,5 years of age	2.6784	2.0987	1.276	0.2019
No-self regulation of amount of eaten food	-1.1238	1.8366	-0.612	0.5406
Hasty swallowing of food	1.4717	1.5204	0.968	0.3331
Special diets	2.4260	2.3647	1.026	0.3049
Child's gastrointestinal disorders	0.3028	1.6734	0.181	0.8564
Child's food intolerances/allergies	-3.1784	1.8635	-1.706	0.0881 .
Child's other allergies	-0.2139	1.3559	-0.158	0.8747
Child's dermatitis	-1.0850	1.7233	-0.630	0.5289
Null deviance: 33.104	Residual deviance: 23.200		AIC: 45.2	

Tab. 3.19: Logistic regression model with discriminating variables for ASD with high and low cognitive level.

Again, a stepwise backward procedure on this model were performed with the R software, resulting in a reduced model with 2 variables as follows (Tab. 3.20)

	Deviance	**AIC**
Child's food intolerances/allergies 1	31.150	35.150
Food selectivity	31.755	35.755
Null Deviance: 33.1	Residual Deviance: 29.07	AIC: 35.07

Tab. 3.20: Stepwise backward procedure on logistic regression model.

Finally, a logistic regression analysis on the reduced model was performed (Tab. 3.21):

	Estimate	Std.Error	z value	p-value
(Intercept)	1.14190	0.69023	1.654	0.098
Child's food intolerances/allergies 1	-1.33652	0.95457	-1.400	0.161
Food selectivity	-0.04925	0.03403	-1.447	0.148
Null deviance: 33.104	Residual deviance: 29.071		AIC: 35.071	

Tab. 3.21: Logistic regression on reduced model.

Although stepwise backward analysis provided a reduced model that discriminates better than the null model between high and low cognitive level, no variables seem to be directly related to differences in functioning among ASD children.

3.5. Results: comparison between different symptoms gravity among ASD

In this section, a possible connection between interview's variables and symptoms gravity among ASD children is explored. Symptoms gravity is referred as ADOS scores.

3.5.1. Description of interviews results

Parental age at child's birth

Following charts show the relationship between maternal or paternal age at child's birth and the ADOS scores of their children (Fig 3.31)

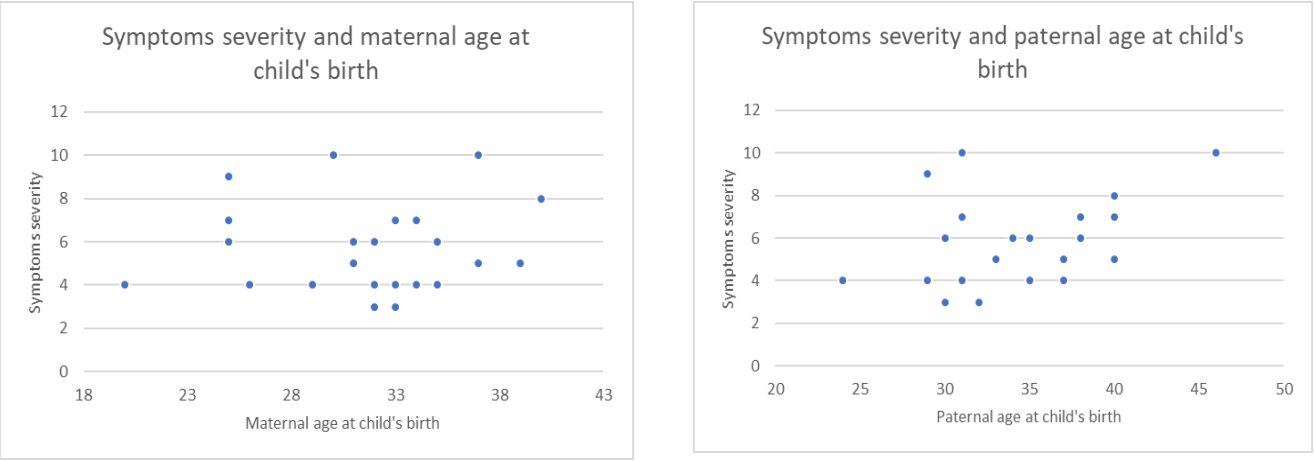


Fig. 3.31: Relationship between parents 'age at child's birth and ADOS scores.

Type of delivery and lactation

ADOS scores were found similar in average regardless the type of delivery and lactation, with the only exception of formula milk exclusive, which was the case of only 1 participant who had 4 as ADOS score (Fig. 3.32).

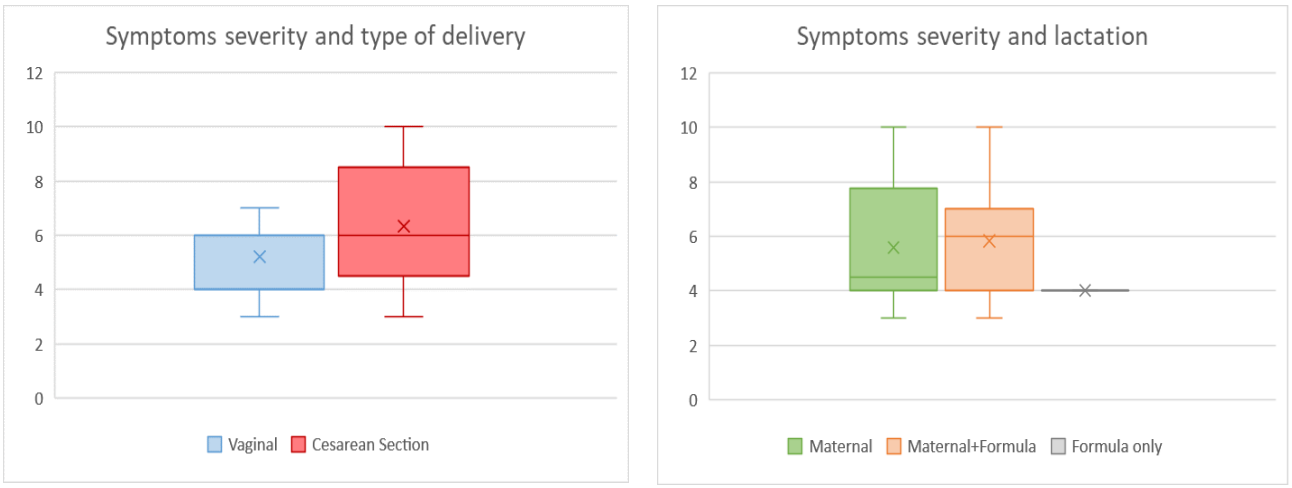


Fig. 3.32: ADOS scores and type of delivery and lactation

In addition, following charts show the relationship between ADOS scores and duration of breast feeding, exclusive and total (Fig. 3.33).

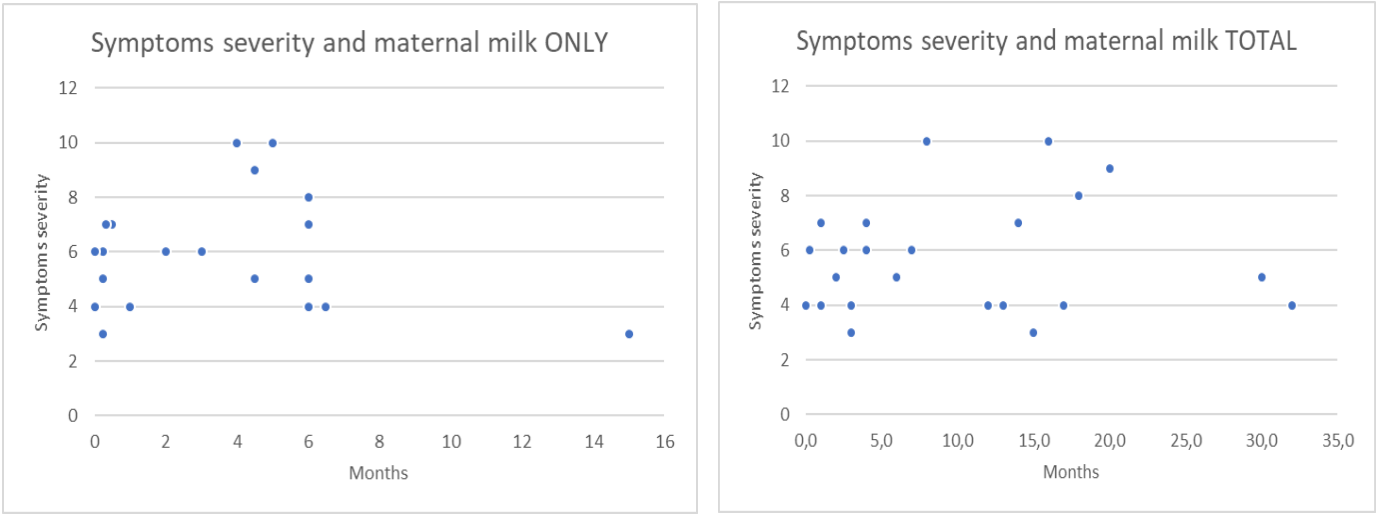


Fig. 3.33: ADOS scores and duration of breast feeding.

Food selectivity and other aspects related to nutrition

Following chart shows the relationship between ADOS scores and food selectivity, reported as percentage of refused foods out of a list included in the interview.

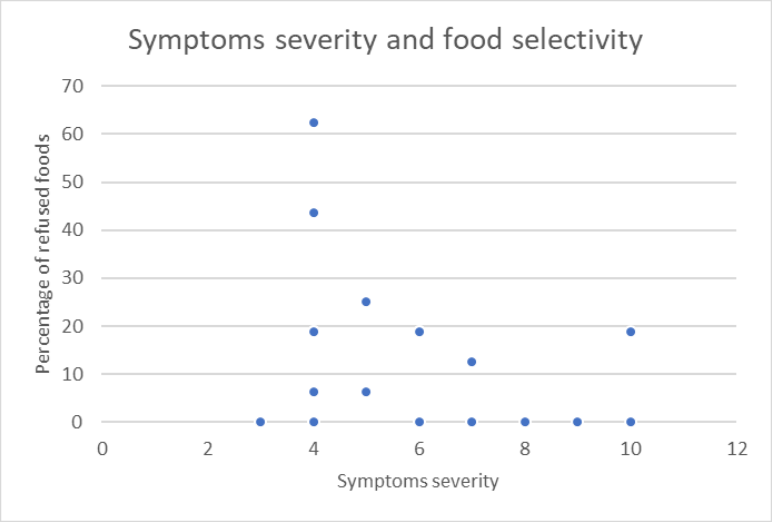
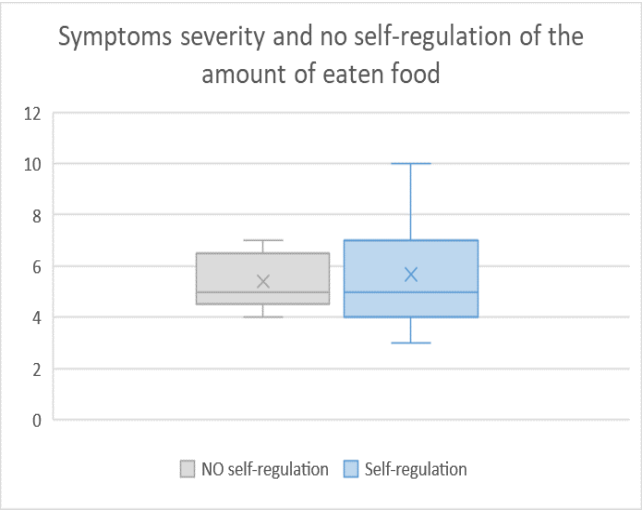
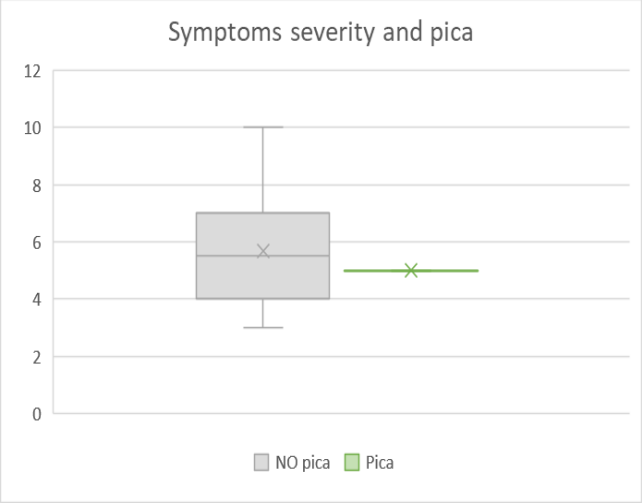


Fig. 3.34: ADOS scores and percentage of refused foods.

Moreover, a similar average ADOS score was found in case of presence/absence of self-regulation of the amount of eaten food, pica and shift to a restricted diet at 2-2,5 years of age (Fig. 3.35).





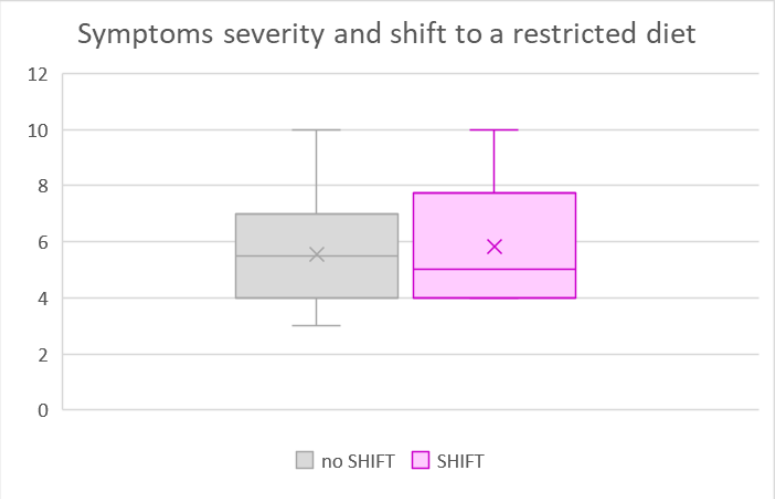


Fig. 3.35: ADOS scores in presence/absence of self-regulation of eaten foods, pica or shift to a restricted diet at 2-2,5 years of age.

In contrast, ASD children on special diets and children who tended to swallow food hastily showed a lower average ADOS score (Fig.3.36)

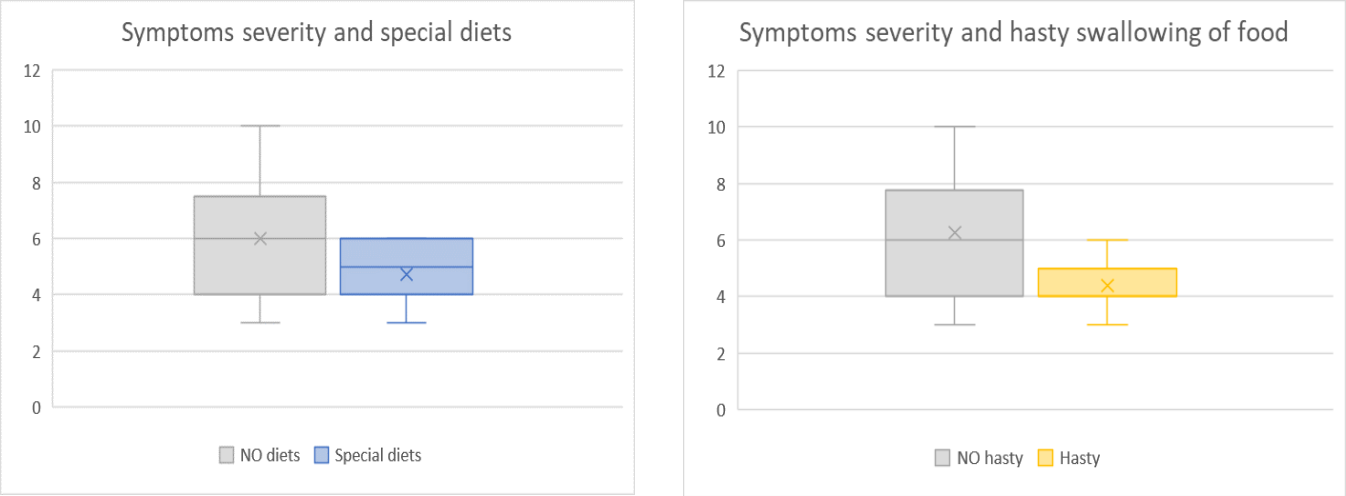


Fig. 3.36: ADOS scores in presence/absence of special diets and tendency to swallow food hastily.

Gastrointestinal disorders

Regarding gastrointestinal disorders, ADOS average score was higher among children without these problems (Fig. 3.37).

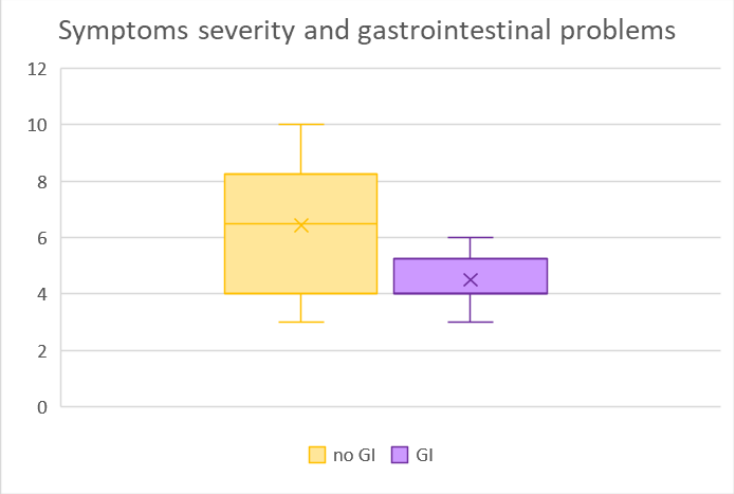


Fig. 3.37: Gastrointestinal disorders in children and ADOS scores.

Also parents and grandparents without persistent gastrointestinal problems tended to have children/grandchildren with higher ADOS scores (in average) (Fig. 3.38).

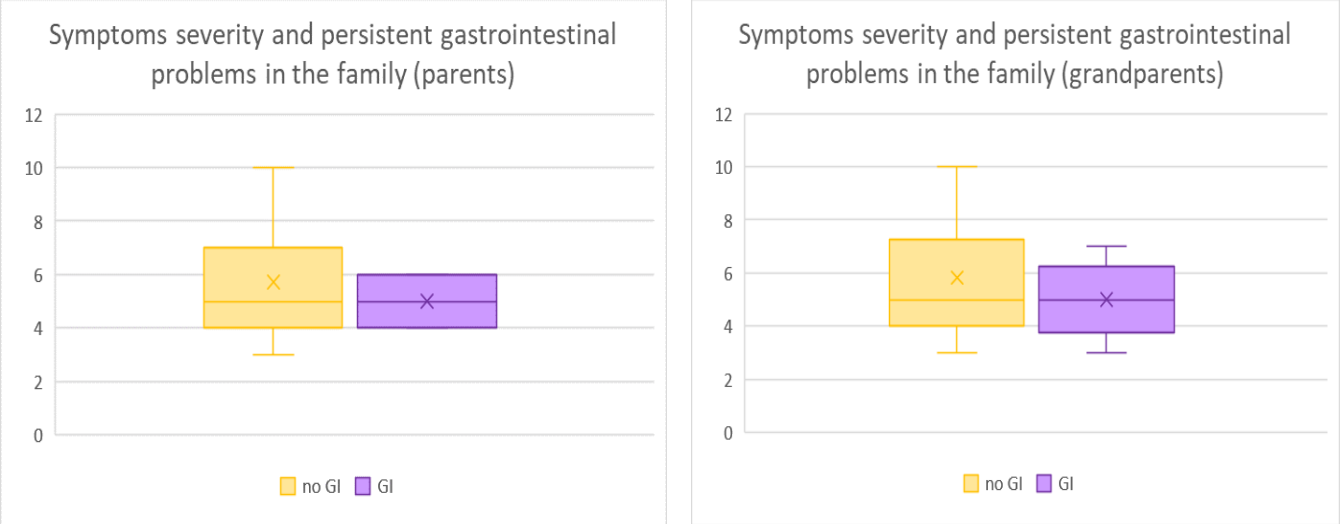


Fig. 3.38: Gastrointestinal disorders in parents and grandparents related to children's ADOS scores.

Immunological aspects

ASD children without any intolerance/allergy and children without dermatitis had a slightly higher average ADOS score (Fig. 3.39)

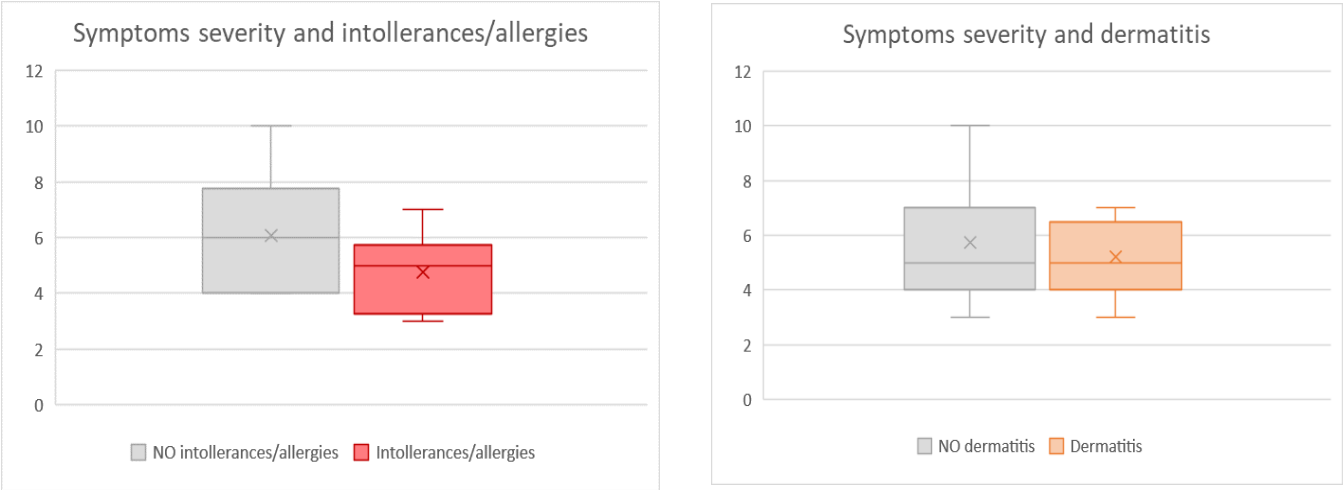


Fig. 3.39: ADOS scores related to presence/absence of intolerances/allergies and dermatitis.

Regarding immunological aspects in the parents, an almost similar ADOS average score was found in the children regardless the presence of food or milk intolerances/allergies in the parents, whereas a higher symptoms gravity was related to parents without other types of allergies (Fig. 3.40)

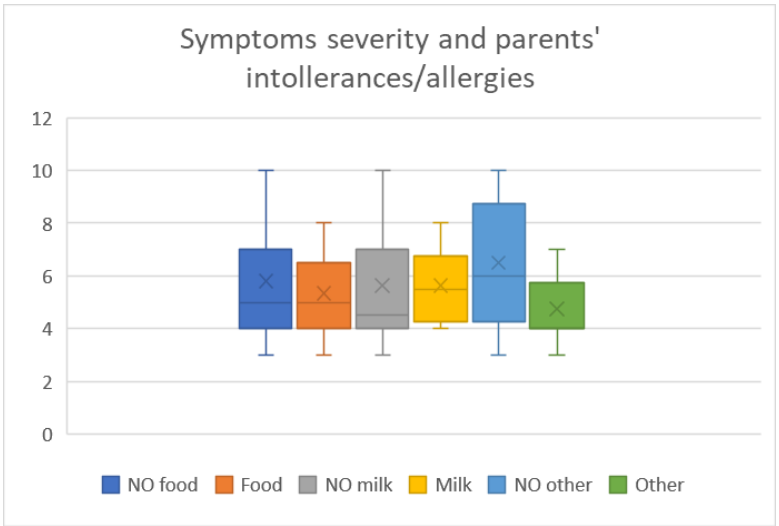


Fig. 3.39: ADOS scores related to presence/absence of intolerances/allergies in the parents.

As far as immunological aspects in the grandparents were concerned, higher symptoms gravity were found in case of no intolerances/allergies, both to food or not. In addition, the presence of autoimmune diseases was related to a slightly lower ADOS average score (Fig. 3.41).

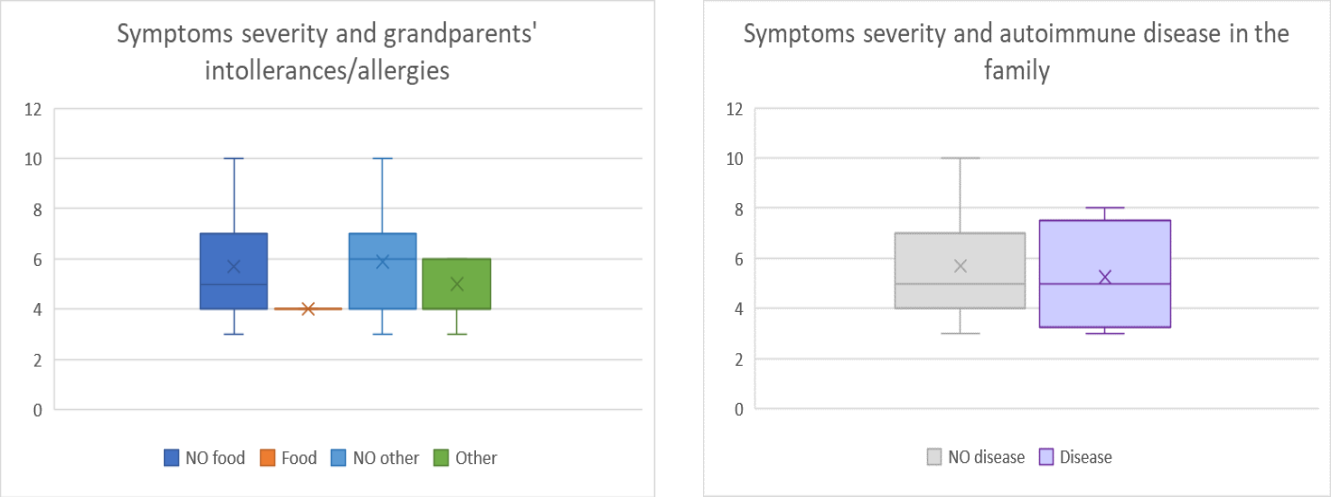


Fig. 3.41: ADOS scores related to intolerances/allergies in grandparents and to autoimmune diseases in the family.

3.5.2 Multiple regression with predisposing variables

Since ADOS scores are a numerical variable, a multiple regression on a model with symptoms gravity as dependent variable and possible predisposing aspects as independent variables was tested using R software.

Following table displays the independent variable that were chosen and the output of this analysis in R (Tab 3.22).

	Estimate	**Std. Error**	**t value**	**p-value**
(Intercept)	4.841835	3.858927	1.255	0.238
Maternal age at child's birth	-0.296094	0.176805	-1.675	-0.296094
Paternal age at child birth	0.315197	0.176243	1.788	0.104
Type of delivery 1	-0.570272	1.718321	-0.332	0.747
Exclusive breast feeding	0.046302	0.222758	0.208	0.840
Total breast feeding	0.118168	0.087119	1.356	0.205
Parents' persistent gastrointestinal disorders	-0.008087	1.991879	-0.004	0.997

Grandparents' persistent gastrointestinal disorders 1	-0.098747	1.417765	-0.070	0.946
Parents' food intolerances/allergies 1	0.204088	3.363695	0.061	0.953
Parents' milk intolerance/allergy	0.595169	3.068405	0.194	0.850
Parents' other allergies	-3.165571	1.814968	-1.744	0.112
Grandparents' food allergies	2.396247	3.028054	0.791	0.447
Grandparents other allergies	-0.790965	1.582570	-0.500	0.628
Autoimmune diseases1	-1.503191	2.437733	-0.617	0.551
Residual standard error: 2.016 Multiple R-squared: 0.5838 Adjusted R-squared: 0.04276 F-statistic: 1.079 p-value: 0.4607				

Tab. 3.22: Multiple regression model with predisposing variables

A stepwise backward procedure on this model were performed with the R software, resulting in a reduced model with 4 variables as follows (Tab. 3.23)

	Sum of Sq	**RSS**	**AIC**
Total breast feeding	12.145	59.972	29.980
Parents' other allergies	18.875	66.703	32.533
Maternal age at child's birth	20.483	68.310	33.104
Paternal age at child birth	24.055	71.883	34.328

Tab. 3.23: Stepwise backward procedure on multiple regression model.

Finally, a multiple regression analysis conducted on the reduced model provided the following output (Tab. 3.24):

	Estimate	**Std.Error**	**t value**	**p-value**
(Intercept)	4.40808	2.65706	1.659	0.11353
Total breast feeding	0.09151	0.04166	2.197	0.04067
Parents' other allergies	-2.05262	0.74960	-2.738	0.01306
Maternal age at child's birth	-0.33415	0.11714	-2.853	0.01019

Paternal age at child birth	0.34976	0.11314	3.091	0.00601
Residual standard error: 1.587 Multiple R-squared: 0.5101 Adjusted R-squared: 0.4069 F-statistic: 4.946 p-value: 0.006652				

Tab. 3.24: Multiple regression on the reduced model.

According to this analysis, the total duration of breast feeding, even together with formula or during weaning, and paternal age at child's birth seem to be positively related to a higher gravity of symptoms, whereas maternal age at child's birth shows the opposite effect.
Also the presence of non-food allergies in the parents seems to be related to lower ADOS scores.

3.5.3 Multiple regression with discriminating variables

Similarly, a multiple regression analysis was performed to explore the effect of possible discriminating variables on symptoms gravity.
Following table displays the independent variable that were chosen and the output of this analysis in R (Tab 3.25).

	Estimate	**Std. Error**	**t value**	**p-value**
(Intercept)	7.03575	0.72645	9.685	2.6e-07
Food selectivity	-0.02976	0.04177	- 0.713	0.489
Less selectivity at school	-0.88265	1.60815	-0.549	0.592
Shift to a restricted diet at 2-2,5 years of age	0.83840	1.32121	0.635	0.537
No-self regulation of amount of eaten food	0.73836	1.33683	0.552	0.590
Hasty swallowing of food	-1.46816	1.04885	-1.400	0.185
Special diets	0.53217	1.56864	0.339	0.740
Child's gastrointestinal disorders	-1.50359	1.22140	-1.231	0.240
Child's food intolerances/allergies	-0.92918	1.22114	-0.761	0.460
Child's other allergies	-0.01354	1.20764	-0.011	0.991
Child's dermatitis	-0.17310	1.32047	-0.131	0.898
Residual standard error: 2.05 Multiple R-squared: 0.4404 Adjusted R-squared: 0.009915 F-statistic: 1.023 p-value: 0.4746				

Tab. 3.25: Multiple regression model with discriminating variables.

A stepwise backward procedure on this model was performed with the R software, resulting in a reduced model with 2 variables (Tab. 3.26)

	Sum of Sq	**RSS**	**AIC**
Hasty swallowing of food	9.480	75.929	31.642
Child's gastrointestinal disorders	12.427	78.875	32.555

Tab. 3.26: Stepwise backward procedure on multiple regression model.

To conclude, a multiple regression analysis was conducted on the reduced model (Tab. 3.27):

	Estimate	**Std.Error**	**t value**	**p-value**
Hasty swallowing of food	-1.3971	0.8071	-1.731	0.0981
Child's gastrointestinal disorders	-1.5294	0.7718	-1.982	0.0608
Residual standard error: 1.779 Multiple R-squared: 0.3193 Adjusted R-squared: 0.2545 F-statistic: 4.926 p-value: 0.01761				

Tab. 3.27: Multiple regression on the reduced model.

In this analysis, child's gastrointestinal problems seem to be related to a lower symptoms gravity, although corresponding p-value is slightly above the statistically significance (p-value=0.06).

3.6. Discussion

Considering that microbiota development happens in a timespan also sensitive for the development of the nervous system (Koshaleva et al., 2016) and that there is a two-way communication system between gut and brain on which the microbiota has an important influence (Cryan et al., 2012), an implication of microbiota in ASD has been hypothesized.

Therefore, factors that influence microbiota could play a role also in ASD, or at least in a subgroup.

According to the findings of this study, however, it emerges no statistically significant association between ASD and delivery mode, despite the fact that some studies have reported it as risk factor for

ASD (Curran et al., 2015). The small number of participants in this study might be a possible explanation for this discrepancy. Nevertheless, this aspect should be better addressed in further studies.

Regarding the type of lactation, it did not appear statistically different between ASD and TD children. However, within the ASD group, the total duration of breastfeeding, even combined with artificial milk and weaning, seems to be associated with lower level of cognitive functioning and higher gravity of autistic symptoms. This is an unexpected finding because of the protective role of breast feeding on microbiota development (Al-Farsi et al., 2012).

Moreover, according to the literature (Bandini et al., 2010), a higher food selectivity was found in ASD children. Also a possible association between food selectivity and IQ was explored, with the idea that a higher cognitive level could lead to a greater awareness in food choices. However, no statistically significant association was found. On the other hand the small number of participants must also be considered as a possible explanation.

In addition, it has been hypothesized that a greater severity in autistic symptomatology could cause a stronger food selectivity, considering that having narrow interests is one of the diagnostic criteria for ASD. However, even in this case, the two conditions do not seem related.

Instead, a different interesting result was highlighted. In fact, a notable percentage of ASD children had a normal diet at the beginning and started to restrict their food repertoire more and more starting between 2 and 2,5 years of age, whereas no TD children had the same behavior. Of course it is common for each child to start to show his/her food preferences at one point, but it tends to happen later in typically developing children and also not in such a dramatic way. Further studies are needed to better explore this aspect, also on a statistical level: if confirmed, it could be considered an early sign of the development of the pathology.

Furthermore, some ASD participants were less selective at school compared to at home, like the TD group. It could be important to be aware about that because it seems that the food choices of the children are not as rigid as expected and maybe it could be possible to introduce also at home some foods that the child is used to eating at school.

According to the literature (Buie et al.,2010) , also a high incidence of gastrointestinal disorders were found in the ASD group, while no TD participants were reported to have this kind of problems. This condition may be linked to an altered activities of the microbiota. Therefore, this should be addressed in-depth in studied that enroll participants with a clear diagnosis of the disorder and its manifestations. Furthermore, these studies should use sufficiently sensitive techniques, such as metaproteomics. In the next chapter of this work the attempt to proceed in this direction will be described.

Nevertheless, in this study gastrointestinal problems were found to be almost statistically related to lower autistic symptoms gravity. Giving an explanation for this result is challenging: it could be

argued that children with less severe symptoms might make better account of their condition and get more frustrated than children with more severe symptoms, which are more isolated from the world around them. This could take them to a sort of somatization. To explore this issue, further studies focused on the assessment of the secretion profile of stress hormones, such as cortisol, over a long period, should be conducted. On the other hand, this does not exclude a possible alteration in the activity of microbiota in the subgroup of subjects with less severe symptoms. This aspect should be better investigated through new studies that combine for example metaproteomics analysis and clear cognitive-behavioral assessment.

A role for gut microbiota in ASD could be plausible also because of its role in shaping the immune system (Matelski and Van de Watere, 2016). In fact, in this study we found a higher incidence of food allergies among ASD children and a higher food and milk intolerances in their parents. These could be considered signs of a possible altered activation of the immune system and could account for the benefic effect that some children have thanks to gluten- and casein free diets. Interestingly, celiac diseases seem to be more common in families of TD children, instead.

To conclude, parents' age at child's birth seem to have an effect on ASD symptoms gravity. In fact, increased paternal age is positively related to higher gravity, whereas increased maternal age has the opposite effect. Further studies are needed to better assess this aspect.

3.7. Conclusion

As already mentioned several times, the issue of having a well-characterized sample is critical for complex studies as those who attempt to find the causes behind the onset of ASD. In fact, these disorders include highly different phenotypes. Thus, an etiological explanation could be valid only for a subgroup and not necessarily for all.

The interview that was developed for this study allows to collect a wide range of information that can be used, together with IQ, ADOS scores and other cognitive-behavioral features, to identify subgroups. This is crucial also for conducting studies that attempt to relate phenotype characteristics with biological aspects, such as for example studies on gut microbiota in ASD.

Moreover, the possibility to highlight the relationship between extremely different aspects (such as for example gastrointestinal disorders and symptoms severity) allows to move forward in understanding the complex mechanisms of this pathology.

CHAPTER 4: GUT MICROBIOTA and ASD FAMILIES

4.1 Introduction

As discussed previously in this thesis, studies on microbiota composition in ASD have shown contrasting results. A possible explanation could be the lack of control on interfering variables such as age and gender of participants, different dietary habits, antibiotics / probiotics / prebiotics intake and presence of gastrointestinal disorders (Mayer et al., 2014). In fact, all these aspects can have a role in shaping microbiota composition (Kashtanova et al., 2016).

In addition, studies conducted so far ignore possible differences in the autistic phenotype of the subjects, such as different cognitive level and severity of autistic symptoms. These aspects, together with the variables mentioned above, could be useful to define subgroups within children with ASD and to assess whether there are differences in the microbiota between subgroups and compared to typical developing children.

Moreover, another important factor influencing microbiota composition is the genetics of each individual (Zang et al., 2010; Goodrich et al, 2014). Therefore, it may be helpful to involve in this kind of studies even the family of the ASD child, i.e. parents and a typically developing sibling, if present. In fact, if similarities are found in the microbiota among ASD children and the other family members, that would speak against a possible involvement of microbiota in the ASD: in this case, a similar composition would be a probable consequence of affinities related to diet, to gastrointestinal problems or to shared genetics.

Another crucial point to address is the kind of biological analysis conducted in previous studies. In the past, in fact, cell cultures were the only way to obtain information about microbiota, but unfortunately they allowed the growth only of some bacterial strains. This obviously represented a big limit (Finegold et al., 2002; Song et al., 2004; Wang et al., 2015).

But in the last two decades a new and more sensitive technique has emerged: metagenomics. This technique makes it possible to acquire a picture of the composition of the microbiota by sequencing a highly variable portion of bacterial DNA, obtainable from fecal samples and attributable unequivocally to a certain taxon (Wang et al., 2015).

Nevertheless, even this technique has some weaknesses. In fact, it allows to recognize which bacteria are present and in what concentration, but it does not offer any information on the degree of activation of metabolic pathways in these bacteria. But these processes are actually the basis of the bacterium ability to exert an effect on the host.

Nowadays, an even newer technique, called metaproteomics, allows to assess the enzymatic bacterial proteins present in fecal samples and, in this way, to highlight which pathways are activated (Xiong W. et al., 2015; Zahng et al., 2016).

Thus, for this study metaproteomics techniques were used to analyze fecal samples from ASD children and their families. Moreover, other information was collected, such as about diet and gastrointestinal problems of each subject, and also regarding the cognitive level of ASD children.

The protocol of this study was approved by the Ethical Committee of Bambin Gesù Hospital, Rome.

4.2 Method

4.2.1 Participants

10 families were involved in this study, each of them consisting of 1 ASD child and the two biological parents. In 7 families also a typically developing sibling was present.

Most of the families (7) were recruited in summer 2015 at a summer camp for ASD children, whose name is "Terapia in vacanza" (Therapy on holiday), organized in Serrada, Trento, by the Laboratory of Observation, Diagnosis and Education, University of Trento. 3 more families jointed this project in December 2015.

9 ASD children were regular patients of the Laboratory for Observation, Diagnosis and Education of the University of Trento, Italy and had already been subjected to an assessment of autistic symptoms by Laboratory's psychologists well experienced in the use of the Autism Diagnostic Observation Schedule - Second Edition (ADOS, Lord et al., 2001).

Furthermore, also the cognitive profile of each child had already been evaluated through intelligence scales, such as The Leiter International Performance Scale, Third Edition (Leiter-3, Leiter, 1940) and The Wechsler Intelligence Scale for Children (WISC, Wechsler, 2003), and / or through The Griffith Mental Development Scales (GMDS, Griffith, 2006).

Finally, 1 family involved in this study had contact to the Laboratory only because of the summer camp. In this case, reference was made to the evaluation documents provided by the parents at the time of enrolling for the summer camp.

Because of technical problems occurred during the biological analysis, it was possible to obtain results only about 8 whole families and 1 father of a further family.

Following tables summarize information regarding only this group of participants: participant identification codes and family structure (Tab.4.1), age (Tab 4.2), gender of ASD children and their siblings (Tab. 4.3) and ASD children cognitive level (Tab. 4.4).

	Code	Family member		Code	Family member
Family 1	A1	*ASD son*	**Family 6**	A19	*ASD son*
	A2	brother		A20	sister
	A3	mother		A21	mother
	A4	father		A22	father
Family 2	A5	*ASD son*	**Family 7**	A23	*ASD son*
	A6	sister		A24	mother
	A7	mother		A25	father
	A8	father	**Family 8**	A26	father
Family 3	A9	*ASD son*		A27	mother
	A10	mother		A28	*ASD son*
	A11	father		A29	sister
Family 4	A12	*ASD son*	**Family 9**	A30	father
	A13	mother			
	A14	father			
Family 5	A15	*ASD son*			
	A16	brother			
	A17	mother			
	A18	father			

Tab. 4.1 Identification codes and family structures.

Group	Size	Age (average + SD)	Age (minimum)	Age (maximum)
ASD	8	6.74 ± 2.08 years	4 years	10.66 years
siblings	5	8.57 ± 3.27 years	4.42 years	12.75 years
mothers	8	40.3 ± 5.65	28.75	46.91 years
fathers	9	43.48 ± 8.80	32.58	64.33

Tab.4.2 Participants' age.

		siblings		
		male	**female**	**none**
ASD	**male**	2	3	3

Tab.4.3: Children's gender.

Cognitive level	
High	Low
4	4

Tab.4.4: ASD children's cognitive level.

All parents signed an informed consent.

4.2.2. Food diary

In order to better control the effect of diet on microbiota, parents were asked to fulfill a 5 day-food diary, recording the amount of what they and their children had eaten.

To assess the amount of the main nutrients (proteins, fat, cholesterol, simple carbohydrates, complex carbohydrates and fiber) taken by each participant every day, the Food Composition Database for Epidemiological Studies in Italy (www.bda-ieo.it) was used. This is the most updated repository related to Italian foods.

Nevertheless, if the food brand was provided by the parent, nutrient composition was obtained directly from the brand official webpage.

For foods that were not present in either of the two databases, a search was conducted in the food composition database of CRANEA (Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria - Council for Agriculture Research and Agricultural Economy Analysis, www.nut.entecra.it) or, if again not present, in the FatsecretItalia Database (www.fatsecret.it), which is the food composition database connected to a famous free smartphone App for estimating calories intake.

4.2.3 Interview

For this study also the previously described interview (Chapter 2) was used to collect various information about factors that can influence gut microbiota composition, such as the presence of gastrointestinal disorders.
All interviews took place at the camp "Terapia in Vacanza" or at the Laboratory and were recorded after having obtained the parents' permit.

4.2.4. Fecal samples collection

Parents were instructed about how to collect 1 fecal sample from themselves and their children and were asked to bring the samples as soon as possible to the camp or to the Laboratory.
Since a -80°C freezer facility was present neither at the camp "Terapia in Vacanza" or at the Laboratory, fecal samples were frozen initially at -20 °C and then transferred at -80°C at the end of the whole collection. This procedure is commonly used and does not affect the quality of the samples (Wang et al., 2011).
Subsequently, all samples were sent in dry ice to the Human Microbiome Unit, Bambino Gesù Children's Hospital, Rome, for metaproteomics analysis.

4.2.5 Metaproteomics protocol

Bacterial pellet extraction

Samples were weighed and aliquoted (about 0.5 g). 2 ml of PBS were added to the aliquots and agitated at 37 ° C for 10 minutes. Subsequently, a centrifugation at 1500 rpm was performed, lasting 15 minutes. The supernatant was taken and centrifuged at 15,000 rpm for 15 minutes.
After removing the supernatant, three washings were performed by adding 2 mL of PBS each time, centrifuging for 10 minutes. The recovered bacterial pellet was stored at -80 ° C until the sample was processed.

Proteins precipitation

Bacterial pellets were resuspended in 200 µL of Sample Buffer (SB, 7M urea, 2M thiourea, Trisbase 40mM, CHAPS 4%, dithiothreitol 50 mM) previously heated at 37 ° C. Samples were sonicated with a needle sonicator for 20 seconds with an intensity of 60% for 7 cycles. Subsequently, they were

incubated at 37 ° C for 1 hour. A centrifugation at 15,000 rpm was performed for 30 minutes by taking the supernatant by transferring it to a new test tube. Six volumes of an organic solution (50% EtOH, 25% Acetone, 25% MeOH) were added (stored at -20 ° C) to induce protein precipitation after one night at -20 ° C. After centrifugation at 4 ° C, 15,000 rpm for 45 minutes, the pellets were washed with the same solution and finally resuspended in 100 µL Diluition Buffer (DB, 6M urea, Tris HCl 100 mM, pH 7-8), sonicating for 5 minutes and incubating at 37 ° C for 20 minutes. The remaining insoluble material was separated by centrifugation at 15000 rpm for 15 minutes and the supernatant was transferred to a new test tube.

Proteins Dosage: BCA Protein Assay

The Pierce™ BCA Protein Assay Kit was used to determine the total protein concentration of the individual samples. The spectrophotometer absorbance at the 562 nm was measured and the concentration was estimated in a working range from 20 µg / mL to 2000 µg / mL.

In this study, 96-well microplates were chosen. The measurement was done in double reading and calibrated through a calibration line constructed using known BSA solutions (1, 2, 4, 8 and 10 µg / µL). 190 µL of reagent solution was added to each well and, depending on the estimated protein concentration, 2 to 10 µL of sample solution. The plate was then covered and incubated at 37 ° C for 30 minutes.

Tryptic digestion in solution

For the digestion of a quantity of about 100 µg of protein, the corresponding sample amount was taken, bringing the final volume of the 30 µL solution with H_2O.

To reduce disulfide bridges, 2.5 µL of 1,4-Dithiothreitol (DTT) (0.1M) was added, leaving the solution for 1 hour at 37 °. Subsequently, 3 µL of Iodoacetamide (IAA) (0.2M) was added to alkylate the cysteine residues by incubating at room temperature in the dark for 1 hour.

Additional 0.5 µL DTT were added to eliminate excess alkylation.

After checking that the pH was around 7-8, a trypsin solution was added to H2O (0.5 µg / µL), considering a protein / protease ratio of about 50. Digestion was continued at 37 ° C for All night interrupting it by adding 1 µL of 10% formic acid.

Purification and enrichment

Extract purification and enrichment were performed using micropipette tips containing a small silica C-18 septum. These must be conditioned prior to their use. Thus, the following buffers were prepared: A) acetonitrile (50%, 1% formic acid) and B) formic acid (1%). The stationary phase was conditioned

with solution A and then washed with B. Peptides were loaded onto the silica by letting the sample solution flow through the tips several times. Finally, peptides were eluted with solution A.

LC-MS / MS analysis

Three biological replicates of the triptic digest were analyzed using an nHPLC-MS / MS approach by a Eksigent Ekspert NanoLC 400 chromatograph (Sciex, Toronto, ON, Canada) interfaced with a SCIEX TripleTOF 5600+ mass spectrometer. Two μL (1 μg protein) of tryptic digest of each sample were injected and pre-concentrated for 5 min on a Eksigent Trap column (350 mm x 0.5 mm Chrom XP C18.3 mm, 120 N nano LC) with a flow of 5 mL / min. The peptide elution gradient was carried out on a C18-Acclaim PepMap100 (25 cm, 75 mm ID, 5 mm ps, Thermo Fisher Scientific, Waltham, MA, USA) with a flow rate of 0.3 μL / min, at a temperature of 40 ° C, with eluents: (A) H_2O / CH_3CN 98: 2 + 0.1%; (B) CH_3CN / H_2O 98: 2 C 0.1% formic acid and a gradient of 5 to 25% B in 120 min. MS data was acquired in information-dependent acquisition mode (IDA).

4.2.6 Data processing and bioinformatics

Raw data was processed through the ProteinPilot 4.0 software and database research was carried out using the NCBInr database, containing all the protein sequences known for the kingdom *bacteria.*
Protein lists of the three technical replicates were merged together, removing duplicates.
Protein sequences in FASTA format were retrieved from the NCBI protein and uploaded on the web open source service WebMGA for functional analysis (Wu et al., 2011). Proteins were classified in Clusters of Orthologs Groups (COG) and by molecular functions.

4.2.7 Data analysis

Data analysis was conducted in cooperation with the Unit of Predictive Models for Biomedicine and Environment (MPBA) of Bruno Kessler Foundation, Trento, Italy.
At first, Wilcoxon-Mann-Whitney test was conducted on COG abundance between ASD children and all others participants, and then between ASD children and parents and siblings separately to better assess possible effects related to age differences between groups.
Furthermore, random forest analysis (Breiman, 2001) was performed considering all COGs together.
Finally, network analysis (Jurman et al., 2015) were also conducted. Networks were constructed by calculating Pearson's Correlation (PC) between each pair of COG (nodes). Maximum correlation

corresponds to a value of 1. Only links between COGs with PC> 0.9 were considered. First, a comparison between ASD children vs all other subjects were performed. Then, also differences in COGs pattern among ASD children were explored, with regard to different cognitive level, type of delivery and presence of gastrointestinal disorders.

In addition, differences related to nutrient intake between ASD children and the other family members were assessed. In fact, if present, these could cause differences in microbiota activity and could act as confounding variable for the actual relationship between microbiota and ASD. Therefore, average intake of each nutrient was calculated for each participant over the 5 days of food diary. Hence, Wilcoxon-Mann-Whitney test was performed for each nutrient comparing the ASD children group and their relatives. Finally, also a random forest analysis was conducted considering all nutrients together.

4.3 Results

4.3.1 Univariate statistical analysis on COGs

Following chart (Fig 4.1) shows COG abundances for each participants (reported with the identification code):

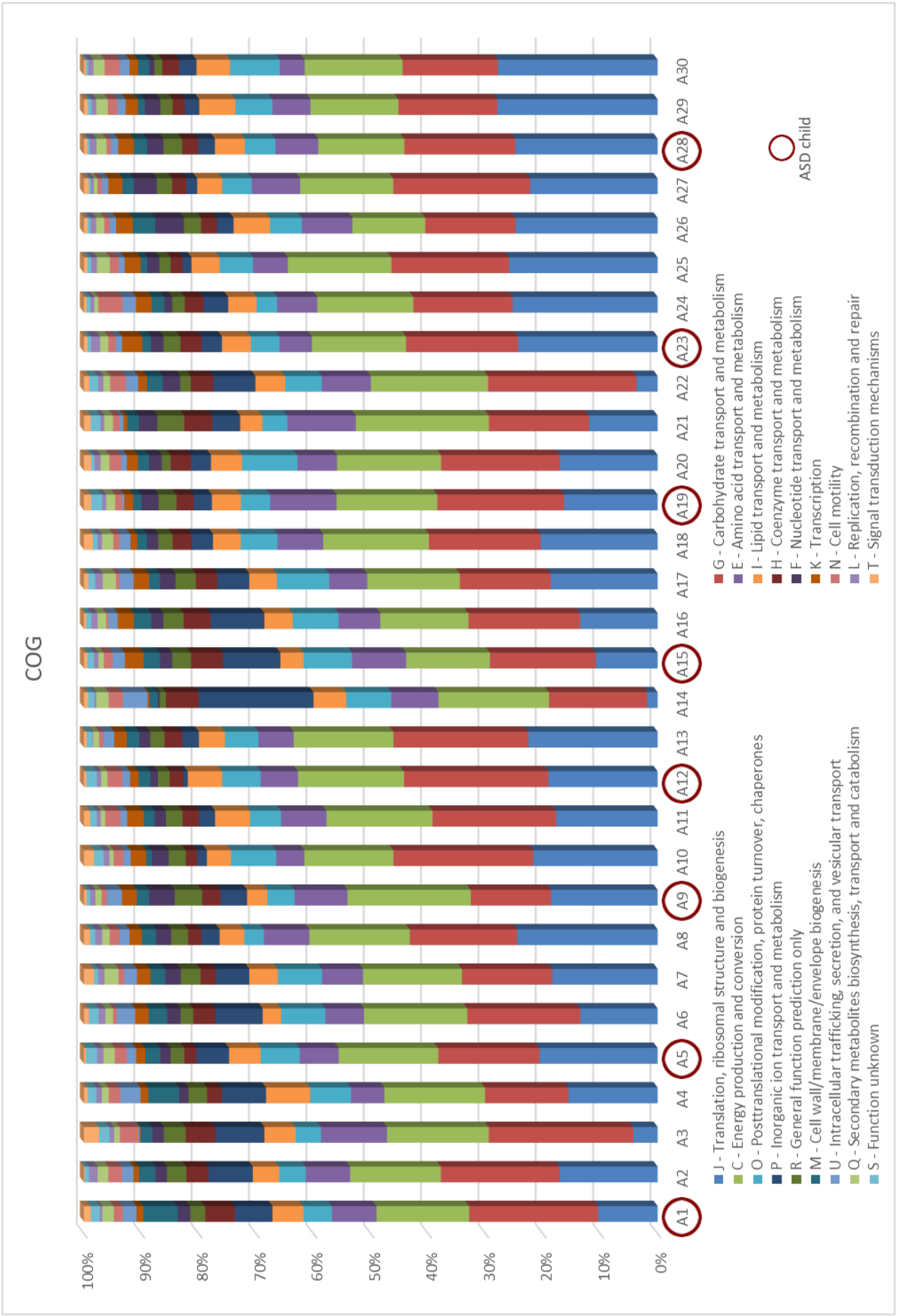


Fig 4.1: COGs abundance in each subject. ASD children are highlighted in red circles.

Wilcoxon-Mann-Whitney test did not provide any statistical significance in the comparison between ASD children and all other participants taken together, as shown in Tab. 4.5.

ASD children vs parents + siblings		
COGs	*p-value*	*statistic (W)*
J - Translation, ribosomal structure and biogenesis	0,496444135	73
G - Carbohydrate transport and metabolism	0,452933474	71,5
C - Energy production and conversion	0,452732232	71,5
E - Amino acid transport and metabolism	0,672828631	78,5
O - Posttranslational modification, protein turnover, chaperones	0,39836876	69,5
I - Lipid transport and metabolism	0,43863224	71
P - Inorganic ion transport and metabolism	0,74250246	80,5
H - Coenzyme transport and metabolism	0,742169777	80,5
R - General function prediction only	1	88
F - Nucleotide transport and metabolism	0,925163382	85,5
M - Cell wall/membrane/envelope biogenesis	0,981252501	89
K - Transcription	0,851032937	83,5
U - Intracellular trafficking, secretion, and vesicular transport	0,249235909	63
N - Cell motility	0,396945885	69,5
Q - Secondary metabolites biosynthesis, transport and catabolism	0,742225314	80,5
L - Replication, recombination and repair	0,723319593	80
S - Function unknown	0,635834526	98,5
T - Signal transduction mechanisms	0,162962603	58

Tab.4.5: Wilcoxon-Mann-Whitney test on COGs for ASD children vs all others.

Moreover, Wilcoxon-Mann-Whitney test did not provide any statistical significance in the comparison between ASD children and only parents, as shown in Tab. 4.6:

ASD children vs parents		
COGs	*p-value*	*statistic (W)*
J - Translation, ribosomal structure and biogenesis	0,510988142	56
G - Carbohydrate transport and metabolism	0,641121712	59,5
C - Energy production and conversion	0,502570704	56
E - Amino acid transport and metabolism	0,70483316	61
O - Posttranslational modification, protein turnover, chaperones	0,683257762	60,5
I - Lipid transport and metabolism	0,502406037	56
P - Inorganic ion transport and metabolism	1	67,5
H - Coenzyme transport and metabolism	0,930192982	66
R - General function prediction only	0,907194772	65,5
F - Nucleotide transport and metabolism	1	68,5
M - Cell wall/membrane/envelope biogenesis	0,906980589	70,5
K - Transcription	0,884029929	65
U - Intracellular trafficking, secretion, and vesicular transport	0,306489019	50
N - Cell motility	0,395722469	53
Q - Secondary metabolites biosynthesis, transport and catabolism	1	68,5
L - Replication, recombination and repair	0,976549976	69
S - Function unknown	0,76871206	73,5
T - Signal transduction mechanisms	0,082327307	38

Tab. 4.6: Wilcoxon-Mann-Whitney test on COGs for ASD children vs all parents.

Again, Wilcoxon-Mann-Whitney test did not provide any statistical significance in the comparison between ASD children and all siblings, as shown in Tab. 4.7:

ASD children vs siblings		
COGs	*p-value*	*statistic(W)*
J - Translation, ribosomal structure and biogenesis	0,724164724	17
G - Carbohydrate transport and metabolism	0,271594161	12
C - Energy production and conversion	0,55709939	15,5
E - Amino acid transport and metabolism	0,769080645	17,5
O - Posttranslational modification, protein turnover, chaperones	0,127428127	9
I - Lipid transport and metabolism	0,523698524	15
P - Inorganic ion transport and metabolism	0,340685192	13
H - Coenzyme transport and metabolism	0,462363854	14,5
R - General function prediction only	0,768770129	22,5
F - Nucleotide transport and metabolism	0,712498805	17
M - Cell wall/membrane/envelope biogenesis	0,8832987	18,5
K - Transcription	0,883138334	18,5
U - Intracellular trafficking, secretion, and vesicular transport	0,338681291	13
N - Cell motility	0,659230371	16,5
Q - Secondary metabolites biosynthesis, transport and catabolism	0,284382284	12
L - Replication, recombination and repair	0,207688719	11
S - Function unknown	0,505351711	25
T - Signal transduction mechanisms	1	20

Tab. 4.7: Wilcoxon-Mann-Whitney test on COGs for ASD children vs all siblings.

4.3.2 Multivariate statistical analysis on COGs

Since single COGs did not seem to be significantly different between ASD children and typically development subjects, a possible combined effect was hypothesized. Thus, a random forest analysis was performed.

Nevertheless, the best performance was obtained considering only 1 single COG, with a MCC = 0.24, as shown in the following table (Tab. 4.8) However this COG represents a group with unknown function.

STEP	MCC	MCC_MIN	MCC_MAX
1	0.24	0.13	0.34
2	0.18	0.06	0.29
3	0.19	0.07	0.29
4	0.14	0.03	0.25
5	0.14	0.05	0.24
6	0.12	0.03	0.23
7	0.02	-0.03	0.09
8	-0.01	-0.04	0.02
9	0.01	-0.04	0.07
10	-0.01	-0.04	0.02
18	-0.03	-0.06	-0.01

Tab. 4.8: RF analysis for COGs distribution in ASD children and their families.

Nevertheless, also models with until 4 COGs could be considered, since their MCCs are in the confidence interval of the highest MCC obtained. This could open more intriguing explanation scenarios than considering only 1 variable, and even with unknown function. In fact, these additional COGs would be “Energy production and conversion”, “Transcription” and “Coenzyme transport and metabolism” (in the same order as in the RF output).
However, the RF performance remains low.

4.3.3 Network analysis on COGs

Networks were constructed by calculating Pearson’s Correlation (PC) between each pair of COGs (nodes). Maximum correlation corresponds to a value of 1. Only links between COGs with PC> 0.9 were considered. The intensity of links colors is proportional to the correlation modulus.

ASD children vs parents and siblings

First of all, a comparison between ASD children and all other subjects (nonASD group) was performed (Fig. 4.2 and 4.3)

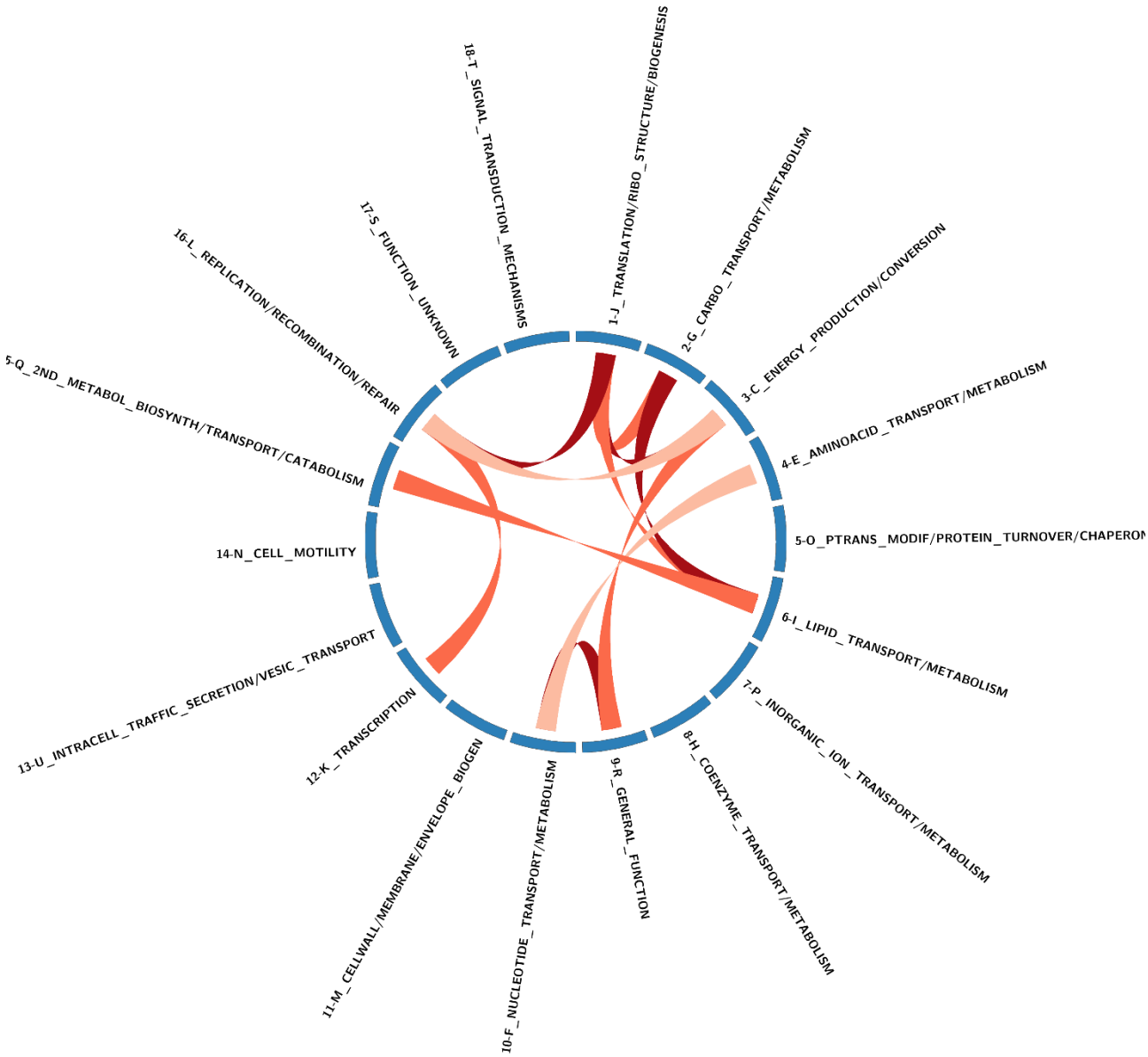


Fig. 4.2: COGs network in ASD children

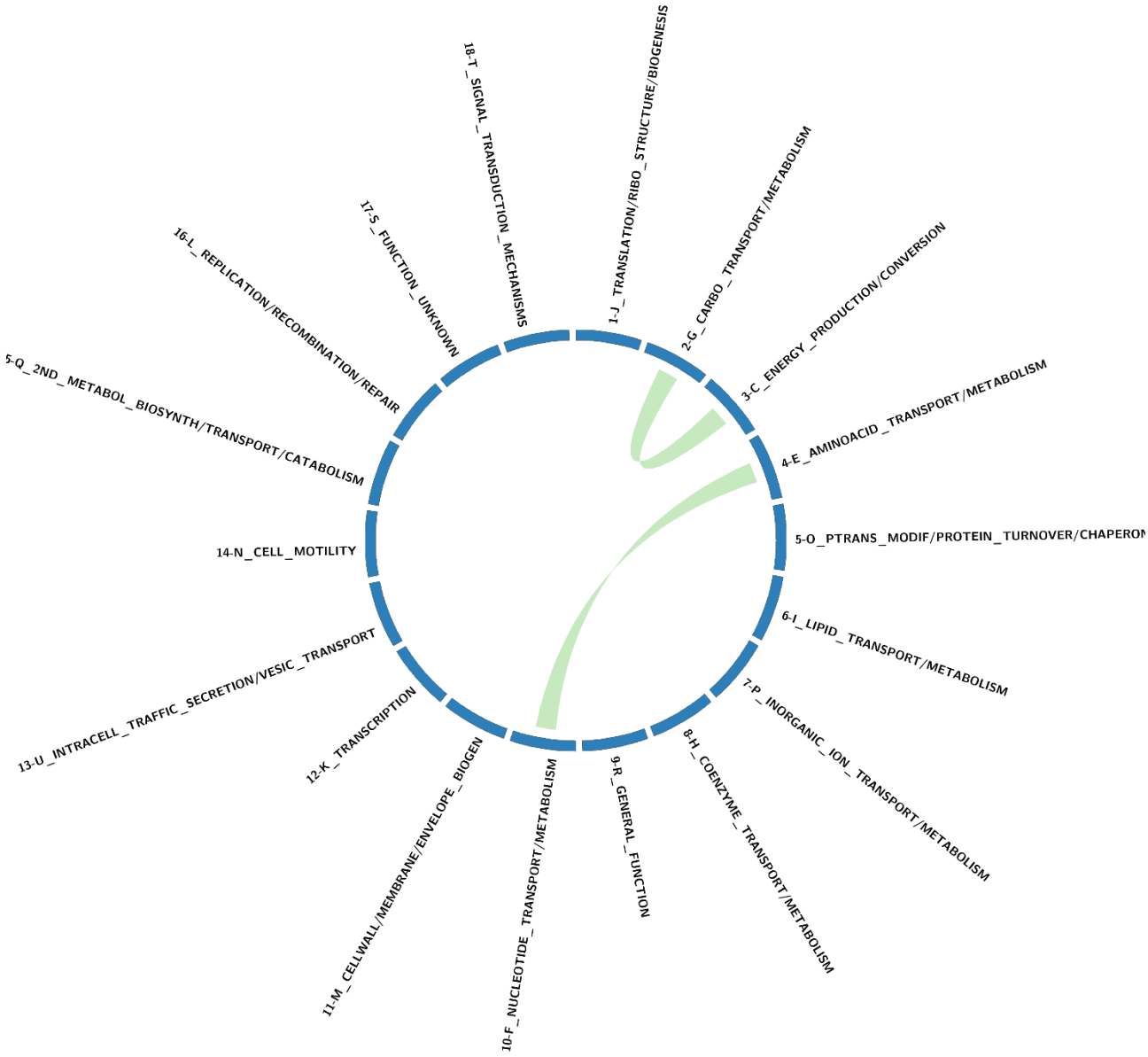


Fig. 4.3: COGs network in nonASD group.

Although network analysis reported a HIM of 0.15 (HIM=0 in case two networks are equal, whereas HIM=1 in case they are completely different), the network for ASD group showed more edges (11) compared to the network for nonASD group (2 edges).
Moreover, the only edge shared by both groups is between “Nucleotide transport metabolism” and “Aminoacid transport metabolism”.

In addition, a network analysis was performed considering different cognitive level of ASD children. In fact, among ASD participants, 4 children were high functioning (IQ equal or above 70) and 4 children were low functioning (IQ less than 70).

Thus, both groups were compared to the nonASDgroup.

Following figure shows the high functioning children CIRCOS (Fig. 4.4)

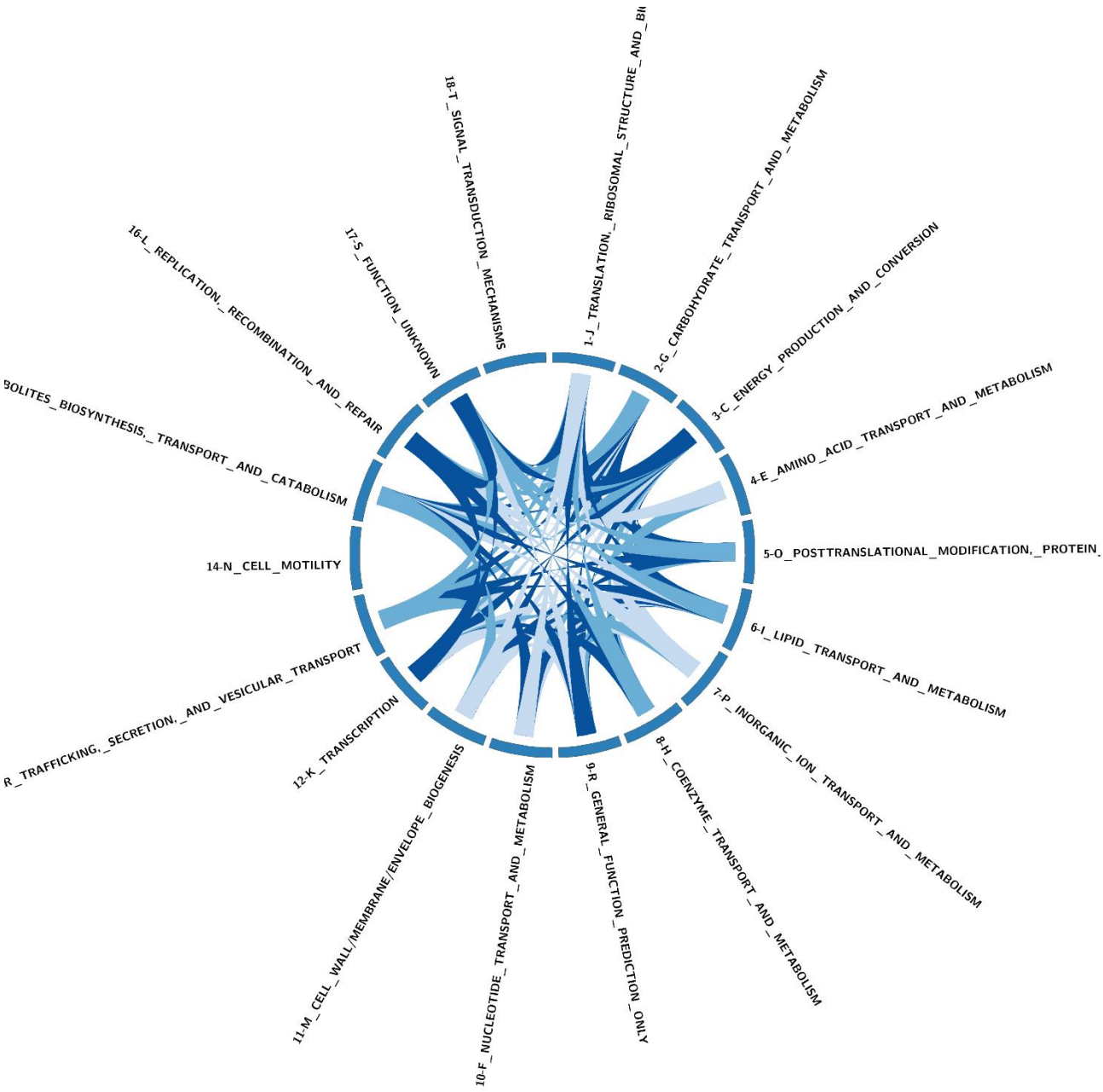


Fig. 4.4: COGs network in high functioning ASD group.

High functioning ASD network includes also the two edges of the nonASD group network, together with further 73 edges. The comparison between high functioning ASD children and nonASD group provided a HIM = 0.40.

Instead, following figure shows the low functioning children CIRCOS (Fig. 4.5).

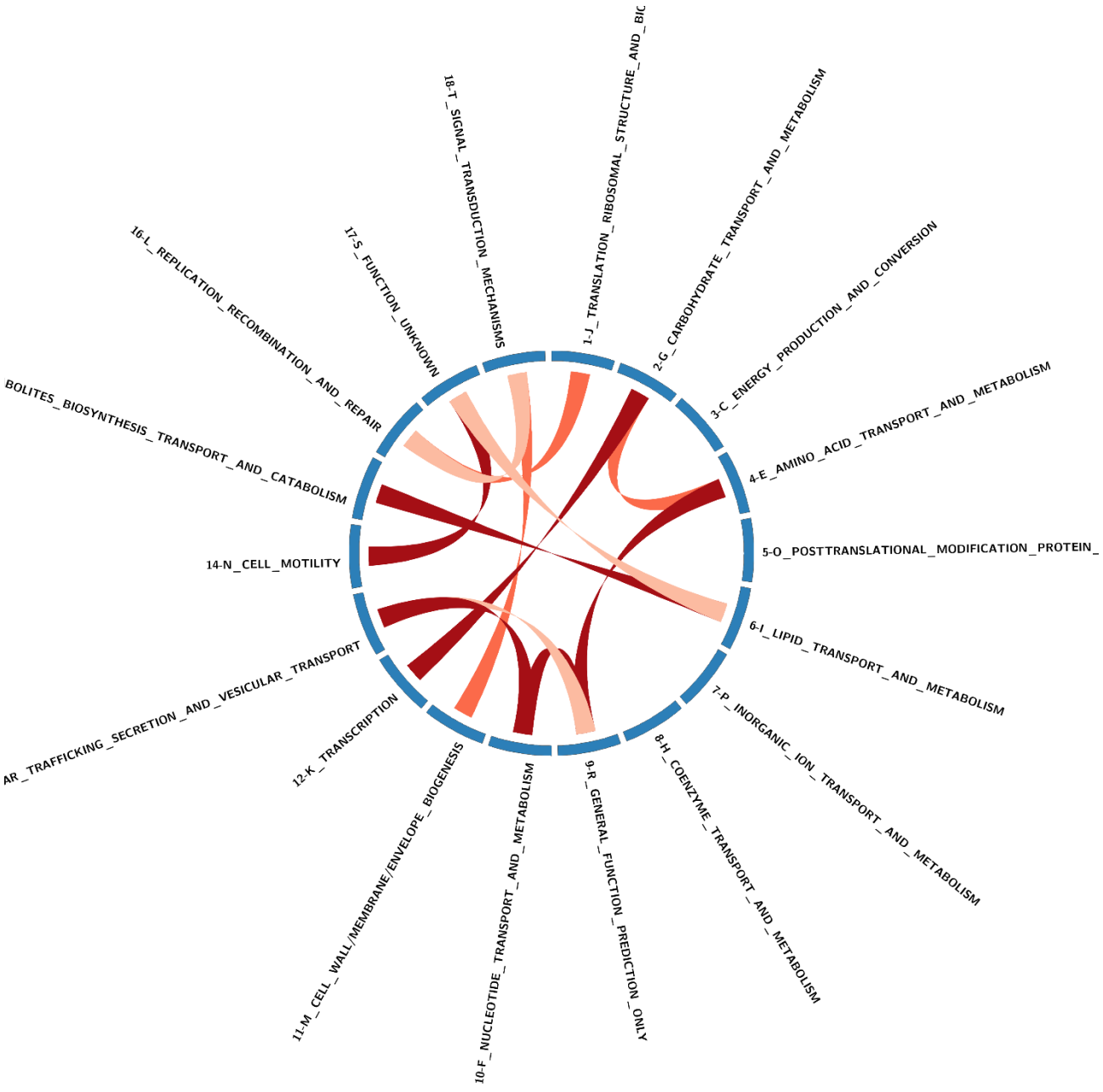


Fig. 4.5: COGs network in low functioning ASD group.

This network does not include the two edges of the nonASD group network, but presents 12 other different edges.

The comparison between low functioning ASD children and nonASD group provided a HIM = 0.20.

Subgroups among ASD children

A network analysis comparing high and low functioning ASD children was also performed, resulting in a HIM = 0.44.
In particular, both networks share following 5 edges (Tab. 4.9), whereas high functioning group has 70 further edges and low functioning group 7 other different edges.

Node1		Node 2	
16	L - Replication, recombination and repair	1	J - Translation, ribosomal structure and biogenesis
12	K - Transcription	2	G - Carbohydrate transport and metabolism
15	Q - Secondary metabolites biosynthesis, transport and catabolism	6	I - Lipid transport and metabolism
17	S - Function unknown	6	I - Lipid transport and metabolism
10	F - Nucleotide transport and metabolism	9	R - General function prediction only

Tab. 4.9: Shared edges between high and low functioning ASD children.

Moreover, in light of the well-known influence of type of delivery on microbiota development (as already discussed in Chapter 2), network analysis on COGs were performed comparing ASD children born by vaginal delivery and by C-section (Fig. 4.6 and Fig 4.7.). A HIM = 0.46 was obtained.

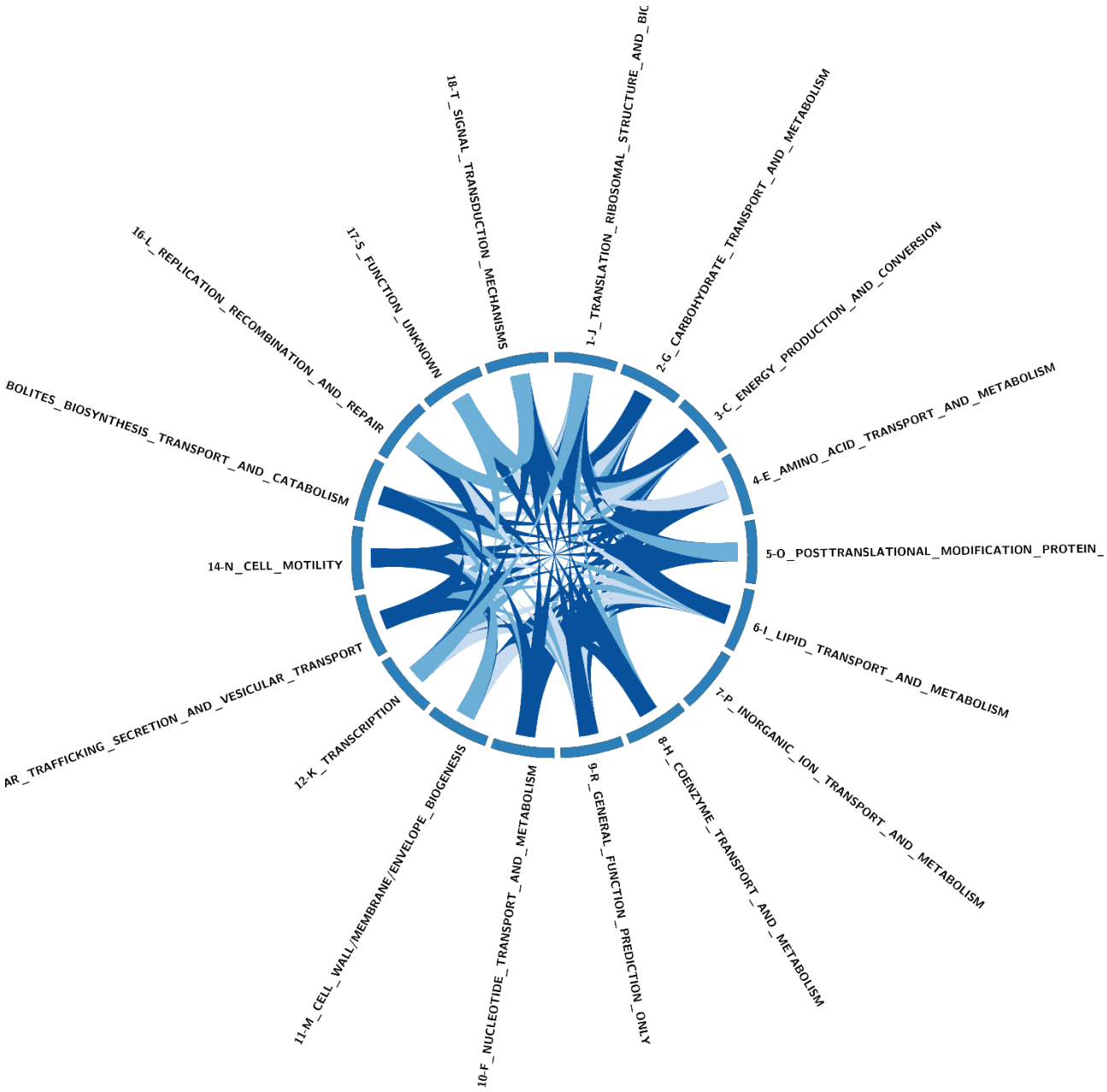


Fig. 4.6: COGs network in ASD children born by vaginal delivery.

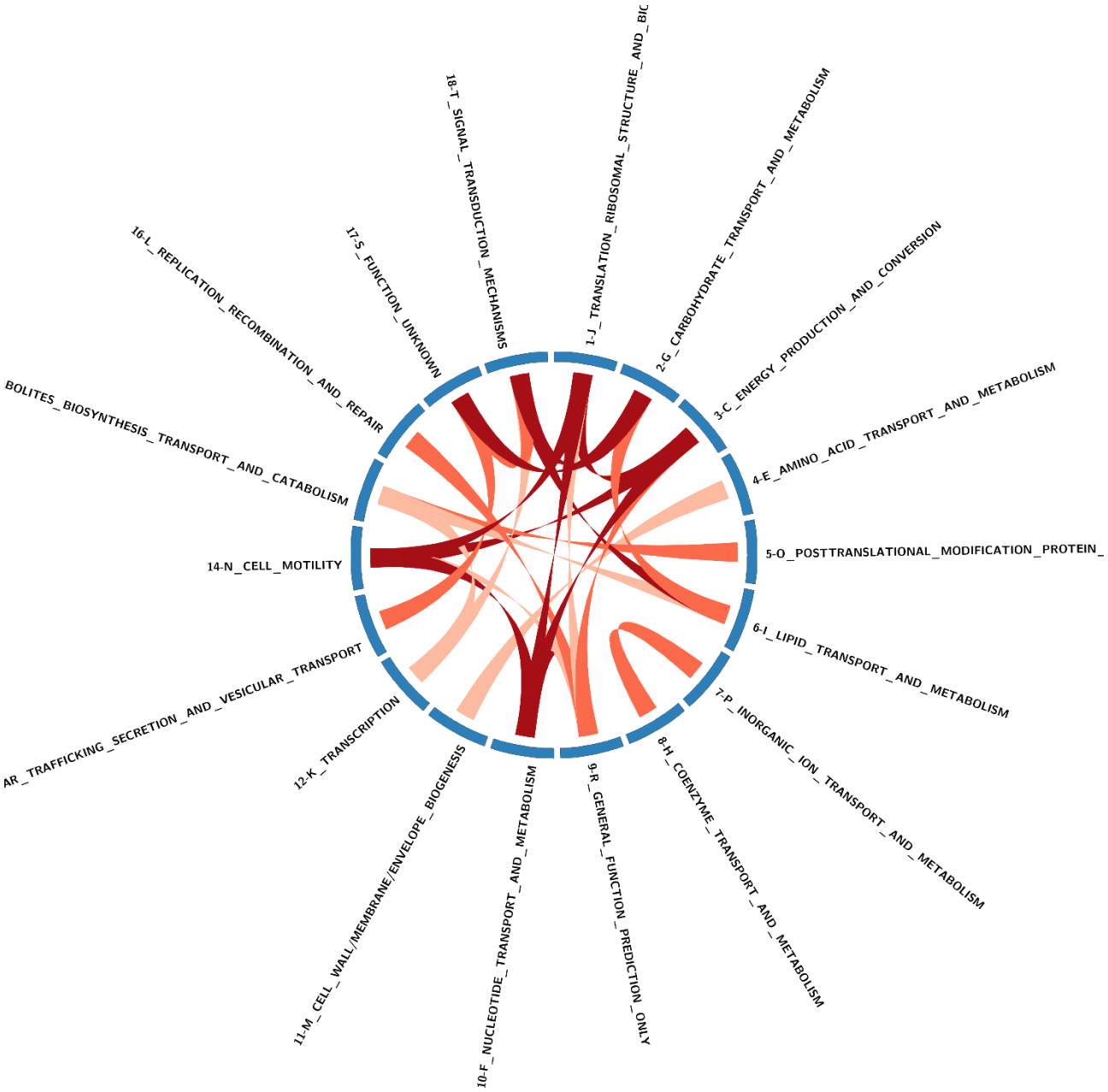


Fig. 4.7: COGs network in ASD children born by C-section

In particular, both networks share following 16 edges (Tab. 4.10), whereas vaginal delivery group have 74 further edges and C-section group 7 other different edges.

Node1		Node 2	
3	C - Energy production and conversion	1	J - Translation, ribosomal structure and biogenesis
9	R - General function prediction only	1	J - Translation, ribosomal structure and biogenesis
10	F - Nucleotide transport and metabolism	1	J - Translation, ribosomal structure and biogenesis
6	I - Lipid transport and metabolism	2	G - Carbohydrate transport and metabolism
17	S - Function unknown	2	G - Carbohydrate transport and metabolism
18	T - Signal transduction mechanisms	2	G - Carbohydrate transport and metabolism
9	R - General function prediction only	3	C - Energy production and conversion
10	F - Nucleotide transport and metabolism	3	C - Energy production and conversion
11	M - Cell wall/membrane/envelope biogenesis	4	E - Amino acid transport and metabolism
15	Q - Secondary metabolites biosynthesis, transport and catabolism	5	O - Posttranslational modification, protein turnover, chaperones
15	Q - Secondary metabolites biosynthesis, transport and catabolism	6	I - Lipid transport and metabolism
18	T - Signal transduction mechanisms	6	I - Lipid transport and metabolism
14	N - Cell motility	9	R - General function prediction only
16	L - Replication, recombination and repair	9	R - General function prediction only
14	N - Cell motility	10	F - Nucleotide transport and metabolism
18	T - Signal transduction mechanisms	12	K - Transcription

Tab. 4.10: Shared edges between ASD children born by vaginal delivery and by C-section.

To conclude, a network analysis on COGs were performed comparing ASD children with and without gastrointestinal disorders, hypothesizing that microbiota activity could be related to this condition (Fig. 4.8 and Fig. 4.9). A HIM = 0.36 was obtained.

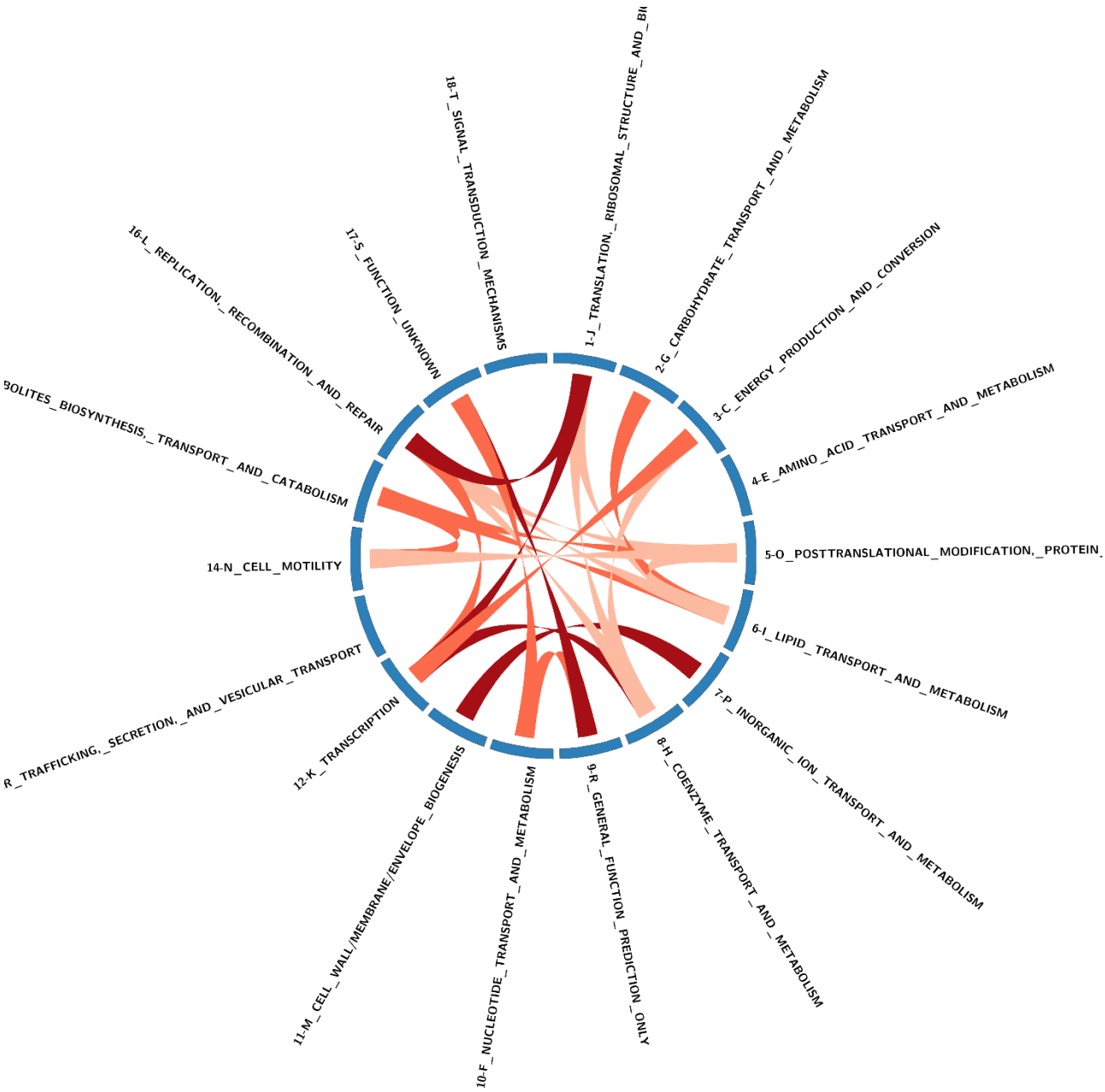


Fig. 4.8: COGs network in ASD children with gastrointestinal problems.

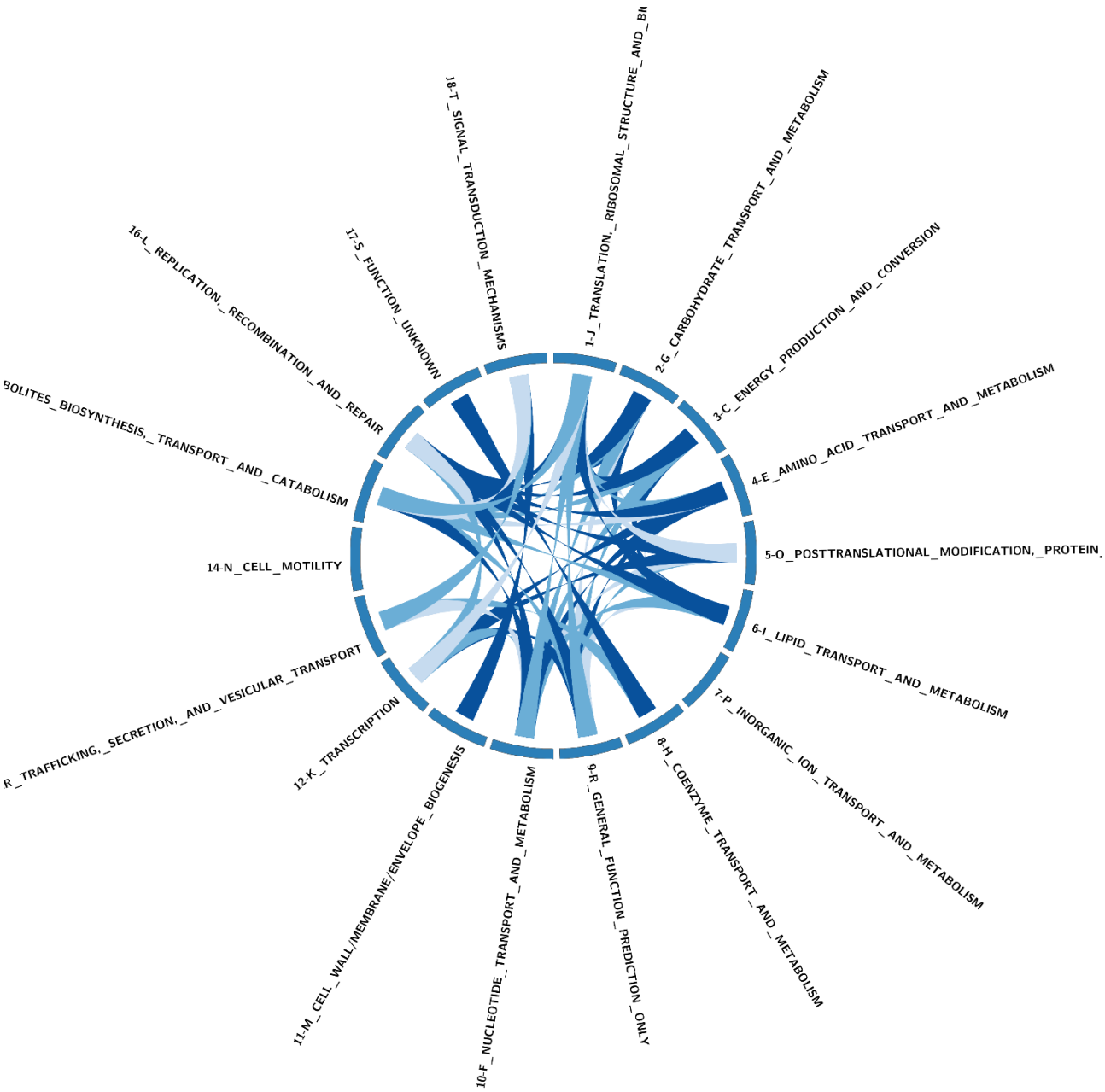


Fig. 4.9: COGs network in ASD children without gastrointestinal problems

Both networks share following 9 edges (Tab. 4.11), whereas the network related to ASD children with gastrointestinal problems has other 11 edges and the network related to ASD children without gastrointestinal problems has 44 further edges.

Node1		Node 2	
6	I - Lipid transport and metabolism	1	J - Translation, ribosomal structure and biogenesis
12	K - Transcription	1	J - Translation, ribosomal structure and biogenesis
16	L - Replication, recombination and repair	1	J - Translation, ribosomal structure and biogenesis
6	I - Lipid transport and metabolism	2	G - Carbohydrate transport and metabolism
16	L - Replication, recombination and repair	5	O - Posttranslational modification, protein turnover, chaperones
15	Q - Secondary metabolites biosynthesis, transport and catabolism	6	I - Lipid transport and metabolism
16	L - Replication, recombination and repair	6	I - Lipid transport and metabolism
10	F - Nucleotide transport and metabolism	9	R - General function prediction only
16	L - Replication, recombination and repair	12	K - Transcription

Tab. 4.11: Shared edges between ASD children with and without gastrointestinal problems.

4.3.4 Univariate statistical analysis on nutrient intake

In addition, differences related to nutrient intake between ASD children and nonASD group were assessed. In fact, if present, these could cause differences in microbiota activity and could act as confounding variable for the actual relationship between microbiota and ASD.

First of all, average intake for each nutrient was calculated for each participant over the days of food diary (Fig. 4.10).

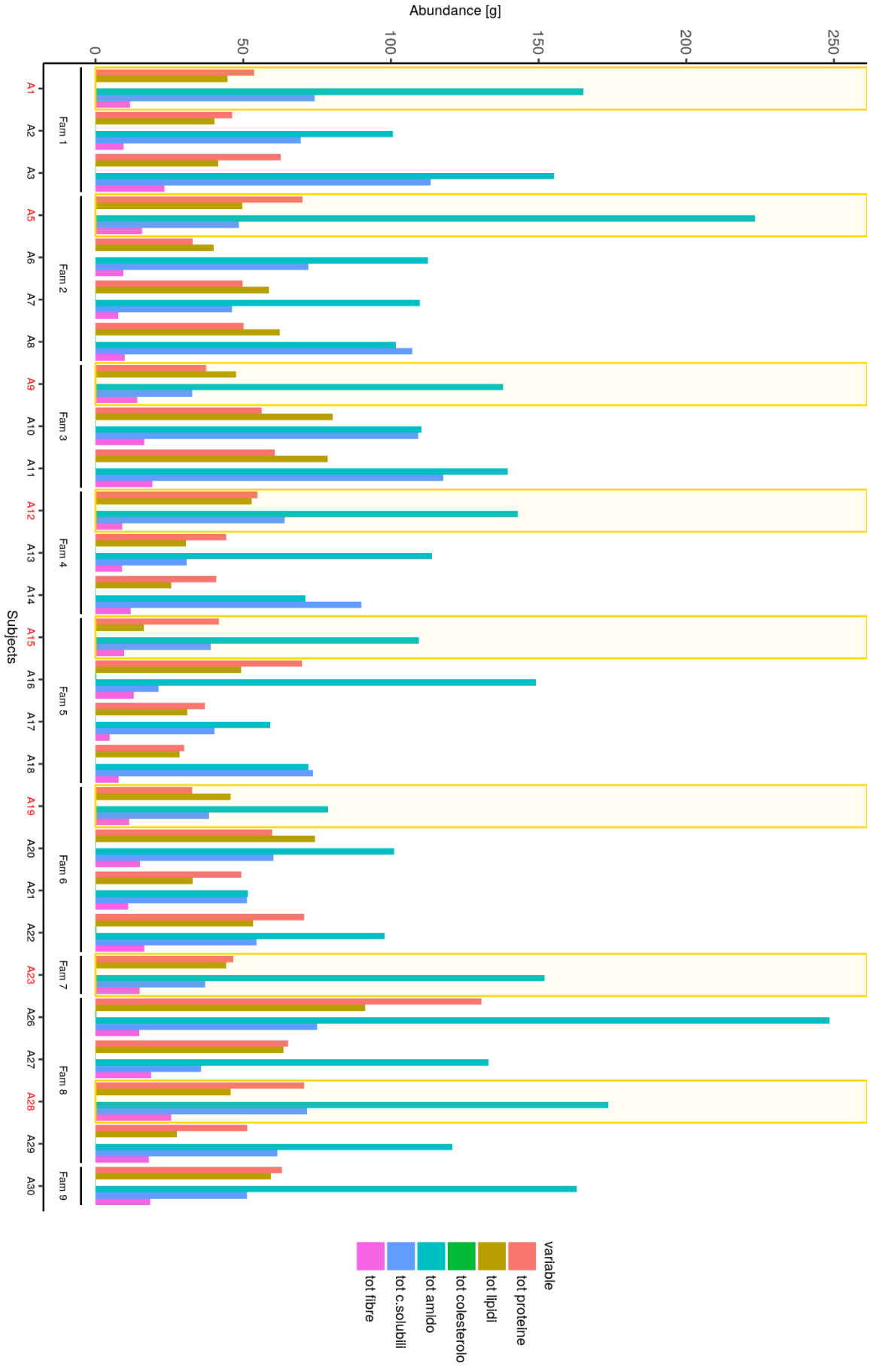


Fig. 4.10: Nutrients intake

Hence, Wilcoxon-Mann-Whitney test was performed for each nutrient comparing the ASD children group and the nonASD group (Tab. 4.12).

ASD children vs parents + siblings		
nutrients	*p-value*	*statistic (W)*
	p-val	statistic
fat	0,696434129	68
cholesterol	0,584535207	65
starch	0,10633875	45
Soluble carbohydrates	0,066197764	111
fiber	0,179533574	50

Tab. 4.11: Wilcoxon-Mann-Whitney test on nutrients intake for ASD children vs nonASD group.

4.3.5 Multivariate statistical analysis on nutrient intake

Finally, also a random forest analysis was conducted considering all nutrients together.
The best performance was obtained considering 4 type of nutrients: proteins, starch, cholesterol and fat (in the same order as in the RF output), with a MCC = 0.31, as shown in the following table (Tab.4.12).

STEP	**MCC**	**MCC_MIN**	**MCC_MAX**
1	-0.04	-0.13	0.05
2	0.17	0.032	0.29
3	0.22	0.11	0.34
4	0.31	0.17	0.44
5	0.29	0.16	0.42
6	0.29	0.15	0.44

Tab. 4.12: Random forest analysis for nutrient intake in ASD children and nonASD group.

4.4 Discussion

In recent years, metaproteomics are experiencing a notable development thanks to improvements in peptide separation efficiency and the use of highly accurate mass spectrometers. Thus, these techniques allow to assess in a rapid way the activity of gut microbiota providing COGs abundances (Kolmeder et al., 2013).

In this study, no statistically significant differences in single COGs were found between ASD children compared neither to their parents together with their siblings, nor to parents and to siblings separately. Even a random forest analysis showed a low performance (MCC=0.24).

Nevertheless, network analysis provided more interesting results. Following table summarizes HIM values related to the comparisons that were performed (Tab. 4.13).

	HIM
ASD children vs nonASD group	0.15
High functioning ASD vs nonASD group	0.40
Low functioning ASD vs nonASD group	0.20
High- vs low functioning ASD children	0.44
ASD children born by vaginal delivery- vs C-section delivery	0.40
ASD children with- vs without gastrointestinal problems	0.36

Tab. 4.13: Networks analysis related to different groups comparisons.

Even though all HIM values are rather low, high functioning ASD children seem to differ from the non ASD group more than low functioning ASD children.

Moreover, the nonASD group shows a very small number of edges (2) compared to the group of ASD children considered together (11), and the group of high functioning (75) and low functioning (12) ASD children. Therefore, a less variability in microbiota activity among ASD children could be hypothesized.

Considering only the ASD group, similar HIM values were found for comparisons using cognitive level (HIM=0.44) and type of delivery (HIM=0.40) as criteria, whereas the presence of gastrointestinal problems seems to have a slightly lower effect on COGs diversity (HIM=0.36).

To conclude, no statistically significant differences were found in single nutrients intake between ASD children and the nonASD group. Also in this case, RF analysis showed a low MCC value (0.31), although slightly higher if compared to RF analysis on COGs (0.24). These results suggest to exclude a possible influence of different dietary habits on COGs among the participants in this study.

4.5 Conclusion

Gut microbiota composition and activity is a complex system influenced by many different factors, as already discussed in this thesis.

Therefore, although metaproteomics techniques allow to conduct analysis with increasing sensitivity, a reliable evaluation of these results requires a large number of participants. For this reason, this study should be considered a pilot study.

Nevertheless, possible differences in microbiota activity between high and low functioning ASD children could suggest the existence of subgroups among ASD children not only on a cognitive level (as already recognized), but also on the physiological one.

In this sense, also other phenotypes differences, such as gravity of autistic symptoms, should be taken into consideration in the future. In particular, regarding this topic, it would be interesting to assess also the presence of autistic traits in parents and siblings of ASD children (Broader Autism Phenotype, Dowson et al., 2002) and related possible differences in COGs.

To conclude, demonstrating the existence of a peculiar microbiota activity in ASD or at least in a subgroup could allow to identify a biomarker of this complex pathology and could help to better understand the molecular mechanism of autism spectrum disorders.

CHAPTER 5: CONCLUSION

5.1 General discussion

Although this is a pilot study, it adds some new elements in the scenario of research on microbiota in ASD.

First of all, the use of metaproteomics instead of the more common metagenomics. This choice allows to assess the actual metabolic activity of microbiota.

Another new aspect of this work is the attempt to characterize ASD participants very well, with regard not only to cognitive-behavioral characteristics, such as IQ and symptoms gravity, but also taking into consideration different variables that can influence microbiota. For this purpose, also a new interview was developed.

Another point is the idea to involve not only ASD children but also their biological parents and siblings to better evaluate genetic proximity.

Thus, possible differences between high functioning and low functioning ASD children compared to each other and to the group of parents and siblings were found. Further studies with a larger sample size should be conducted to better assess these findings.

Considering the results of the present work, it would be also worth for the future to better address the tendency of a certain percentage of ASD children to show a rapid reduction of the variety of food after an initial completely normal diet. This could be considered an early symptom of the disease and helps perhaps to come to an earlier diagnosis.

Moreover, it could be interesting to assess whether the food selectivity profile remains the same also at school, which is not the case of some of ASD children involved in this study. This information could allow to better understand if food selectivity depends on the characteristics of the single food rather than on a rigid pattern of thinking, following which some foods are associated with the home context and others not. This is interesting because it opens new perspectives of intervention with the aim to introduce new foods in the restricted diet of the child: if a single food is eaten at school it could be possible to obtain that the child eats it at home as well.

Furthermore, it is critical to keep in mind that autism spectrum disorders include different cognitive-behavioral phenotypes. Therefore, it is important to conduct studies on the microbiota dividing participants in subgroups as uniform as possible for cognitive-behavioral characteristics and other variables that can affect the microbiota. This makes necessary to involve a large number of participants, which has not been possible for this study. Thus, it would be fundamental to create a research network willing to work together with the same procedures in order to overcome this limitation.

Unfortunately, this is not the only limitation this work is affected by. In fact, since there are many factors that can interact and influence microbiota composition and activities, considering all them with a good level of precision is not easy.

For example, regarding to the diet, the use of food diaries or food questionnaires have critical aspects: people are asked to estimate the quantity and frequency of various eaten foods but mistakes are around the corner, e.g. because of the memory or personal representations of the different quantities (e.g. a cup). Not to mention the little care of some people in reporting the information in a complete manner, without the possibility for the researcher to recover this information after in time. A partial solution might be to use the so called 24h-recall: each participants is called by the researcher every 24 hours on the phone and must list what he/she ate during the last 24 hours, providing also quantities. This improves the completeness of the reported data, but not necessarily the precision, which remains dependent on the accuracy in estimating by the interviewee.

The same goes for the interview, where parents are asked to remember things also about the past of their child. Moreover, they are asked about the presence of gastrointestinal problems in their children. For this aspect, an evaluation made by a clinician would be more reliable.

Again with respect to the interview, the total duration is rather long (from 45 min to 2 hours) depending on how much the parents have to tell. The section dedicated to the dietary questionnaire tends to weigh it down enough and would be worthwhile to make a reflection on whether to amend the list of foods and transform this session in a questionnaire filled by the parents separately.

But it will be even better to create a food frequency questionnaire validated for ASD children on the Italian population: this could be a direction in which to extend this work in the future and it would also allow to render homogenous the evaluation of food selectivity, an aspect that lacks a clear definition and a proper assessment tool.

5.2 Concluding remarks

Unfortunately, so far there are no diagnostic biomarker for ASD, or at least for a predisposition, that could be used earlier than the diagnosis based on cognitive-behavioral aspects. In fact, this diagnosis can be performed starting from 2-3 years of age, but it often takes place later, especially in milder cases. But it is well known that an earlier diagnosis means an earlier possibility to start with therapies with better outcomes for the child.

If there were actually alterations in the microbiota of ASD children, these could serve as a biomarker. It could also be possible to develop treatments with probiotics to restore the microbiota to that typical one of a child of the same age and geographical origin.
This is especially true in the case of children with both ASD and gastrointestinal problems: probiotics could improve gastrointestinal discomfort and reduce in this way some problematic behaviors often associated with gastrointestinal disorders, for example in no speaking ASD children.
Furthermore, it could be even possible to create preventive interventions with appropriate doses of probiotics soon after birth. But the road is still very long.
In fact, given the numerous variables involved, studies on the microbiota require to involve very large numbers of participants: this increases the costs and the time needed for the analysis.
I have had direct experience in these 3 years of work: it takes a long time to find people willing to take part into to the study, especially if the intention is to involve the whole family, like I did.
It also depends on the timing of analysis conducted by specialized laboratories and this often means a long wait to get the results.
Furthermore, even the single analysis is still quite expensive. It may be possible that the costs will be reduced in the future, but at the present it takes a large availability of funds to engage the number of participants required to obtain sufficiently robust results.
Nevertheless, more research in this field could open very intriguing perspectives.

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