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MicroRNA profiling in body fluids of patients affected by Autism Spectrum Disorder, Tourette syndrome and Arnold-Chiari syndrome

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Abstract

The growing prevalence of neurodevelopmental disorders (NDDs) is leading to a significant social impact and a considerable interest in the scientific community. In the last decades, there has been an increasing need for society to invest in early diagnosis and provision of appropriate healthcare and educational services. However, the multifactorial and heterogeneous nature of NDDs makes the etiopathogenesis poorly understood and the diagnostic process challenging**,** especially in cases of overlapping clinical symptomatology. Consistent evidences indicate that complex and dynamic interactions among genetic, epigenetic and environmental factors influence the etiology of neurodevelopmental disorders. Morphological abnormalities of the central nervous system also represent a significant problem of child neurology, which dates to the embryonic or foetal period. However, the etiology of neural malformations is still unknown and the molecular basis has not yet been fully clarified.

Using liquid biopsies could be an extremely valuable and innovative approach to strengthen the standard clinical diagnostic processes and to further explore the molecular basis of such elusive disorders, for example Autism Spectrum disorder (ASD) and Tourette syndrome (TS), as well as Arnold-Chiari (AC) syndrome. In fact, the use of serum or saliva provides a more effective and suitable alternative to the conventional tissue biopsies, which often lack or are not easily accessible to researchers for the study of neurodevelopmental anomalies.

Over the past few years, circulating microRNAs (miRNAs) have attracted increasing attention for their consistent presence in all body fluids, their specific expression patterns related to both physiological and pathological conditions, as well as their remarkable stability in the extracellular environment. For all these reasons, circulating miRNAs have been progressively emerging as promising diagnostic, prognostic and predictive biomarkers in neurodevelopmental disorders.

Based on these premises, we conducted two research projects aimed to profile circulating miRNA expression (i) in saliva from ASD patients and (ii) in serum of AC, TS and comorbid ACTS patients, compared to neurologically unaffected individuals. Moreover, the ASD project aimed to verify the existence of a molecular correlation between salivary miRNA dysregulation and oral microbiota alterations, as well as their potential contribution to ASD pathogenesis.

Many studies proposed the use of circulating miRNAs as promising biomarkers for several pathologies, but very few studies profiled salivary miRNAs in ASD, much fewer ones explored serum miRNAs in TS context, no one in AC syndrome and the very rare comorbid condition between AC syndrome and TS (ACTS). In addition, while mounting evidences suggest a key role for the gut microbiota in ASD, the etiopathogenetic contribution of microorganisms living in the oral cavity has not been satisfactorily explored.

In our saliva study, in order to investigate salivary ASD-related miRnome and microbiome alterations, we performed a combined approach of miRNA expression profiling by NanoString technology, followed by validation experiments in qPCR, and 16S rRNA microbiome analysis on saliva from 53 ASD and 27 neurologically unaffected control (NUC) children.

We revealed that miR-29a-3p and miR-141-3p were upregulated, while miR-16-5p, let-7b-5p, and miR-451a were downregulated in ASD compared to NUCs. Microbiome analysis on the same subjects revealed that *Rothia, Filifactor, Actinobacillus*, *Weeksellaceae, Ralstonia, Pasteurellaceae* and *Aggregatibacter* increased their abundance in ASD patients, while *Tannerella, Moryella* and TM7-3 decreased. Neuropsychological scores related to typical ASD cognitive impairments, especially for anomalies in social interaction and communication, were statistically associated to variations of both miRNAs and microbes. Among miRNA/bacteria associations, the most relevant was the negative correlation between salivary miR-141-3p expression and *Tannerella* abundance. This interesting finding suggests the occurrence of a potential cross-talking between circulating miRNAs and resident bacteria in saliva of ASD patients.

To evaluate the diagnostic accuracy of miRNAs and microbiome taxa, we performed univariate and multivariate ROC curve analyses. The first analysis led to moderate but significant Area Under Curves (AUCs) values, while the second one, which evaluated the combination of all miRNAs and bacteria, resulted in a remarkable increase of the predictive performance to discriminate ASD patients from NUCs. In particular, the combination of the 5 salivary miRNAs and 4 bacterial species (i.e., *Filifactor*, *Moryella*, *Tannerella* and *TM7-3*) significantly represented the best-fit performing model for ASD diagnosis, suggesting a potential application as non-invasive biomarkers. Pathway enrichment analyses were computed to investigate the potential biological impact associated with the differential expression of the miRNAs reported in this study. The computational data would suggest a functional involvement of differentially expressed (DE) salivary miRNAs in molecular signaling pathways related to the development of cognitive functions and often reported to be dysfunctional in ASD.

In our serum study, we aimed to identify serum microRNA expression profiles as molecular fingerprints for AC syndrome, TS and ACTS, by using NanoString technology. For this aim, 10 AC patients, 11 ACTS patients, 6 TS patients, and 8 unaffected controls (NC) were recruited. Nine miRNAs resulted significantly differentially expressed: let-7b-5p (upregulated in ACTS vs TS); miR-21-5p (upregulated in ACTS vs AC; downregulated in AC vs TS); miR-23a-3p (upregulated in TS vs NCs; downregulated in AC vs TS); miR-25-3p (upregulated in AC vs TS and NCs; downregulated in ACTS vs AC); miR-93-5p (upregulated in AC vs TS); miR-130a-3p (downregulated in ACTS and TS vs NCs); miR-144-3p (downregulated in ACTS vs AC; upregulated in AC vs TS); miR-222-3p (upregulated in ACTS vs NCs); miR-451a (upregulated in AC vs TS and NCs; in ACTS vs NCs). The altered expression of miRNAs was statistically correlated to neuroimaging and neuropsychological anomalies, especially for low intelligent quotient scores, cranial disproportions and behavioral impairments. Furthermore, computational analyses indicated that DE miRNAs are involved in AC syndrome and TS pathomechanisms.

Our liquid biopsy-based investigation approach aims to pave the way to valuable, non-invasive, unbiased molecular tools for such elusive neurodevelopmental disorders and neurological malformations and to provide some clues into their molecular basis.

Table of contents

List of Figures and Tables

Chapter 1. A focus on Neurodevelopmental Disorders

Neurodevelopmental disorders (NDDs) represent a very wide range of disabilities, resulting from anomalies in the development of the central nervous system which lead to impairments in cognition, communication, behaviour and/or motor function [1].

Deficits manifest early in childhood and are often subject to maturational changes, mostly persisting during adult life [2]. According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), the wide range of developmental impairments includes autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), intellectual disability, communication disorders, specific learning disorders, motor disorders, tic disorders and Tourette syndrome (TS), with onset in the developmental period, and major neurocognitive disorders later in life (American Psychiatric Association, 2013).

These disorders have an overall influence on social, occupational and academic functioning in the daily life of affected children [1], leading to an accumulation of co-occurring adversities over time. The impact on families and society is not far behind: parents with children affected by NDDs, particularly mothers, often report a lower quality of life, increased stress, instability, depression and anxiety, as well as more physical health problems [3, 4]. In the last decades, there has been an increasing need for society to invest in early diagnosis and in the provision of appropriate healthcare and educational services. The rising awareness has led, in part, to the alarming and increasing prevalence especially of ASD and ADHD.

It has been reported that NDDs affect more than 3% of children worldwide and commonly affect males more than females [5]. The exact prevalence rates differ among countries and may depend on geographical differences in healthcare services, as well as, in diagnosis provision. An incorrect diagnosis due to low accuracy could be a huge issue. The current diagnostic process is limited to a careful evaluation performed by a child and adolescent neuropsychiatrist expert, who conducts intellectual, neuropsychological and adaptive behaviour assessments.

The first step of the assessment process is to delineate the framework of the child's early developmental history and current concerns on symptomatology, through the assistance of reports given by parents and teachers and following the DSM-5 criteria [6]. However, it is possible that the accuracy of parental responses and clinical interpretations could be affected by many factors and, to complicate the issue, in clinical practice, no biomarkers are currently available to objectively support the diagnose of NDDs or to differentiate among them. In addition, it is widely accepted that delineating psychiatric disorders is challenging because of the complexity and heterogeneity of phenotypes, as well as the frequent co-occurrence of disorders. Overall, a robust, unbiased NDD diagnosis is still necessary worldwide for early prognostic and therapeutic interventions.

Many individuals affected by NDDs often manifest the occurrence of more than one disorder, notably for TS and ASD (88% and 70% of individuals, respectively, with at least one co-occurring condition). Comorbid conditions complicate the clinical picture of the patient compared to a single "pure" disorder, due to an increased symptomatologic severity, a worsening of the quality of life, a poor long-term prognosis and a minor effort on interventions [7, 8].

The therapeutic strategies often involve a combination of pharmacological and non-pharmacological interventions, together with home- and school-based programs. However, the multiple layers of NDD complexities make treatments still challenging [9, 10].

The complex understanding of NDDs is mainly due to the high heterogeneity, not only in terms of therapy responses, but also in terms of the overlapping of clinical symptomatology among the disorders, as mentioned above, and in terms of etiopathogenesis, which is multifactorial [1]. Cognitive development is a complex result of a combination of genetic, epigenetic and environmental factors, but the pathogenesis mechanisms are still not clearly understood [11]. Myriad cellular and molecular processes finely and strictly regulate brain development over an extended period of time [12]. In particular, genome-wide profiling studies revealed spatio-temporal differences in gene expression across brain regions and developmental time points [13]. The high vulnerability to abnormal and accidental events, during prenatal and postnatal neurodevelopment, can lead to a more susceptibility

for CNS disfunctions in cognition and behaviour, resulting in neuropsychological and neuropsychiatric diseases [14].

In the last decades, many advancements have shed new light on the NDD genetic architecture [14, 15]. Copy number variants (CNVs) have been considered one of the strongest risk factors for ASD and other NDDs, and due to their size and recurrence, they affect the function of many genes involved in neurodevelopment [15-17]. Furthermore, a large number of single nucleotide polymorphisms (SNPs) in non-coding regions have been demonstrated to be susceptible for NDDs [15]. It is worth noting that these recurrent mutations are not predisposing factors strictly associated to one single disorder, but with many ones, suggesting that the genetic etiopathology of NDDs is highly heterogeneous [15].

Various environmental factors contribute to different possible scenarios of NDD phenotypic expression. According to many experimental and epidemiological studies, the exposure to environmental toxicants during pregnancy (e.g., prenatal nicotine exposure, monomer of bisphenol A, persistent organic pollutants and heavy metals) have a negative effect on neurodevelopment, notably increasing the risk of NDD insurgency. Although the molecular mechanisms have not been clarified, it has been suggested that epigenetics may work as the linking mechanism between environmental toxicants and neurodevelopmental disorder occurrence [18].

Further investigation through *in vitro*, *ex vivo*, *in vivo* models are still needed to contribute and clarify the etiopathogenesis and risk factors of these broad-based conditions.

Morphological abnormalities of CNS also represent a significant problem of child neurology, which dates to the embryonic or foetal period. Among the most frequent congenital anomalies, neural tube defects may arise from failure of the neural tube to spontaneously close during the first weeks of embryonic development [19]. Other structural defects in the cerebellum, called Chiari malformations, are considered congenital conditions, except for some acquired forms of the condition. Chiari malformations have been supposed to be the result of a developmental defect of occipital somites originating from the paraxial mesoderm, which lead to a volumetrically-reduced posterior cranial fossa, an overcrowding of the cerebellum and a downward cerebellar herniation [20].

However, the etiology of Arnold-Chiari (AC) syndrome is still unclear and debated: exploring the molecular basis could provide valuable insights into the mechanisms underlying the etiopathogenesis of the disorder and deal with the complexity of differential diagnosis in case of comorbid conditions.

1.1. Autism Spectrum Disorder

1.1.1. A clinical framework: definition, symptomatology and prevalence

Autism spectrum disorder is the comprehensive designation for a group of multifactorial neurodevelopmental conditions characterized by two core symptoms: (i) impairments in social communication and interaction, (ii) restrictive and repetitive patterns of behaviour, interests or activities (American Psychiatric Association, 2013).

According to the DSM-V, the clinical definition is currently used to conceptualize the broad spectrum of the neurodevelopmental disorders previously described in DSM-IV under the umbrella of the Pervasive Developmental Disorders, as all members of a single and broader diagnostic category [21]. The broadening of ASD concept allowed to break down the boundaries among the distinct clinical entities, which show similarity in the core symptoms. However, the considerable clinical heterogeneity remains one of the hallmarks of ASD [22]. Indeed, the wide spectrum of impairments and the different clinical manifestation among ASD people lead to a large variability of ASD phenotypes: different areas of functioning may be affected with a high variability and symptoms may widely vary among ASD individuals, into a range from mild to severe, according to the classification of ASD severity [22, 23]. Despite the onset of ASD signs typically emerge within the first 3 years of life, the symptoms can be subtle and no easily detectable before school age [22, 24]; moreover, they typically persist during adolescence and adulthood [25].

In daily life, people with ASD typically manifest impairments on important functioning as dealing with a conversation, interpreting nonverbal interactions, socialising with peers, adapting to situations or focusing on appropriate items (American Psychiatric Association, 2013). Early detection and medical treatments, as well as, educational interventions through an appropriate healthcare service, are crucial for improving the quality of life of affected children and for alleviating family distress [26].

In the past two decades, the prevalence of ASD has been increased. According to Autism and Developmental Disabilities Monitoring Network (ADDM) estimates, in the US, the rates more than

doubled between 2000-2002 and 2010-2012; in 2014, US surveillance reports from the Centres for Disease Control and Prevention (CDC) estimated a prevalence of 1.68% in 8-years old children, representing a 150% increase compared to the year 2000 [27]. A combination of a greater public awareness and the reclassification of disorders, as well as, the changes in diagnostic criteria and the improved assessment process, may impact the resulting higher prevalence [28, 29]. It is worth noting that differences in race, ethnicity, culture, socio-economic status and the healthcare service provision may affect the diagnosis as possible bias, thus they may influence the epidemiologic estimates. In addition, ASD is more commonly reported in boys than girls, with a 4:1 male-to-female ratio, suggesting a contribution of sex-differential genetic and hormonal factors to the incidence [30].

1.1.2 Psychopathological and medical comorbidities in ASD patients

Co-occurring conditions have been commonly identified in individuals affected by ASD, with a high prevalence [31]. In 2010, a surveillance study revealed that over 2,000 8 year-old children with ASD, 83% showed co-occurrence with other developmental disorders (e.g., language disorder, ADHD and intellectual disability), 10% with psychiatric disorders (e.g., anxiety disorder, obsessive- compulsive disorder, depression, bipolar disorder, sleep disturbance), 16% with neurological disorders (e.g., epilepsy, tic disorder) and at least 4% with medical disorders (e.g., down syndrome, fragile X syndrome) [32].

Medical comorbidities also include immune system anomalies and gastrointestinal disturbances [22]. Due to the crosstalk between immune and neural systems, the role of immune system dysfunction in ASD has been widely explored over the past few years. Numerous studies identified circulating antibodies targeting brain proteins in ASD children [33], elevated levels of plasma cytokines associated with impairments in communication and behaviour [34], altered levels of immunoglobulins [35] and neuroinflammation [36].

The association of ASD with a great prevalence of immune and gastrointestinal dysregulations led to investigations on the gut microbiome of ASD patients, which is emerging as a key regulator of intestinal physiology, neuroimmunity and host behaviour.

Many studies have reported a dysbiosis of the gut microbiota in ASD patients [37]. The role of the gut microbiota is extremely important: it represents a barrier against the proliferation of pathogenic organisms [38], playing a key role in the functioning of the host immune system [39], modulating gene expression [40] and reducing inflammation [41]. Alterations of brain structure and function development are related to modifications of the gut microbial composition because interactions between the Central Nervous System (CNS) and the gut microbiota (the so-called gut-brain axis) are already established during fetal life and are maintained in adults.

The understanding of the complex relationship between co-occurring disorders and their characterization is still limited. Comorbid conditions may significantly complicate the diagnosis reflecting in an extremely high clinical heterogeneity, thus, an appropriate and careful clinical evaluation is decisive, above all in these cases [42]. The co-occurrence may lead to the worsening of ASD-related symptoms and a high risk of mortality [43]. Finally, the comorbidity burden requires a broad multidisciplinary management to achieve more specific and efficacious interventions.

1.1.3. Current ASD Diagnosis and potential bias

The early recognition of ASD is essential for starting tailored interventions as soon as possible.

The better comprehension of early features of ASD has contributed to acquire reliable diagnoses in early childhood [44-46]. The first signs rely on difficulties to pay attention, making eye contact, social smiling, sharing interests. However, symptoms are not clear before the first 3 years of life and sometimes may be detected later [47]. Commonly, pediatricians evaluate early symptoms on 18-24 months children using screening instruments such as the CHAT (Checklist for Autism in Toddlers), through reports from parents, and then the ASD suspicion is confirmed or excluded by a childhood neuropsychiatrist [24]. Currently, the clinical diagnosis is conducted following the diagnostic criteria,

revised in 2013 and updated with the DSM-V, as well as the ASD severity categorization. The diagnostic criteria rely on observations of behavioural features, while the severity classification accounts for the effect of symptoms on adaptive functioning, resulting in the levels of support that an affected individual is needed (APA, 2013).

The "gold standard" diagnostic process for ASD evaluation is the combination of both Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R) [48]. ADOS consists of a semi-structured assessment on social communication, interaction, play, based on the direct observation of the child behavioural features by specialists in child psychiatry [49]. On the other hand, ADI-R consists of a structured interview conducted by neuropsychiatric experts to parents and caregivers about social communication, interaction and stereotyped behaviour, together with the developmental history of the child [50].

Despite the good discrimination capacity in the evaluation of ASD, these tools may stumble because of several bias from many contextual factors. Firstly, since the clinical assessment strictly relies on the direct observation of child behaviour and interviews addressed to parents, it may be vulnerable to subjectivity. Secondly, the occurrence of comorbid conditions, the variability in clinical presentation and symptomatology may seriously complicate the differential diagnosis. Thirdly, the gender, age, ethnicity, cultural barriers and socioeconomic status, thus the access to healthcare services, may affect the diagnosis's accuracy and, in other cases, the provision [22, 51].

The use of ASD biomarkers could considerably reduce the influence of the above-mentioned contextual factors and support the clinical discrimination process in an unbiased way. However, despite there has been a growing interest [52], the identification of potential biomarkers is still challenging, but necessary.

1.1.4. Exploring the etiology of ASD

Despite the widespread research, the causes of ASD are still poorly understood and are still subject to numerous debates [21, 23]. A growing body of evidences supports that the etiology of ASD is the complex result of a combination of genetic, epigenetic and environmental factors [53-56].

The genetic architecture of ASD is very complex and highly heterogeneous, however it makes a very strong contribution to the etiology of the disorder [57].

Indeed, twin studies indicated that monozygotic twins show a higher concordance rate for ASD risk (70-90%) compared to dizygotic twin pairs (up to 30%) [58-60], revealing a significant genetic heritability [61]. In addition, family studies showed that siblings or first-degree relatives of ASD affected individuals have a higher percentage of risk for ASD in comparison to the general population [57, 62].

Many genetic and disparate variants have been associated to ASD. Rare genetic risk factors from ASD-related syndromes (e.g., Fragile X syndrome, tuberous sclerosis, Rett syndrome, Timothy syndrome), rare chromosomal aberrations, rare *de novo* and some inherited copy number variations (CNVs) and highly penetrating gene mutations [63-66], have been estimated to have an overall contribution of approximately 25% to ASD risk [63]. Moreover, genome-wide association studies (GWAS) have independently identified single nucleotide polymorphisms (SNPs) associated at few different loci [53, 67-69]. However, it has been reported that these individual common variants have a small effect size, thus, a small substantial impact on ASD risk [57].

These findings indicate that no singly causative gene and no common risk loci have been identified for ASD, rather, the complex genetics of ASD is a product of multigenic contributions from rare variants, with large effect sizes, and common variations, with modest effect sizes [57, 70]. The striking degree of genetic heterogeneity seems to be strongly related to the clinical variability of ASD. So far, hundreds of genes have been suggested as candidate genes associated to ASD [71] and converge in important biological processes and pathways crucial for the neurobiology of ASD [72-75]. The most

frequently reported genes are those involved in synaptic functions, such as postsynaptic cell adhesion molecules neuroligins (NLGN4X and NLGN3), postsynaptic scaffolding proteins (SHANK2 and SHANK3) and presynaptic cell adhesion molecule neurexin 1 (NRXN1) [65, 75].

Beyond the genetic risk contribute, epigenetics plays a significant role in the etiology of ASD. Epigenetic mechanisms contribute to regulate the spatiotemporal expression of many genes and may work as a link in the gene-environment interaction [76, 77]. Mutations in genes involved in epigenetic regulation [78-82] and the maternal immune responses during pregnancy [83, 84] may widely affect several epigenetic mechanisms (e.g., DNA methylation, histone modifications, chromatin remodelling) and, subsequently, have a strong impact on ASD phenotypes [77].

Over the last decades, the alarming and increasing estimates of ASD prevalence led to deeply search for prenatal, perinatal and neonatal environmental risk factors [85]. Among these, the aforementioned maternal infection, inflammation, immune activation during pregnancy and all the other prenatal factors belonging to the fetal environment (e.g., sex steroids, diabetes, hypertension), as well as maternal and paternal age, diet, smoking, alcohol use, toxic exposures (e.g., air pollutants, heavy metals, pesticides) have been associated with ASD phenotypes, thus claimed to strongly influence the risk of ASD [56].

1.1.5. Macroscopic and microscopic abnormalities in ASD neurobiology

Multiple crucial biological processes occur during specific stages of neurodevelopment, including cell proliferation and differentiation, migration, maturation, as well as synaptogenesis and neural network formation (Figure 1.1) [86-88].

Working up a timeline, ASD has been considered a multistage and progressive disorder that begins from abnormal prenatal brain development and leads to postnatal heterogeneity in neural circuits, behaviour and clinical outcomes [86].

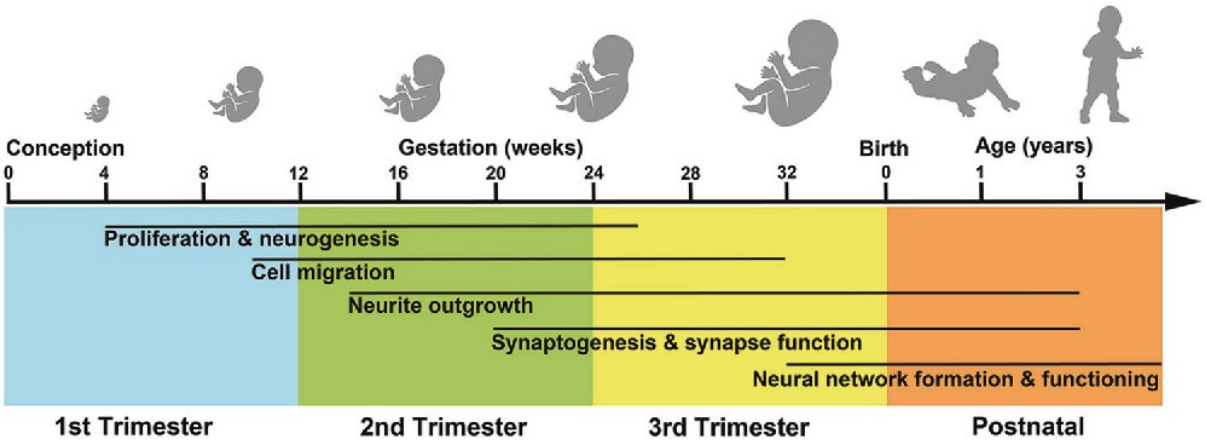


Figure 1.1. Multiple neurobiological processes occurring during fetal and post-natal brain development that are crucial for ASD pathogenesis (from *Courchesne E. et al., Mol Psychiatry, 2019*).

Alterations of signalling pathways that govern these events during development and, consistently, perturbations of gene regulatory circuits, have been widely associated with ASD [88, 89]. This cascade of pathogenic processes can begin during the 1st and 2nd trimesters, with anomalies in rates of cerebral cell proliferation, followed by neural migration and cortical laminar organization alterations [86]. It has been suggested that the overabundance of cortical neurons may represent a possible biological mechanism to support the evidence of early brain overgrowth of about 25–30% of ASD children, which proceeds during the first two years of life and may slow down during late childhood [86, 90, 91]. During the 1st trimester, the maternal immune activation (MIA), in cases of prenatal infections, may also contribute to cause brain neuroanatomical overgrowth by upregulating the cell cycle gene expression and neuronal proliferation [87]. Consequently, aberrations in cytoarchitectural organization in ASD brains, due to dysregulation of cell migration, laminar organization, neurite outgrowth, leads to a 10-fold decrease in spontaneous neural activity and impairments in synaptogenesis [87, 92, 93]. Neuroanatomical studies have reported anatomic anomalies in specific ASD brain regions such as the cerebellum, the cerebral cortex, the amygdala and hippocampus, which contribute to sensory, motor, synaptic, social cognition, language ASD-related impairments and aberrant connectivity. In particular, neuroimaging techniques have consistently revealed that ASD adult individuals display patterns of short-range "over-connectivity" and long-range or inter-regional

"under-connectivity" [89, 94]. Moreover, it has been reported that alterations in the ratio of excitatory and inhibitory cortical activity (E/I imbalance) may result from the early impairments in initial neural circuit formation or maintenance and contribute to social and cognitive deficits [90, 95]. Specifically, alterations in glutamatergic and GABAergic systems, early in development, lead to increasing the ratio of excitation/inhibition, contributing to the development of ASD [96].

Differences in how and when the prenatal disruptions may occur across ASD individuals, combined with the individual-dependent background genetics, will lead to early heterogeneity of pathophysiology and post-natal high clinical variability. Many studies in different model systems (e.g., human, mouse models) shed the light on ASD complexity at molecular level, by analyzing ASD-related genetic variants [88]. In fact, behind the macroscopic *scenario*, numerous ASD risk genes converge in specific regulatory networks, impacting multiple stages of neurodevelopment (Figure 1.2) [86].

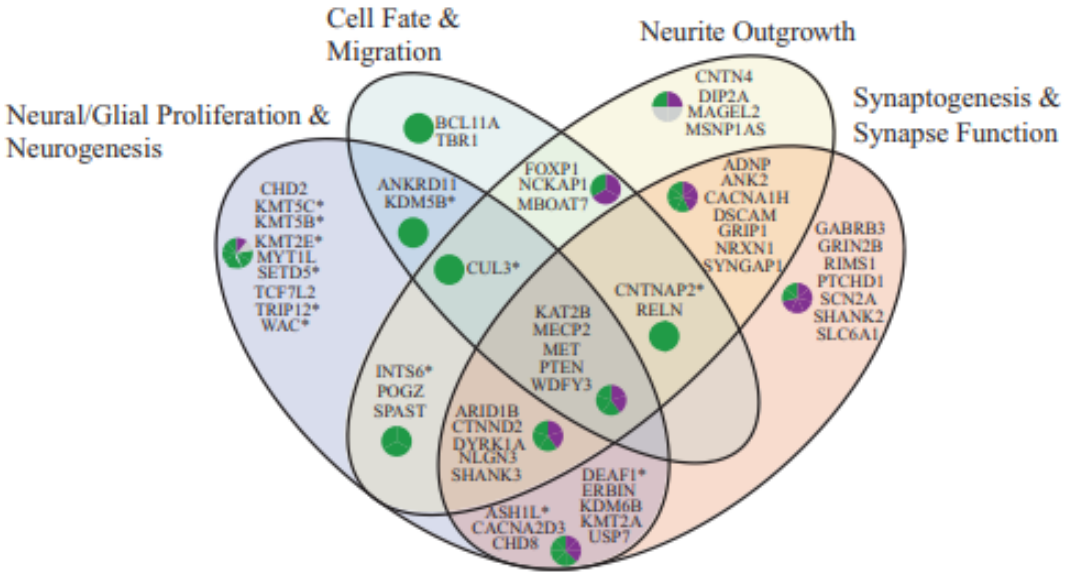


Figure 1.2. Collocation of 58 high-confidence ASD genes in four domains of neural development processes (from *Courchesne E. et al., Mol Psychiatry, 2019*). The distribution shows that most of high-confidence ASD genes are pleiotropic, thus their regulation affects multiple stages of neurodevelopment. The small pie-charts indicate the contribution of the high-confidence ASD gene expression to two clusters (green and purple) resulting from a hierarchical clustering of neocortex developmental transcriptome. The grey areas in the pie-charts represent no contribution of the high-confidence genes during the developmental period.

Interestingly, most of the commonly reported high-confidence ASD risk genes have a peak expression during prenatal brain development and, spatially, in different brain regions typically abnormal in ASD (Figure 1.3) [86].

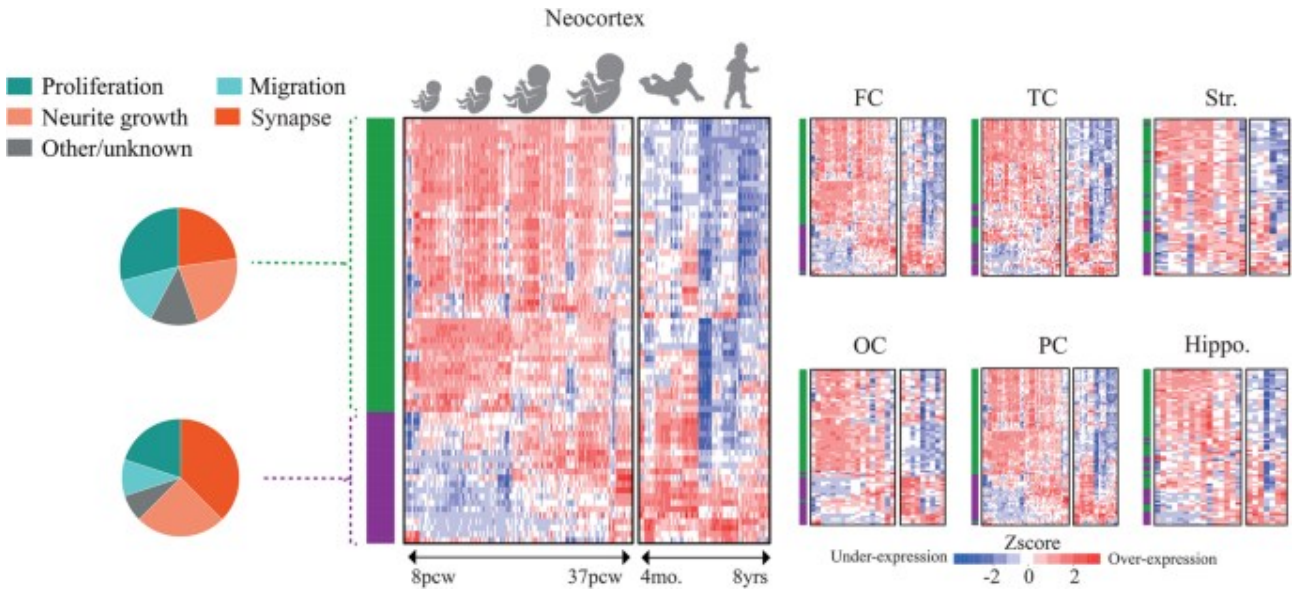


Figure 1.3. Spatio-temporal expression of high-confidence ASD genes (from *Courchesne E. et al., Mol Psychiatry, 2019*). Heat maps show the developmental expression patterns (x-axis) of 69 high-confidence ASD genes (y-axis) in different stages of development (prenatal and post-natal) and in specific brain regions. The largest heat map on the left displays a hierarchical clustering of neocortex developmental transcriptome with two main clusters of genes (in green and purple). The two pie charts, on the far left, indicate the contribution of the high-confidence ASD genes to the clusters. About the green cluster, proliferation and neurogenesis hcASD genes make the largest contribute, while synapse development and function hcASD genes make the largest contribution to the purple cluster. The clustering pattern occurs in the different neocortex regions (FC: frontal cortex; OC: occipital cortex; PC: parietal cortex; TC: temporal cortex) and with less degree, in hippocampus (Hippo) and striatum (Str).

For example, CHD8 contributes to the regulation of many processes in ASD pathogenesis such as neurogenesis, neuronal differentiation, cell adhesion, axon guidance [86], also perturbing the regulation of other risk genes [97]; MECP2 modulates synapse development, cortical cell proliferation, neurogenesis [98, 99]; SHANK3 regulates neurogenesis, excitatory synapses morphology and prefrontal connectivity [100, 101]; PTEN contributes to the disruption of neuronal production and abnormal dendritic arborization [102]; NRXN1, NLGN3, NLGN4 control synaptic cell adhesion and function, leading to imbalances of synaptic transmission [103, 104]. ASD-related genetic variants are enriched in pleiotropic genes converging in commonly ASD dysregulated signaling pathways such as WNT, NOTCH, PI3K/AKT, RAS/ERK. These pathways are highly

interconnected and modulate key neurodevelopmental mechanisms, ranging from cell proliferation to synapse development and function (Figure 1.4) [89, 105].

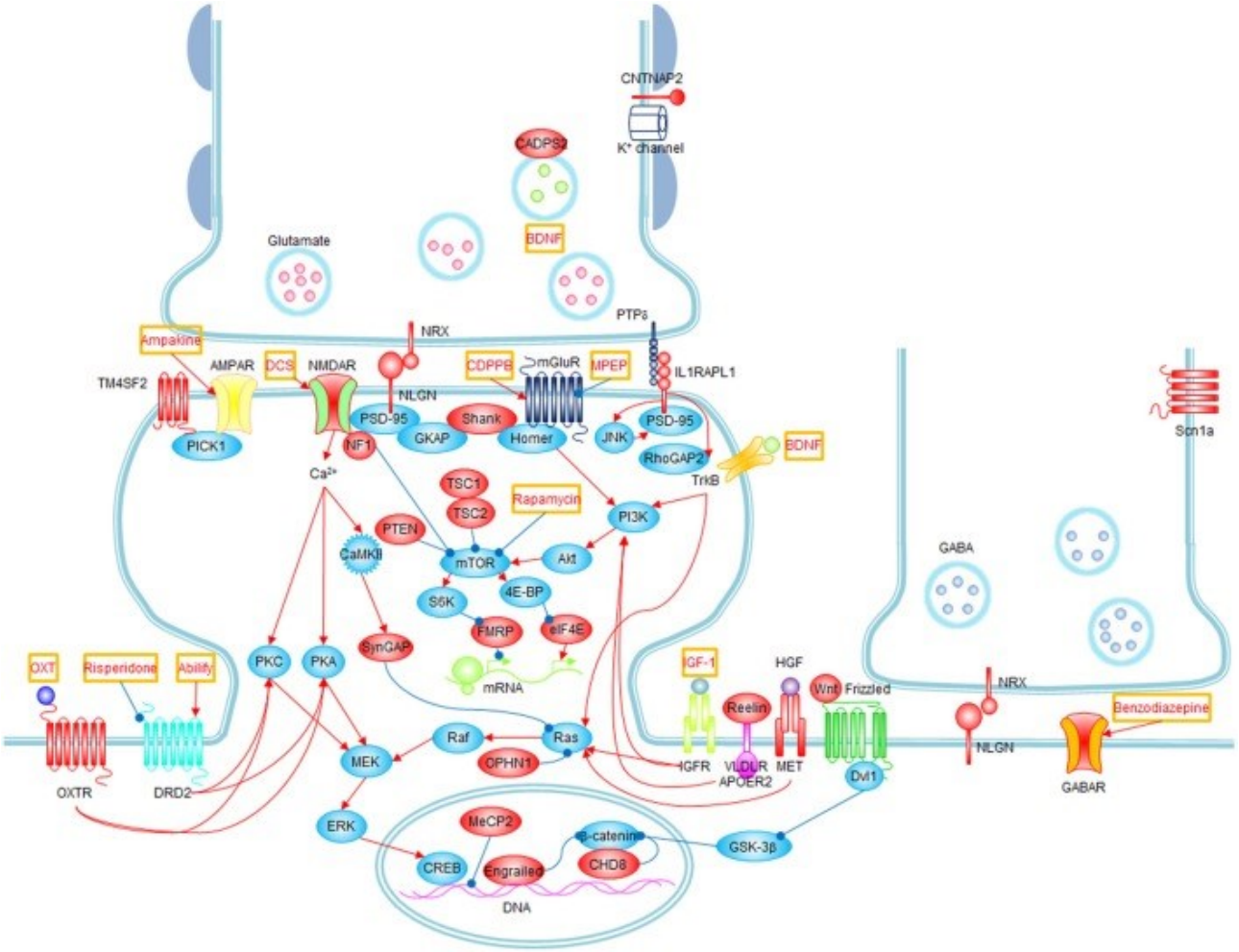


Figure 1.4. Signaling pathways associated to ASD (from Won H. et al., *Frontiers in Molecular Neuroscience,* 2013). In red, molecules whose mutations have been related to ASD; in red and blue arrows, respectively, stimulations and inhibitions; the red texts in orange boxes indicate the potential treatments and their target molecules.

Lastly, the increasing understanding of molecular and cellular mechanisms involved in ASD pathophysiology has provided a framework that may help in investigating new and efficacious therapeutic targets, hopefully at individual level in the near future.

1.1.6. The emerging interplay between Microbiome and ASD

The high prevalence of comorbidity of gastrointestinal (GI) problems reported in ASD patients (up to 70%) [106] has increasingly focused the attention to a potential role of the gut in the etiology of ASD [107]. In particular, many scientific reports suggest that one of the key modulators of the gut-brain bidirectional communication in social behaviour-related disorders (e.g., ASD) is the gut microbiota, in addition to endocrine- and immune-derived components [108-111] (Figure 1.5).

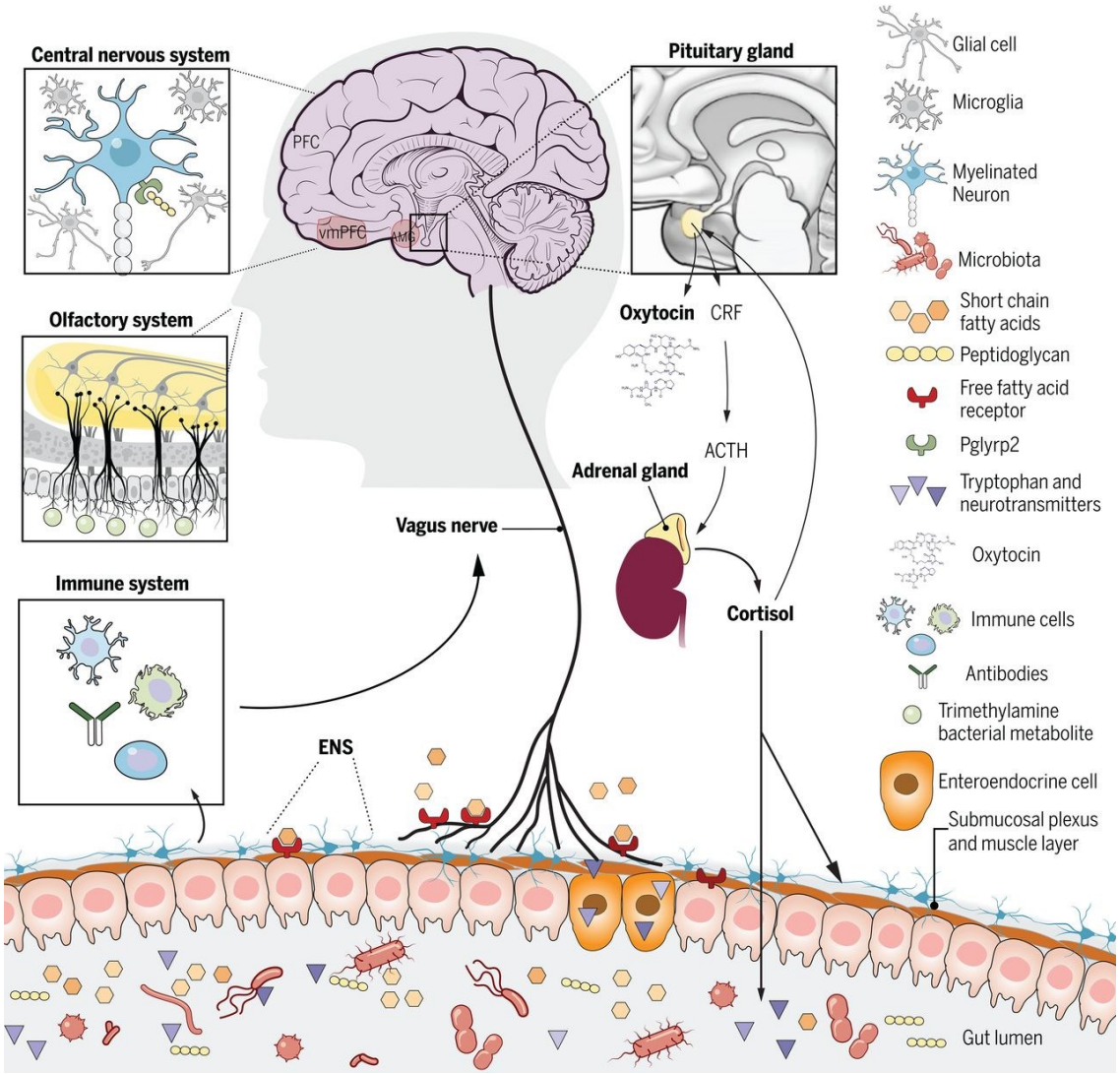


Figure 1.5. Multiple biological pathways of social behaviour regulation mediated by gut microbiota (from *Sherwin E. et al., Science, 2019*). Microbial metabolites such as short-chain fatty acids (SCFAs) influence brain physiology, by binding and activating free fatty acid receptors (FFARs) expressed on the vagus nerve. On the other hand, bacterial peptidoglycan, expressed on the cell wall, is able to influence the development of social behaviour by activating specific brain pathogen recognition receptors, such as PGLYRP2. Other metabolites act as olfactory pheromones and affect the social interaction. Biological processes such as neuroinflammation, serotonin turnover, myelination, oxytocin secretion, are also regulated by gut microbiota. The release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (pituitary gland inset)

occurs in cases of stress, and in turn, induces the release of cortisol in humans. Cortisol exposure has been related to a decrease of the bacterial abundance.

Therefore, any microbial shift in diversity and abundance may result in disturbances of the so-called gut-to-brain axis (GBA). Intriguingly, reports on gnotobiotic animals and probiotic studies have shown that microbiome dysregulation can directly induce behavioural and neuropathological endophenotypes in human ASD [112-115], making the brain-gut-microbiome axis a compelling area of investigation in ASD [108]. Many papers have reported a dysbiosis of the gut microbiota in ASD patients compared to typically developing children [37, 116, 117]. In addition, many factors could strongly influence the microbial community such as diet, medication regimens, medical comorbidities, geographic location and both acute and chronic GI symptoms (Figure 1.6) [108].

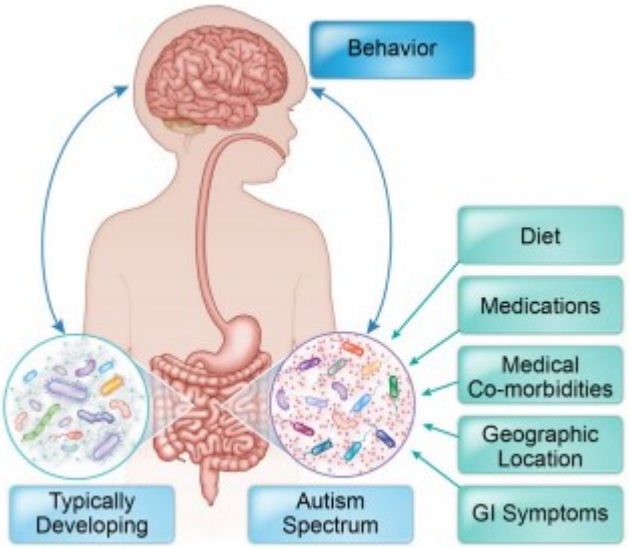


Figure 1.6. Different factors influence the microbial composition in ASD children, which plays a key role in the bidirectional brain-gut axis (from *Saurman V. et al., Dig Dis Sci, 2020*).

It is still unclear whether microbiome alteration is caused by ASD symptoms or whether it contributes to the ASD gut phenotypes. Interestingly, even if the colonization of the GI tract with microbiota starts prenatally and the diversity quickly increases, the microbiota composition stabilizes around the age of 2 to 3, which is the same critical time window for brain development: this suggests that prenatal maternal stress, infections or diet may have a strong impact on the pathophysiology of ASD [118].

Despite several reports of gut microbiota dysbiosis in ASD patients, there is little consensus across independent studies on specific bacterial species that are similarly altered [119]. In other words, to date there is no univocal microbial signature identified for ASD.

While mounting evidences suggest a key role for the gut microbiota in ASD, the etiopathogenetic contribution of microorganisms living in the oral cavity has been overlooked. Investigating on the oral cavity would strengthen and extend the notion of a microbial gut-brain axis in ASD, since the oral cavity is the start of the gastrointestinal tract.

The oral cavity represents the major gateway of bacteria into the human body: it is an unique and complex habitat [120], which harbors approximately 700 predominant taxa including the species *Prevotella*, *Porphyromonas*, *Treponema*, *Tannerella*, *Fusobacterium*, and *Streptococcus*, as well as less widely known species such as phylotypes members of the phyla SR1, GN02 and TM7 [121]. In addition, there may be biologically relevant interactions between the saliva microbiome and other microbiomes in the human body, including that of the gut [122]. The saliva microbiome is dysregulated in patients affected by systemic diseases, such as liver cirrhosis, rheumatoid arthritis, and pancreatic cancer; interestingly, these alterations are also reflected in the gut microbiome [123-125]. The oral microbiome is modulated in response to several intrinsic and extrinsic factors that may in turn lead to alterations that manifest at distant body sites, including the neural system [126]. Accordingly, oral dysbiosis has been associated with Parkinson's disease, Alzheimer's disease, multiple sclerosis and migraine [127-129]. Several potential pathways have been suggested for oral bacteria to reach the brain and directly influence neuroimmune activity and inflammation [130]. The increased permeability of the blood–brain barrier (BBB) in children with ASD could expose the brain to bacterial metabolites, thereby triggering an inflammatory response and altering metabolic activity within the CNS [130]. The general immune system alterations in the developing brain may result in synaptic disfunctions. However, the mechanisms are still poorly understood.

The collection of saliva samples is easier compared to stool or intestinal mucosal biopsies; thus, it is possible to overcome the challenging sample collection from ASD children, notably supporting the

diagnostic clinical practice [131]. However, very few studies explored the contribution of oral microbiota to ASD [131-133]. Further investigations in this field are necessary: salivary microbiota should be more exploited to unveil the pathological mechanisms of such elusive disease.

1.2. Tourette Syndrome

1.2.1. A clinical framework: definition, symptomatology and prevalence

Gilles de la Tourette syndrome is a neurodevelopmental and neuropsychiatric condition characterized by multiple involuntary motor and vocal tics, as well as behavioral impairments (American Psychiatric Association, 2013). Tics are brief, sudden, recurrent and vary in severity with a waxing and waning course. In addition, anxiety and voluntary suppression may influence the expression of tics [134].

The onset typically occurs in childhood with an average age of 4 to 6 and, to be diagnostically helpful, the tics must have persisted for more than 1 year since onset (DSM-V). Often, tics are preceded by behavioral disturbances that manifest at the age of 3.

In most cases, tics remit by adulthood and patients have complete or nearly complete remission of the disorder. On the other hand, in 10-20 % of cases, TS symptomatology persists or even worsens [135]. In daily life, patients affected by TS commonly manifest physical pain or injuries, social isolation, emotional disturbances and impairments of academic achievements [136]. Moreover, the overall quality of life of TS patients is impaired by the occurrence of comorbid neuropsychiatric conditions (e.g., ADHD; obsessive-compulsive disorder, OCD; anxiety disorder; oppositional defiant disorder, ODD), which should be considered as negative prognostic factors [136, 137].

Pharmacological therapies as well as the neuropsychological interventions like comprehensive behavioral intervention for tics (CBIT), habit reversal training (HRT) and exposure and response prevention (ER) are frequently used for treating TS patients [135, 136]. However, the first-line of intervention should be the socio-education of teachers and caregivers, aimed to improve knowledge and attitudes about TS [8].

The prevalence of TS is estimated to be approximately 3–9/1,000 children, with a male:female ratio of 3:1 to 4:1 [135]. It is worth noting that the rates of prevalence seem to vary between countries, indicating that TS is twice as common in Caucasian children as compared to Hispanic or Afro-

American children (CDC). Many factors may contribute to such variations, including the healthcare service provision which may affect the diagnosis and, therefore, the epidemiologic estimates [136, 138]. In addition, the changes in diagnostic criteria over the past years, the different assessment processes and the high variability in clinical symptomatology may impact the resulting prevalence.

1.2.2. Psychopathological comorbidities in TS patients

Up to 85% of children affected by TS have at least one comorbid condition with other neurobehavioural disturbances. Most commonly, they include ADHD (54%), OCD (50%) and, less frequently, ASD (5-15%) [139]. Comorbid conditions considerably affect patients in terms of psychosocial and psychological outcomes, as well as in terms of prognosis. Indeed, it has been reported that, in adult life, most cases of TS patients manifest mild or inexistent tics, but comorbid status with ADHD or OCD may still persist [140]. The presence of comorbid OCD in TS patients often leads to earlier symptom onset, sensory phenomena and counting, aggressive thoughts [139]. Comorbidity with ADHD has been associated with a higher rate of other comorbidities [134]. They include anxiety, learning disorders, personality disorders, sleep disorders, impaired social cognition and sensory processing difficulties [139]. Overall, these comorbid conditions have to be carefully evaluated and, accordingly, TS children with comorbid disturbances need to be treated with different therapy options.

1.2.3. Current TS Diagnosis and potential bias

Tourette syndrome can be diagnosed through a detailed history, a careful physical and a neurological examination by experienced child psychiatrists and neurologists [135].

As discussed above, the characteristic signs of TS are sudden repetitive motor and vocal tics. They often are preceded by a premonitory urge, which is followed by the physical expression of the tic and

a sense of relief afterwards. Although the involuntary tics may be partially suppressed in most cases, this action can take a huge amount of focus and energy [136].

The high variability of tics may cause some bias in clinical assessment: tics are classified as simple or complex (e.g., echolalia and echopraxia, palilalia and palipraxia, coprolalia), based on their severity [141]; in addiction, it is not always clear whether the symptoms are tics, compulsions, stereotypes or signs of other movement disorders such as chorea [139]. Therefore, quantifying and reporting the history of tics and their impairment, is a useful clinical practice.

The Yale Global Tic Severity Scale (YGTSS) is the gold standard measure of TS and consists of an 11-item clinician-rated semi-structured interview. It is able to evaluate motor and phonic tic severity considering not only the number, frequency, intensity, complexity, but also the impairment that tics provoke in the patient. The score of the YGTSS is 0-100, including the impairment section: higher scores indicate higher severity of symptoms and impairment [142]. In addition, neuropsychological assessment allows to measure intelligent quotient and behavioural problems through the reports from the Child Behaviour Checklist (CBCL) [143] and the Conner's Comprehensive Behaviour Rating Scales [144]. In 2012, the DSM-V specified the diagnostic criteria for TS evaluation (e.g., onset and number of tics) [135].

It is worth noting that, currently, there is no specific objective test available to diagnose TS [136], which would considerably support the clinical discrimination of patients, considering the high clinical variability of TS and the frequent association with comorbid conditions.

1.2.4. Exploring the etiology of TS

As many other neurodevelopmental disorders, TS pathogenesis is highly conditioned by multifactorial interactions among complex neurobiological and genetic mechanisms, prenatal and perinatal infections, as well as environmental factors [145].

The genetic contribution in developing TS is relatively large. In fact, TS is considered one of the most heritable and polygenic, non-Mendelian neuropsychiatric disorders [136].

Based on twin and family studies on the genetic heritability, the concordance for TS has been estimated at 53% for monozygotic (MZ) pairs and 8% for dizygotic (DZ) pairs [146]; moreover, family studies revealed that the risk of TS is between 10 and 100 times higher in first-degree relatives compared with the general population [147]. However, the genetic architecture of TS is still elusive, since a clear genetic cause has not yet been identified [135, 136].

Linkage analyses and candidate gene association studies identified a number of chromosomal regions and gene polymorphisms potentially related to TS. An interesting finding was a premature termination codon in L-histidine decarboxylase gene (HDC) in a large multigenerational family, confirmed by a further large family study, which revealed the role of HDC in the development of TS, through the histaminergic pathway [148-150].

CNV studies have revealed *de novo* or recurrent rare CNVs in multiple genes such as NRXN1, coding for a pre-synaptic cell adhesion molecule and CTNNA3, coding for cytoskeleton modelling molecule [151]: notably, they overlap with those genes observed in other neuropsychiatric and neurodevelopmental disorders often comorbid with TS, such as ASD, OCD and schizophrenia, suggesting a shared etiology [145, 152].

Interestingly, some of the chromosomal aberrations identified in TS patients (i.e., IMMP2L mutations, [153]) have been also implicated in other neurodevelopmental disorders such as ASD and ADHD [152].

A major focus has been emphasised on immunological risk factors associated with TS expression, and mostly associated with ASD as well, such as the activation of autoimmune mechanisms consequently to a streptococcal infection (condition labelled as paediatric autoimmune neuropsychiatric disorder associated with streptococcal infection, PANDAS [154, 155]), maternal autoimmunity, microglial activation, elevated cytokines in blood and/or cerebrospinal fluid [139].

Furthermore, environmental risk factors as pre-, peri- and post-natal adversities (e.g., maternal smoking during pregnancy, alcohol abuse, maternal stress, lower birth weight, perinatal hypoxia) play an important role in TS [156].

Finally, epigenetic mechanisms like methylation and other epigenetic factors may considerably influence the gene expression during sensitive developmental stages and, consequently, affect molecular pathways and the development of neural circuitries [150].

1.2.5. Macroscopic and microscopic abnormalities in TS neurobiology

Although the pathophysiological mechanisms of TS are still largely unknown, there is a general consensus that the tic generation is related to dysfunctions of the striatal GABAergic networks, leading to an excess of striatal dopamine neurotransmitter in the basal ganglia [157, 158]. In particular, it has been suggested that the abnormal release of striatal dopamine may induce a focal excitatory abnormality in the striatum and a consequent disinhibition of the thalamo-cortical circuits, leading to the production of motor tics [159, 160]. Interestingly, the basal ganglia were believed to influence the formation and maintenance of stereotyped repetitive behaviours or habits like tics, obsession and compulsions [160]. A system-level perspective supports the co-working of basal ganglia with cerebellum and cortex to generate motor tic and cognitive behaviours, even if the exact contribution has not been clarified [157, 161, 162]. The altered neural activity and the interregional interplay between all portions of the motor pathways (i.e., the sensorimotor cortex, putamen, pallidum and substantia nigra) and the portions of cortico-striato-thalamo-cortical (CSTC) circuits have suggested a more complex framework of TS pathophysiology, which is also linked to tic severity [163].

Neuroanatomical changes in the cortex and subcortical white matter, thalamus, basal ganglia, globus pallidus and the cerebellum, detected in TS patients through volumetric MRI studies, have been associated with a loss of inhibitory function [164]. In particular, the smaller volume of caudate nucleus in TS children predicted the severity of tics and the symptoms of obsessive–compulsive behaviours in early adulthood [165]. Functional neuroimaging studies revealed anomalies in frontostriatal networks and in frontoparietal connectivity related to the age of TS patients. These results suggested that abnormalities occurred during the neurodevelopmental steps [166].

These findings provided exciting clues to the understanding of TS neurobiology, but numerous neurobiological points are still unsolved.

1.3. Arnold-Chiari syndrome

1.3.1. A clinical framework: definition, symptomatology and prevalence

Arnold-Chiari syndrome is characterized by rare malformations of the cerebellum and brainstem [167]. Also known as Chiari malformations (CM), such anomalies can be divided into four subtypes: Chiari malformation type I (CM-I), Chiari malformation type II (CM-II), Chiari malformation type III (CM-III) and Chiari malformation type IV (CM-IV).

CM-I is characterized by caudal displacement of cerebellar tonsils below the foramen magnum (FM), which can either be both or singularly herniated 3 mm or 5 mm, respectively [168].

It is often associated with a volumetrically reduced posterior cranial fossa (PCF) and syringomyelia [167, 169], a disorder in which a fluid-filled cavity (syrinx) lies within the spinal cord [170]. CM-II describes the herniation of the cerebellar tonsils and vermis, involving the brainstem, fourth ventricle, associated cranial nerves and vasculature [171]. Furthermore, CM-II is commonly accompanied by a myelomeningocele, a birth defect due to incomplete closure of the neural tube, and hydrocephalus [172].

CM-III and CM-IV are rare anomalies in children. CM-III is characterized by a cervical encephalocele, thus the posterior fossa contents herniated by a defect of the upper cervical vertebra [173]. Finally, CM-IV describes the hypoplasia or aplasia of the cerebellum [167, 174].

CM-I is the most common type of Chiari malformation, affecting from 1/5,000 to 1/1,000 individuals, with a prevalence of females more than males (1.3 to 1) [175]. However, the incidence is influenced by the high variability of disease presentation, since many patients may be asymptomatic or they may manifest associated conditions. The age of onset may be also variable, because it ranges from the neonatal period to adulthood, although CM-I typically occurs in middle age [168]. Common debilitating neurologic symptoms affect CM-I patients, such as occipital headaches, ocular disturbances, muscle weakness, motor deficit, vertigo, dysphagia or dysarthria and tingling pains, as well as motor and sensory disturbances [175, 176]. Significant relief from such disturbances may occur after surgery, which consists of decompression of the posterior fossa allowing its expansion and

a consequent improvement of cerebrospinal fluid flow [177]. The pathological condition is defined by the degree of cerebellar tonsillar herniation below the foramen magnum (one 5mm or both 3mm at least), as well as the reduced volume of posterior cranial fossa [175].

These two factors have been proposed to be linked: the small posterior cranial fossa is unable to house a normal hindbrain and leads to the tonsillar herniation (Figure 1.7) [167, 178].

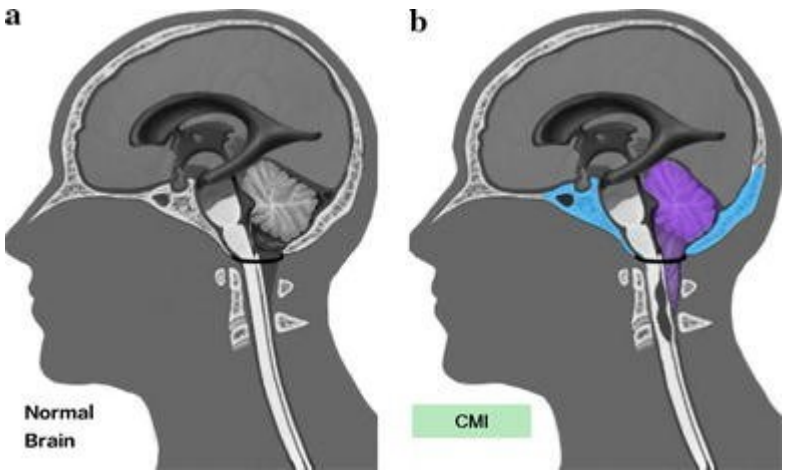


Figure 1.7. Depiction of the midsagittal region of the craniocervical junction (from *Yan H. et al., Eur Spine J 2016).* (a) A non-affected brain has a normal sized posterior fossa and cerebellum; (b) An affected brain with CM-I shows a volumetrically reduced posterior fossa (blue area) which leads to the herniation of the cerebellar tonsils through the foramen magnum (purple area).

As a result, the hydrodynamic of cerebrospinal fluid (CSF) flow is impaired and the direct compression of the neural tissue at the craniovertebral junction or the spinal canal may lead to lower cranial nerve deficits, causing the neurological dysfunctions [175].

1.3.2. Comorbidities in AC patients

It is worth noting that multiple etiologies should be considered in all AC patients. Indeed, the high intracranial pressure by several diseases like idiopathic intracranial hypertension (pseudotumor cerebri), brain tumour or craniosynostosis may strongly influence the cerebellar tonsilar herniation downward, leading to Chiari malformations as a secondary event. Other conditions such as Syringomyelia or hydrosyringomyelia (syrinx) have been detected in 65–80% of CM-I patients.

Furthermore, CM-I has been associated in comorbidity with cranial skull base and bony defects ranging from assimilation of the atlas, proatlas anomalies, basilar invagination and Klippel–Feil anomalies, which can lead to the ventral compression of the brainstem and spinal instability [167]. Several neuropsychiatric disorders may also coexist with AC condition: anxiety [179], epilepsy [180], intellectual disability [181], attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) [182], worsening the patients' quality of life [183]. Interestingly, both ADHD, ASD and CM-I patients show anomalies in brain volume. More specifically, ASD and CM-I patients share the cephalocranial disproportion in neural tissue, which causes cerebral volume alterations. Since the altered cranial growth occurs during the developmental stages of ASD patients, it has been suggested that the ASD typical abnormalities might be caused by similar mechanisms that lead to PCF defects [182]. Unfortunately, the comorbid condition between ASD and CM-I has not ever been easily detected, because some clinical features in ASD patients (e.g., headaches, neck pain, dysarthria) are thought to be caused by ASD and it is not frequently recommended to perform cranial imaging which could assess also for CM-I [184].

Interestingly, Chiari malformations have been reported to be part of secondary tics (tourettism) causes [185]. However, to date, a coexistence of both syndromes in the same individual (i.e., ACTS) has not been described.

1.3.3. Current diagnosis of AC syndrome

The "gold standard" for diagnosis of AC syndrome is based on the cranial magnetic resonance imaging (MRI) and a detailed clinical examination (Figure 1.8) [175].

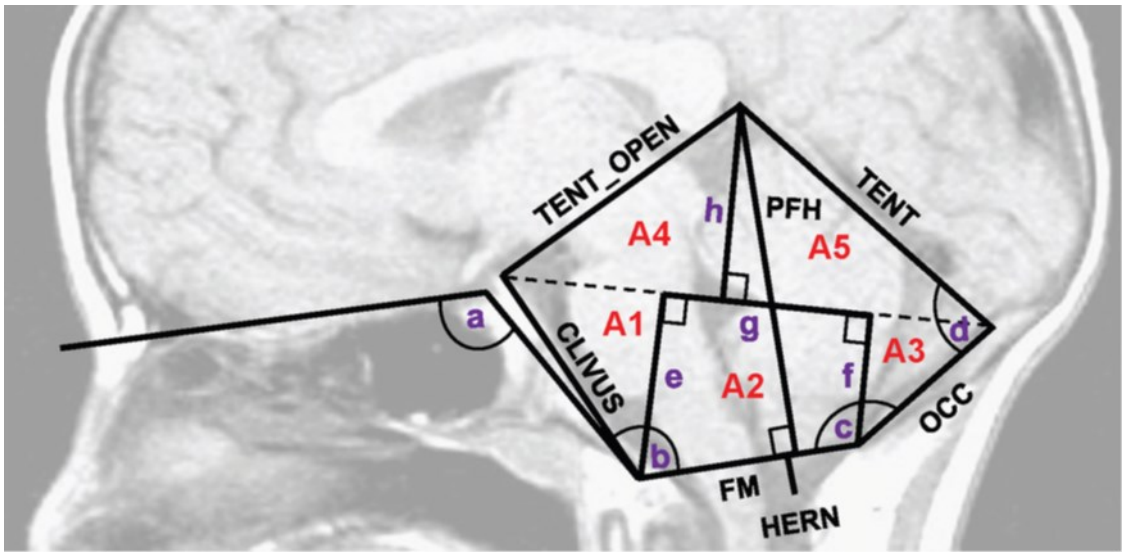


Figure 1.8. Typical measurements of PCF from the midline of a sagittal, T1-weighted MRI (from *Urbizu A. et al., PLoS One 2013).* The PCF area was estimated as the polygon represented. a) basal angle, (b) boogaard angle, (c) occipital angle, (d) tentorial angle, (e) basion to reference, (f) opisthion to reference, (g) trapezoid height, and (h) tentorium to reference. Abbreviations: TENT_OPEN = tentorial opening, TENT = tentorium, OCC = supraoccipital bone, FM = foramen magnum, PFH = posterior fossa height, and HERN = cerebellar tonsillar herniation.

In particular, sagittal T1-weighted head and neck MR scans are used to detect the degree of cerebellar tonsil herniation, which defines Chiari I malformations if both tonsils are herniated 3 mm or one tonsil is herniated 5 mm below the foramen magnum, as well as other associated conditions like syringohydromyelia [186]. This technique allows to reveal Chiari malformations not only in patients who show typical debilitating neurologic symptoms, but often incidentally in those ones who are asymptomatic [168, 175, 187]. In fact, it has been reported that 30% of patients with tonsillar herniation of 5 to 10 mm are asymptomatic. Moreover, cine phase-contrast MRI (cine MRI) of the subarachnoid fluid and CSF hydrodynamics has been commonly used to evaluate the severity of CSF flow disturbances. Early diagnosis and intervention are needed to avoid permanent damage [186].

1.3.4. Exploring the etiology of AC syndrome

The etiology of AC syndrome still remains unclear and has long been subject to numerous debates. As a matter of fact, although Chiari malformations seemed to be sporadic conditions without hereditability, several studies marked the identification of familial aggregation, clustering, twin

studies, co-segregation with known genetic syndromes. These findings supported a genetic contribution to CM-I, even if the specific genetic factors and the type of inheritance have not been identified [168, 175]: many genes have been proposed as candidates for AC syndrome and a polygenic model of inheritance seemed to be the most appropriate [175]. In order to identify susceptibility genes, whole genome linkage screens using CMI multiplex families reported significant evidences for linkage to regions in chromosomes 9, 15 [188], 8, 12 [189], 22 and 1 [190]. In these regions, biological candidate genes for CM-I, such as FBN1 (Fibrillin 1), GDF6 (Growth differentiation factors 6), GDF3 (Growth differentiation factors 3), EP300 (E1A binding protein p300), CREBBP (CREB binding protein), LHX4 (LIM homeobox 4) are implicated in several syndromes associated to CM-I, such as Klippel-Feil, Shprintzen-Goldberg, Marfan, Rubenstein-Taybi syndromes and have an important role in cranial base bone formation [168, 190]. Furthermore, a case-control association study revealed nominal associations for SNPs found in several genes that are involved in the vascular development of the placenta and the early development of paraxial mesoderm, sclerotome development or syndromes associated to CM-I [175]. All these finding revealed that multiple susceptible genetic factors contribute to Chiari malformation etiology and, together with environmental factors, may reflect the phenotypic heterogeneity.

1.3.5. Macroscopic and microscopic abnormalities in neurobiology of AC syndrome

Different biological mechanisms have been proposed to define the critical factors causing cerebellar herniation [171, 191]. Among these, the most accepted are cranial constriction, in cases of a shallow PCF, and cranial settling, spinal cord tethering, intracranial hypertension, intraspinal hypotension, in cases of normal sized PCF and secondary CM-I.

The cranial constriction mechanism consists of a shortness of the basichondrocranium, suggesting a developmental defect of occipital somites originating from the paraxial mesoderm, which lead to a volumetrically-reduced posterior cranial fossa, overcrowding of the cerebellum and a downward cerebellar herniation in the classical form of CM-I [192]. It is unknown whether mesodermal

insufficiency is a primary (genetic) or secondary condition as a result of disturbances during the early gestational or post-natal stages. Cranial settling condition is associated with hereditary disorders of connective tissue, posttraumatic occipitoatlantoaxial joint instability, osteogenesis defects. Intraspinal hypotension is associated to CSF leaks or lumboperitonial shunting. Spinal cord tethering is associated with cord traction in CM-II. Intracranial hypotension is associated with PCF cysts, intracranal mass lesions or hydrocephalus [175].

Overall, the altered expression of critical genes that converge in dysregulated biological processes and pathways (e.g., the development of occipital somite, sclerotome, placenta and the dorso-ventral axis formation) results in micro- and macro-structural and functional abnormalities during the early embryo development stages, contributing to Chiari malformation pathogenesis [175].

Chapter 2. Expression profile of circulating microRNAs as a promising fingerprint for neurodevelopmental disturbances

2.1. Far from "junk": non-coding RNAs are critical regulators of gene expression

For many years, non-coding RNAs (ncRNAs) have taken part in a big revolution of the molecular biology landscape.

In September 1957, a few years after the discovery of DNA three-dimensional structure, Francis Crick gave a lecture in which he presented a conceptualized hypothesis on the link between the base sequences of nucleic acids and those of amino acids in a protein. As he termed, the "sequence hypothesis" pointed out the idea that genetic information is encoded in sequences of units as a specific code, which leads to the protein synthesis through a "flow of information" [193]. Secondly, Crick proposed the "Central Dogma" of molecular biology [194], which stated that once information had gone from DNA, RNA, to the protein, it could not get out of the protein and go back into the genetic code (Figure 2.1) [193].

Figure 2.1. Schematic representation of the Central Dogma of molecular biology (from *Cobb M. et al., PLoS Biol 2017*).

Followed by a lot of subsequent misunderstandings, the unidirectional flow of information postulated by the central dogma was believed to imply that DNA served as an inert storage of information and that RNA served as a passive intermediary of information flow between the genetic material (DNA) and the end product (protein), which, in turn, regulated all cellular biological processes [195]. Also, RNA was believed to passively contribute as a scaffold for the ribosome or to present anticodons in tRNA [196].

Thanks to the modern view of gene expression and the consistent sequencing studies, many evidences rapidly challenged the linear logic and addressed the evolution of our understanding of the central dogma towards new perspectives on RNA function.

The Human Genome Project (HGP), that represented the world's largest collaborative scientific project to date, aimed to sequence the entire human genome, reporting a first draft in 2001 [197] and a final genome visualization in 2004 [198]. Surprisingly, it revealed that only less than 2% of the human genome includes approximately 21,000 different protein-coding genes, more recently corrected in 19,042 genes [199], while the major genome is composed by non-protein-coding sequences [200]. The protein-coding gene fraction in *Homo sapiens* was unexpectedly lower than expected and has long mystified scientists, since it was thought that the number of protein-coding genes positively correlated with the biological complexity of an organism [201]. Actually, the number of human protein-coding genes was surprisingly similar to the one of other less complex vertebrates such as mice (*Mus musculus*, ~20,210), chickens (*Gallus gallus*, ~16,736) and lower than plants (*A. thaliana* ~26,000, rice ~37,000) or protists (*Paramecium tetraurelia* ~40,000, *Tetrahymena thermophila* ~27,000) [202]: all these observations drew up the so-called G-value paradox [203]. The use of alternative splicing in complex organisms could somehow support the concept of biological complexity due to the multiple protein isoforms expressed, even if this could not be the complete explanation. Also, for many decades the G-value paradox gave rise to the concept that the huge amount of human non-coding DNA was composed by non-functional evolutionary debris accumulated over time from diverse origins (mainly represented by repetitive sequences such as transposable elements) and consequently, it was regarded as "junk DNA" [195, 204]. However, it was becoming assumed that the number of protein-coding genes does not reflect the developmental complexity of an organism; conversely, the biological complexity seems to be positively correlated with an increasing number of non-coding DNA fractions compared to the total genomic DNA (Figure 2.2) [205].

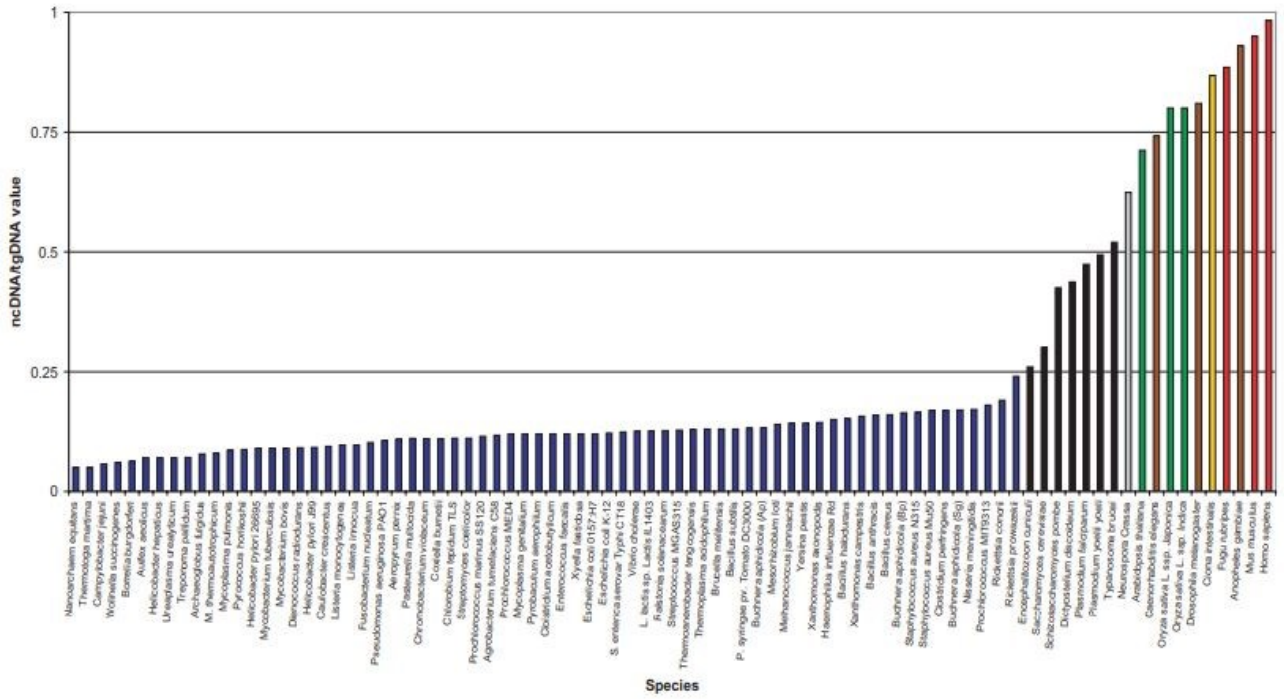


Figure 2.2. Comparison of ratios between non-coding DNA fraction (ncDNA) and the total genomic DNA (tgDNA) between distinct species (from *Taft R. J. et al., Genome Biology, 2003*). The biological complexity is positively correlated with increasing ncDNA/tgDNA ratio. Prokaryotes are labeled in blue, unicellular eukaryotes in black, the multicellular fungus *Neurospora crassa* in grey, plants in green, non-chordate invertebrates in brown, the urochordate *Ciona intestinalis* in orange and vertebrates in red. *Homo sapiens* is the last species represented having the highest amount of ncDNA.

This was in agreement with several findings supported by large-scale projects for the systematic annotation and functional characterization of genes, such as the international Encyclopedia of DNA Elements (ENCODE) project consortium [206]: far from being "junk", at least 80% of mammalian genome is pervasively transcribed, producing many previously undiscovered non-coding transcripts, and holding biochemical functions [207-209].

These findings suggested a more complex view of the transcriptome in eukaryotes and that many gene regulatory processes confer their major biological complexity. In fact, while the amount of non-coding sequences has considerably increased in accordance to the complexity, the repertoire of protein-coding genes has remained almost evolutionarily static (Figure 2.3) [202, 209-211].

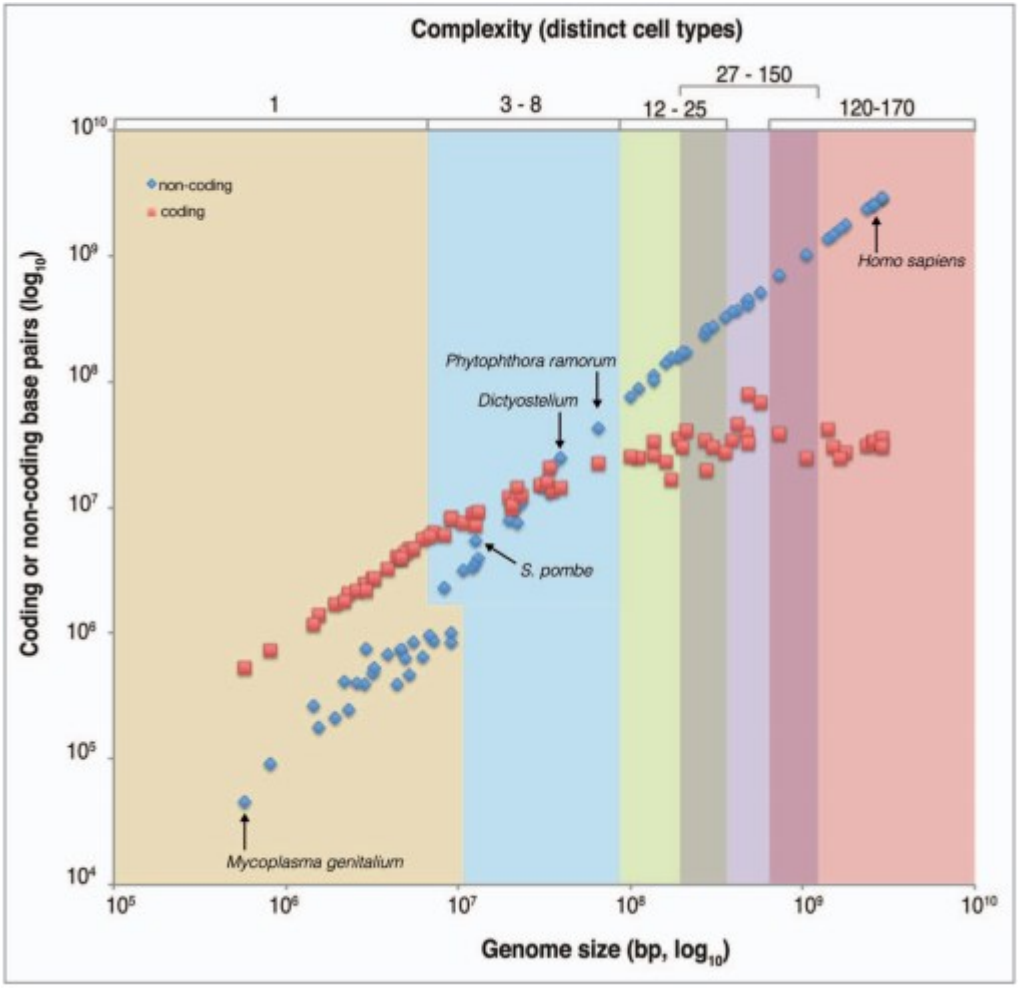


Figure 2.3. Correlation between biological complexity and the genome composition (from *Liu G. et al., Cell Cycle, 2013*). The plot displays 73 organisms as pairs of data points: in red, the total protein-coding sequence bases and in blue, the total non-protein-coding bases, which overall assemble the entire genome size (x-axis). The number of non-coding base pairs increases in an exponentially way, while the number of coding base pairs shows an asymptotic trend compared to the increasing of the number of different cell types.

Non-coding RNAs (ncRNAs) are known to play critical roles in many biological and molecular functions associated with the control of epigenetic mechanisms, transcription, translation and turnover.

The identification and the classification of ncRNAs have been quite difficult, as well as the knowledge of the exact number in the human genome. However, it has been assumed that lncRNAs represent the biggest portion of ncRNAs in human genome, followed by miRNAs (Figure 2.4) [212].

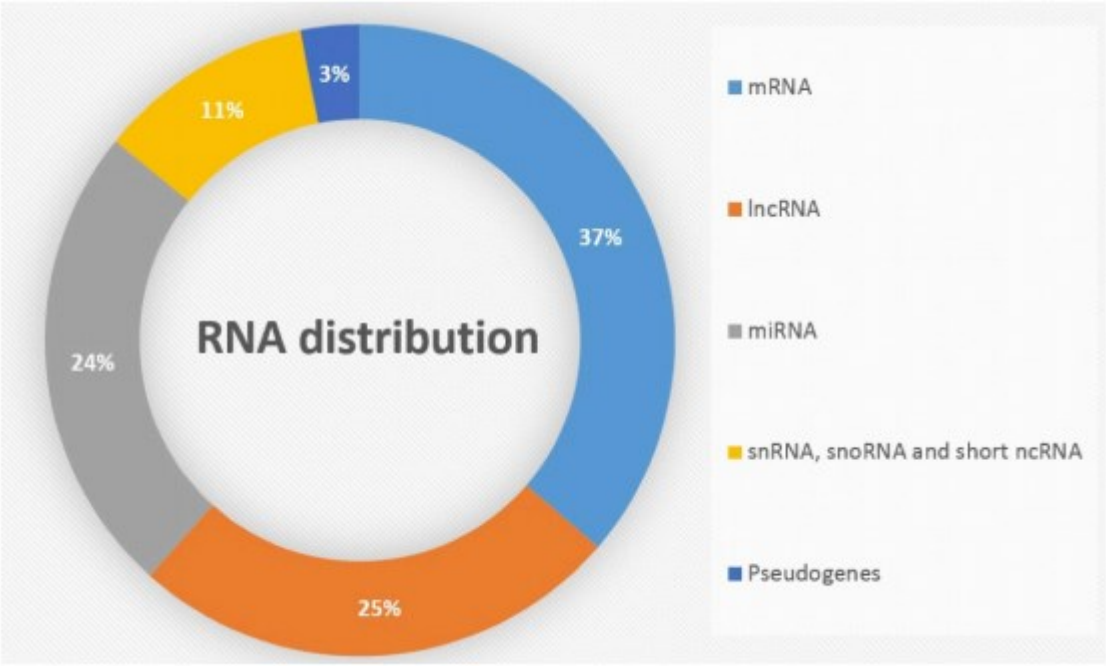


Figure 2.4. Proportions of RNA subtypes in human genome (from *Flippot R. et al., Oncotarget. 2016*). lncRNAs: long non-coding RNAs, miRNAs: micro RNAs, short ncRNAs: short non-coding RNAs, snRNAs: small nuclear RNAs, snoRNAs: small nucleolar RNAs.

Indeed, ncRNAs are characterized by highly variable lengths, the absence of Open Reading frames (ORFs), low or absent evolutionary conservation and a non-preferential localization within the genome.

The two current classifications take account of length and functional features. According to the length, ncRNAs can be divided into long non-coding RNAs (lncRNAs) (>200 nucleotides) and small non-coding RNAs (<200 nucleotides), which comprise microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). According to the functional characteristics, ncRNAs can be divided into infrastructural ncRNAs and regulatory ncRNAs. The first are constitutively expressed and comprise ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). On the other side, regulatory ncRNAs can be expressed in a cell-specific and developmental stage-specific way or as a result of external *stimuli*; they include microRNAs, Piwi-interacting RNAs, small interfering RNAs and long non-coding RNAs [211, 213].

Among small non-coding RNAs, miRNAs represent the most explored class of ncRNAs and play a crucial role in the pathogenesis and pathophysiology of neuropsychiatric and neurodevelopmental disorders [214].

2.2. MicroRNAs: small molecules with big roles

MiRNAs are a class of endogenous, evolutionarily conserved and small single-stranded RNAs (19-24 nt), whose main role is to post-transcriptionally fine-tune gene expression and control pivotal cellular processes [215].

The first miRNA, LIN-14, was discovered in *Caenorhabditis elegans* by Lee and colleagues in 1993 [216]. It was found to post-transcriptionally repress the lin-14 mRNA and, consequently, to critically modulate the developmental transition from the L1 first larval stage to L2. Years later, a novel small RNA, let-7, was found to be crucial for the developmental progression to adult stage in *C. elegans* [217]. At that time, it was thought that the discovery was linked to an exclusive phenomenon belonging to *C. elegans*, but a consistent research on small RNAs, aimed to identify new molecules, uncovered many thousands of small regulators across different species of plants and animals [215, 218]. Then, the new class of small RNA was named "microRNA".

A miRNA sequence repository, miRbase, was released in 2002 for all the information about miRNA sequences, annotation, nomenclature and target prediction [215]. Since that year, the number of deposited human miRNAs has constantly increased, but most of them need a complete characterization and experimental validation [219].

The genome localization of miRNAs-coding genes can notably vary: miRNAs may be originated from protein-coding gene introns, intergenic regions, non-coding gene introns, non-coding gene exons. In addition, miRNAs can be generated as distinct units by the co-expression of clusters of miRNA genes located at adjacent loci on the same chromosome.

MiRNAs undergo multiple biogenesis steps to achieve their mature form. According to the canonical pathway (Figure 2.5, pathway 1), RNA polymerase II (rarely, RNA polymerase III) transcribes long

transcripts denominated pri-miRNA (primary miRNA), which have a double-stranded hairpin structure and a 5′ 7-methylguanosine (m7Gppp) cap, as well as a 3′ polyadenylated tail. Then, pri-miRNA is cleaved by Microprocessor into ~70- to 120-nucleotide-long pre-miRNA (precursor miRNA) with a hairpin structure. Microprocessor is a multiprotein complex containing a nuclear RNase III enzyme denominated Drosha, along with a double-stranded (ds) RNA binding protein denominated DiGeorge syndrome critical region gene 8 (DGCR8) or Pasha. Once pre-miRNA is generated, it is exported to the cytoplasm by exportin-5 (Exp5), a Ran GTP-dependent nuclear transport receptor protein. In the cytoplasm, a cleavage process occurs by another RNase III enzyme, Dicer, together with a dsRNA-binding protein, called Tar RNA Binding Protein (TRBP), which overall process the pre-miRNA into mature ~18- to 23-nucleotide-long miRNA-miRNA* duplex, involving the removal of the terminal loop. Both strands of the mature miRNA duplex are loaded into the Argonaute protein (Ago-2) in an ATP-dependent manner. The two strands of the duplex are later separated: one strand will be the "guide strand" and the second one will be the "passenger strand", depending on several factors such as the thermodynamic stability. The guide strand is integrated into the RNA-induced silencing complex (RISC) by Argonaute protein, which is the catalytically active RNase in the RISC complex. On the other hand, the passenger strand is usually cut by Ago2 and further degraded by the nuclease complex C3PO; for a long time, this strand has been considered non-functional, although several studies recently argued this hypothesis.

The name of the mature miRNA is determined by the directionality of the strand selected: the "5p" derives from the 5' end of the pre-miRNA and the "3p" derives from the 3' end. Finally, the guide strand directs the RISC complex to the mRNA target and negatively regulates it, by binding to complementary sequences.

The non-canonical pathway is Drosha-independent (Figure 2.5**,** pathway 2**)**: pre–miRNA-like hairpins, called "Mirtrons", are produced from spliced and debranched introns by debranching enzyme (Dbr); once exported into the cytoplasm, pre-miRNA is cleaved by Dicer and follows the same steps of the canonical pathway [215, 220].

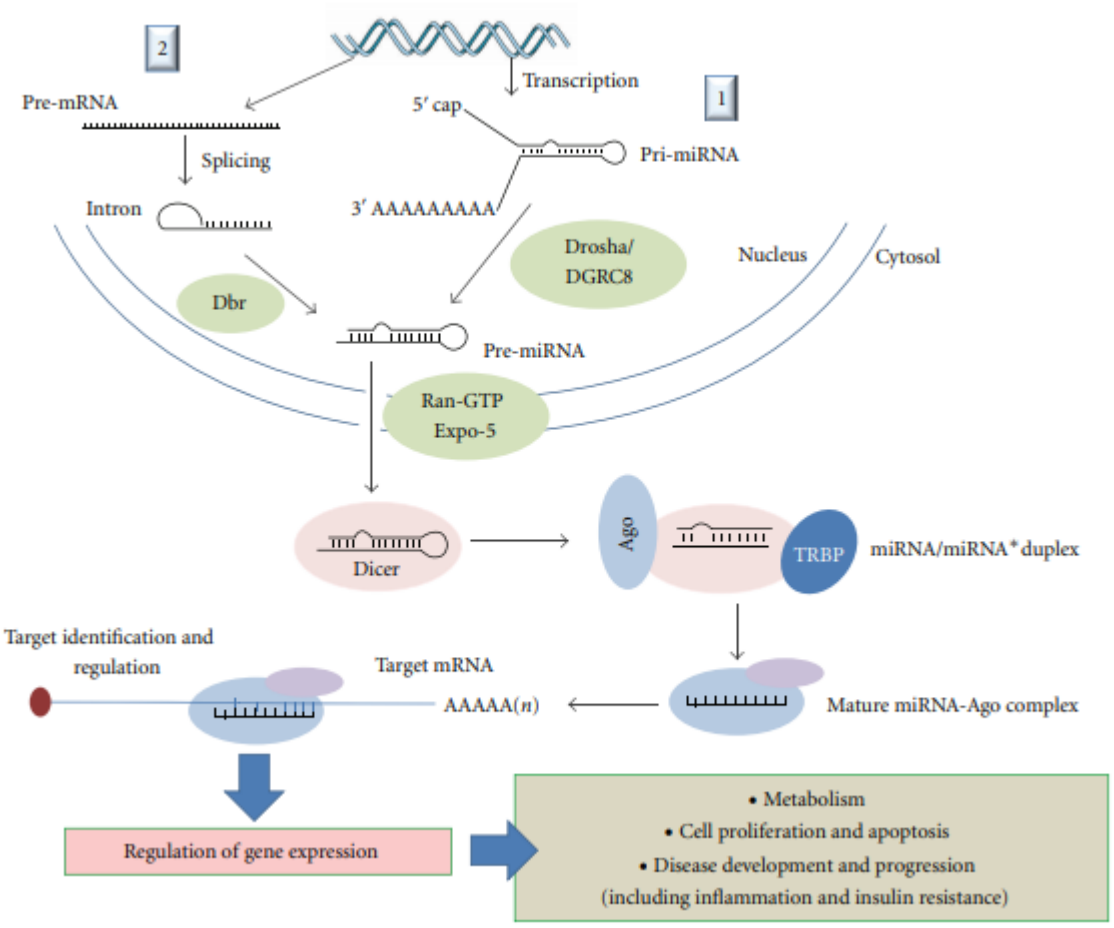


Figure 2.5. MiRNA biogenesis and mechanisms in regulation of gene expression (from *Hirabara S.M. et al., J Biomed Biotechnol, 2012).* (1) Canonical pathway generates pre-miRNA molecules by Drosha/DGCR8 cleavage; (2) Non-canonical pathway produces mirtrons by Dbr debranching, without the involvement of Drosha. Once mature, miRNAs exert a pleiotropic effect on their mRNA targets, negatively modulating their expression and affecting all cellular pathways, from metabolism to disease development and progression.

Once incorporated into RISC complex (miRISC), mature miRNA exerts as functional unit that includes Ago protein. The target mRNA recognition occurs by a specific complementarity of sequence. In particular, the miRNA-mRNA interaction is achieved by the binding between the "seed" region, a specific sequence of 2-7 nucleotides present in the 5' end of the miRNA, and the miRNA-binding sites in the 3'-UTR of mRNA target [221]. However, the stability and specificity of miRNA-mRNA interaction are more allowed by additional base pairing to nucleotides ~13–16 of the miRNA at the 3' end of the mature miRNA, called the "supplementary region" (Figure 2.6A).

MiRNA silence gene expression occurs by different mechanisms, depending on the degree of interaction: a fully complementary miRNA-mRNA interaction leads to the cleavage and degradation of mRNA target; a non-perfect and contiguous complementarity, for example, due to the presence of mismatches within the central region of mature miRNA, leads to mRNA translation suppression [218, 222]. The mRNA decay involves a member of the GW182 protein family, which binds PABPC protein and the deadenylation complexes PAN2–PAN3 and CCR4–NOT, leading to the decapping by the DCP1–DCP2 complex and, finally, the mRNA degradation by the 5′–3′ exoribonuclease 1 (XRN1). The translation initiation repression depends on interfering with the function of eIF4A-I and eIF4A-II factors, as well as the recruitment of CCR4–NOT and the probable ATP-dependent RNA helicase DDX6 (Figure 2.6B) [222].

Furthermore, it is noteworthy that miRNAs are able to build a complex network of interactions: one single miRNA can target multiple mRNAs, thereby silencing hundreds of genes, and one mRNA contains multiple miRNA-binding sites, thus it can be targeted by multiple miRNAs. MiRNAs can also cooperatively regulate one mRNA by binding on multiple and neighboring binding sites (Figure 2.6C) [222-224].

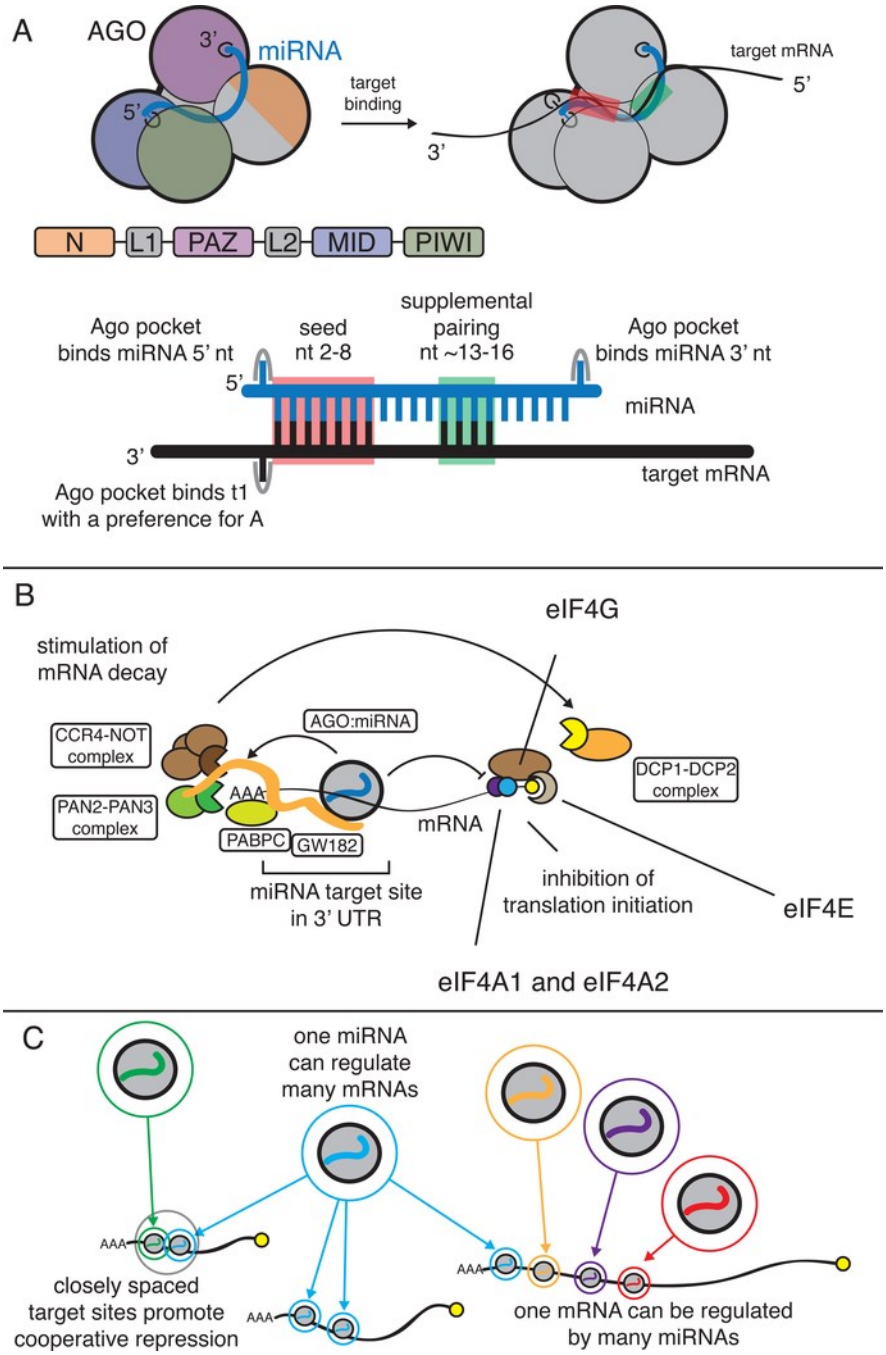


Figure 2.6. Mechanisms of miRNA function and regulation (from *Gebert L.F.R. et al., Nature reviews. Molecular Cell Biology, 2019*). (A) The functional unit of the post-transcriptional silencing: mature miRNA and an Argonaute (AGO) protein, which is constituted by four domains (the amino-terminal domain, N; the Piwi–Argonaute–Zwille domain, PAZ; the middle domain, MID; the P-element induced wimpy testes domain, PIWI; two linker regions, L1 and L2). MiRNA is arranged in a helical conformation by MID and PIWI domains. mRNA recognition is allowed by the binding with miRNA seed region, which is formed by nucleotides 2-8 at the 5' end, and can be supported by a supplemental pairing on the "supplementary region". (B) Mechanisms of translational inhibition and mRNA decay as discussed in the main text. (C) Many complex interactions link multiple miRNAs to a single mRNA: one miRNA can regulate many mRNAs, as well as one mRNA can be regulated by many miRNAs by having multiple miRNA-binding sites; when closely spaced, the mechanism of mRNA repression can be cooperative.

Therefore, these small molecules have notably relevant impact on the correct functioning of cellular pathways and biological processes, such as cell proliferation, differentiation, cell death, migration. In fact, their altered expression has been widely reported as associated with many pathological conditions such as metabolic, infectious, neoplastic diseases, cardiovascular diseases and neurodegenerative, neurodevelopmental and neuropsychiatric disorders [215, 225-229].

2.2.1. The role of miRNAs in neurodevelopment

The human brain contains the richest repertoire of ncRNA species, which are involved in the crucial steps of neural development (e.g., neurogenesis, neuronal differentiation, neuronal migration, neuronal apoptosis, synaptogenesis, neuronal plasticity) [226].

MiRNAs are part of the sophisticated multilayered regulatory networks in the nervous system, with the role of critical regulators of neural structure and function at multiple levels, such as neural circuit development (Figure 2.7) [230].

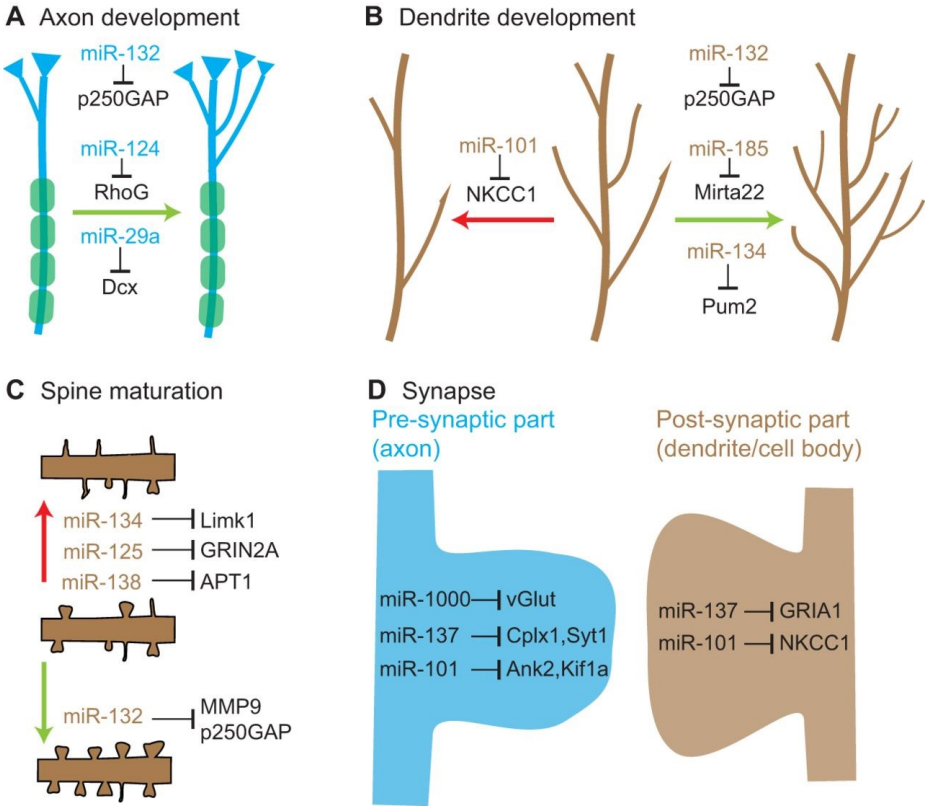


Figure 2.7. Involvement of miRNAs in neural circuit development (from *Rajman M. et al., Development. 2017*). MiRNAs exert a fine regulation (A) at axon developmental level, through the intrinsic and activity-induced axonal branching by miR-29/miR-124 and miR-132 regulation; (B) at dendrite development level, through the inhibition (red arrow) or activation (green arrow) by miR-101, miR-185 and miR-134 regulation and the indicated miRNA-target interactions; (C) at dendritic spine maturation level, through the inhibition (red arrow) or activation (green arrow) by miR-134, miR-125, miR-138 and miR-132 regulation and the indicated miRNA-target interactions; (D) at synapse transmission level, through the coordinate repression on different sets of targets in the pre- and post-synaptic compartments by miR-137 (or the ortholog miR-1000 in Drosophila) and miR-101.

Moreover, it has been reported that miRNAs show specific temporal and spatial patterns of expression during brain development and are abundantly expressed in the adult brain [226, 231, 232]. For example, miRNAs can regulate specific neuronal cell types, at particular stages of development as well as in particular cellular regions.

The correct formation and function of neuronal networks is crucial for cognition and behavior. Dysregulation in neuronal circuit development, maturation and function by miRNA regulatory action, can lead to various brain anomalies, neurodevelopmental and neuropsychiatric disorders (e.g., ASD, schizophrenia, major depressive disorder, anxiety, bipolar disorder), delineating a discriminative molecular signature [230, 233]. Indeed, extensive studies associated the abnormal expression of

miRNAs with the aforementioned pathophysiological states, also suggesting that miRNAs might contribute to their molecular basis [214].

2.2.2. Intracellular miRNA expression in neurodevelopmental disorders

Many miRNA expression profiling studies investigated miRNA dysregulation in different tissues from ASD patients compared to healthy controls, including lymphoblastoid cell lines [234-236], post-mortem brain regions [237-240], olfactory mucosal stem cells (OMSC) and primary skin fibroblasts [241]. Although the exact pathophysiologic role is unclear, most of miRNAs identified are involved in neurogenesis, synaptogenesis, neural and glial differentiation and cognitive and motor function [242, 243]. Among all of them, miR-21-3p and miR-146a-5p have been considered strong candidates for ASD pathogenesis. MiR-21-3p was identified by Wu et al. [239] as upregulated in the postmortem cerebellar cortex of ASD subjects and its overexpression in human neuroprogenitor cells was related to repress multiple M16 hub genes, including DLGAP1, which is a scaffold protein interacting with SHANK3, both involved in postsynaptic density [242]. On the other hand, miR-146a-5p was found to be dysregulated during early embryonic development, thus, it is likely associated to the occurring of ASD at early stages. Moreover, the dysregulation of miR-146a-5p has been correlated with the dysregulation of its validated targets such as NOTCH1, GRIA3, SYT1, NLGN1, which are crucial for brain development and function, as well as for neuroprogenitor cell differentiation, dendritic cell extension and impaired long-term potentiation of the synapses [243].

Among the few studies aimed to investigate the role of miRNAs in TS context, one recognized a rare nucleotide variant (var321) in the 3′ UTR of the mRNA of SLITRK1 gene, specifically in the binding site for miR-189. SLITRK1 mRNA and miR-189 showed an overlapping expression pattern in brain regions which were previously associated to TS [244].

To date, no one study has focused on miRNA expression in AC patients. Only heterozygous deletions including miR-140 gene were reported in a single individual with AC malformations, from an extensive research in CNV database regarding human phenotypes associated with CNVs [245].

2.2.3. Circulating microRNAs in human body fluids: promising non-invasive biomarkers for several diseases, including neurodevelopmental disorders

Although the majority of miRNAs were originally identified in the cellular microenvironment, a consistent number of miRNAs, known as circulating miRNAs, were detected in extracellular environments (i.e., plasma [246] and serum [247]).

Then, circulating miRNAs were recognized in all other mammalian body fluids, including saliva, tears, urine, colostrum, breast milk, peritoneal fluid, amniotic fluid, cerebrospinal fluid, pleural fluid, seminal fluid, synovial fluid, bronchial lavage, follicular fluid and feces [248, 249].

The remarkable stability of extracellular miRNAs in body fluids is a notable characteristic that allows them to survive and withstand adverse conditions for a long time, such as high/low pH, boiling, prolonged storage time and multiple freeze–thaw cycles [218, 249]. Furthermore, it has been demonstrated that extracellular miRNAs are more resistant to the high endogenous RNase activity in the extracellular environment compared to other endogenous RNA species, likely due to some protective mechanisms that are not still well clarified [248]. Their notable stability and the resistance to endogenous RNase activity in the extracellular space have been explained by several hypotheses, which include the binding with lipoprotein complexes like high-density lipoproteins (HDL), Argonaute2 (Ago2), nucleophosmin I or the encapsulation within microparticles as exosomes, microvesicles and apoptotic bodies [249].

Further theories proposed the involvement of the extracellular vesicles to explain the potential mechanisms underpinning the sorting of miRNAs.

According to these theories, once packaged into exosomes and other microvesicles into the cell, miRNAs can be selectively and actively secreted into the bloodstream, through the fusion of the multivesicular bodies and plasma membranes. In addition, miRNAs can passively leave the cell within the apoptotic bodies in response to a damage or an alteration of the cell membrane, in cases of cell death, apoptosis, metastasis or inflammation [250].

Finally, exosomes and the other microvesicles can fuse with a recipient cell membrane, establishing an intercellular communication. Other carriers, such as HDL, apoptotic bodies, Ago protein complexes, can also transport the secreted miRNAs to adjacent target cells or distal tissues. Once into the recipient cell, miRNAs can be cleaved by helicase, bound and activated by an Ago protein, through which exert their regulatory functions on mRNA targets (Figure 2.8) [251].

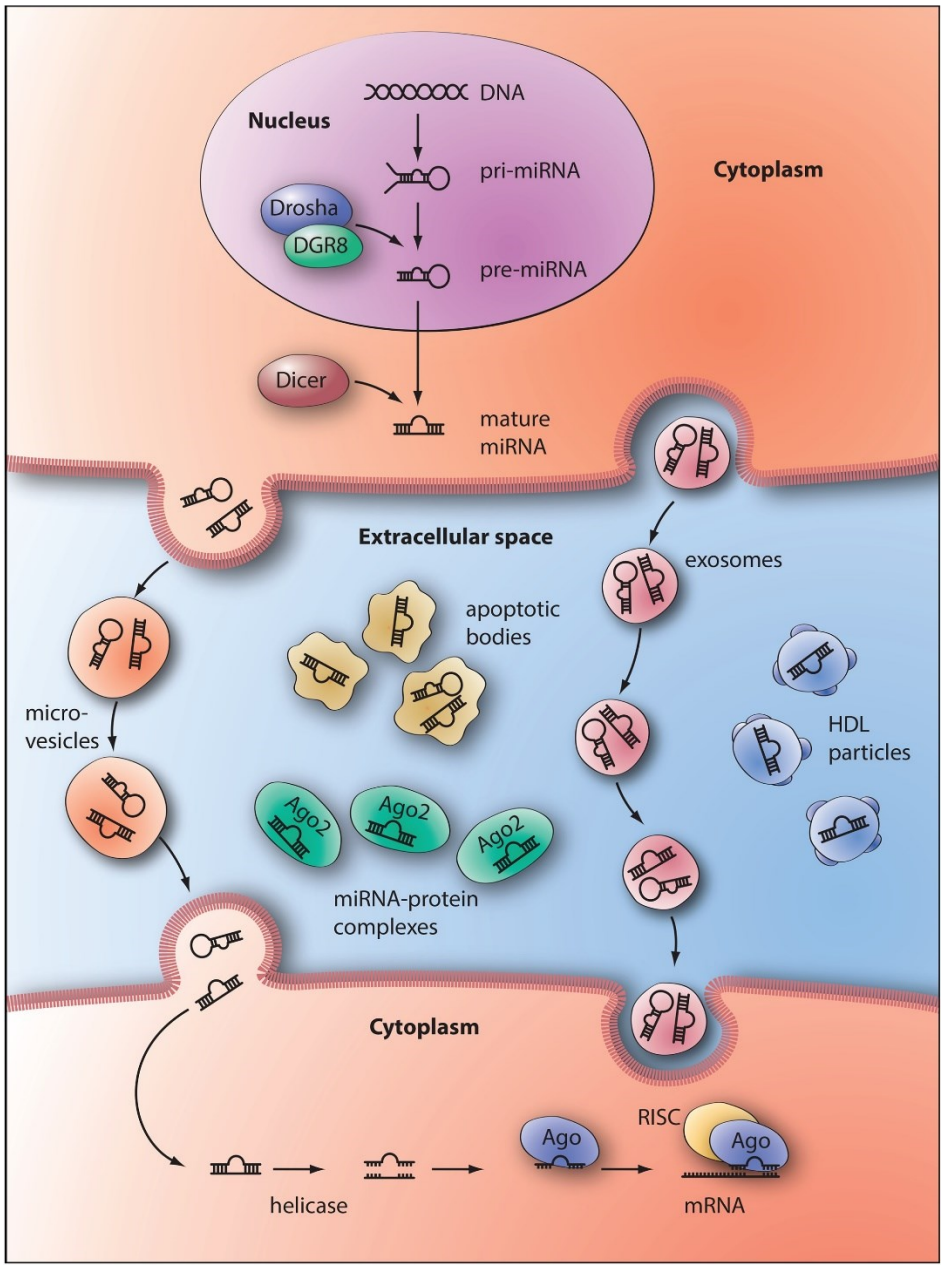


Figure 2.8. Representation of processing, release and transport of miRNAs (from *Vegter E.L. et al., Eur J Heart Fail, 2016*). After biogenesis (see Chapter 2, section 2.2), microRNAs can directly act on cellular mRNA targets or can be released into the extracellular environment and circulation. Exosomes, microvesicles, HDL, apoptotic bodies and Ago2 protein work as carriers. Into the recipient cell, miRNA can be further processed to exert its regulatory function on gene expression, by binding Ago protein and loading into the RNA-induced silencing complex.

Therefore, extracellular miRNAs show an expanded role of influencing biological mechanisms outside and far from the tissue in which they originated. This wide field of action and the potential role as an intercellular communication system, along with the high stability, the consistent presence in all biofluids and their specific expression patterns in relation to both physiological and pathological conditions, have notably attracted many researchers.

A plethora of studies have demonstrated that circulating miRNAs may be accounted as emerging biomarkers for several diseases, such as cardiovascular diseases [252], cancer [253], neurodegenerative diseases [254-257], brain pathological conditions [258], neurodevelopmental and neuropsychiatric disorders [259, 260].

Specifically, several studies recently reported altered levels of circulating miRNA in serum [261-266], and fewer in saliva [267-270] of ASD patients compared to healthy subjects. On the other hand, to date, only two studies have been performed to investigate circulating miRNA levels in serum of TS patients [271], also in comorbidity with ASD [261].

Overall, the expression levels of circulating miRNAs may represent molecular fingerprints associated with both physiological and pathological conditions in the study of ASD, TS, as well as other brain anomalies like AC malformations. Therefore, the molecular signatures may help the differential diagnostic process, including cases of comorbidity.

The use of serum or saliva, as a source of starting biological material, provides a more effective and suitable alternative to the conventional tissue biopsies, which often lack or are not easily accessible to researchers for the study of neurodevelopmental disorders. In particular, saliva collection is the most non-invasive method, especially when children are included in the study. For all these reasons, salivary miRNAs are progressively emerging as promising diagnostic, prognostic and predictive biomarkers in neurodevelopmental disorders [249].

However, we need to take into account several critical issues hindering their reliability for the practical clinical application. Analyzing circulating miRNAs and their dysregulation in pathological

phenotypes is still challenging for several reasons. For example, it is known that the concentration of circulating miRNAs in body fluids is much lower than in cellular environment and it is not clear how accurately circulating microRNAs reflect the transcriptomic framework of pathophysiological conditions of secreting cells [272, 273]. Further studies are notably needed to elucidate their suitability as appropriate biomarkers in clinical practice.

2.2.4. The emerging interplay between microRNAs and Microbiome

Although many questions remain to be addressed, changes in the gut microbiota composition have been widely associated with gut-brain axis related disorders, such as autism. Moreover, it has been proposed that pathogens may modulate the expression of host miRNAs, with the aim to increase their survival [274]. Host miRNAs, in turn, regulate gene expression, consequently fine-tune many biological pathways and have an impact on the survival and composition of gut bacteria [275-277]. Overall, three possible mechanisms of interaction may exist between the host and gut microbiome through the regulation by host miRNAs (Figure 2.9) [278].

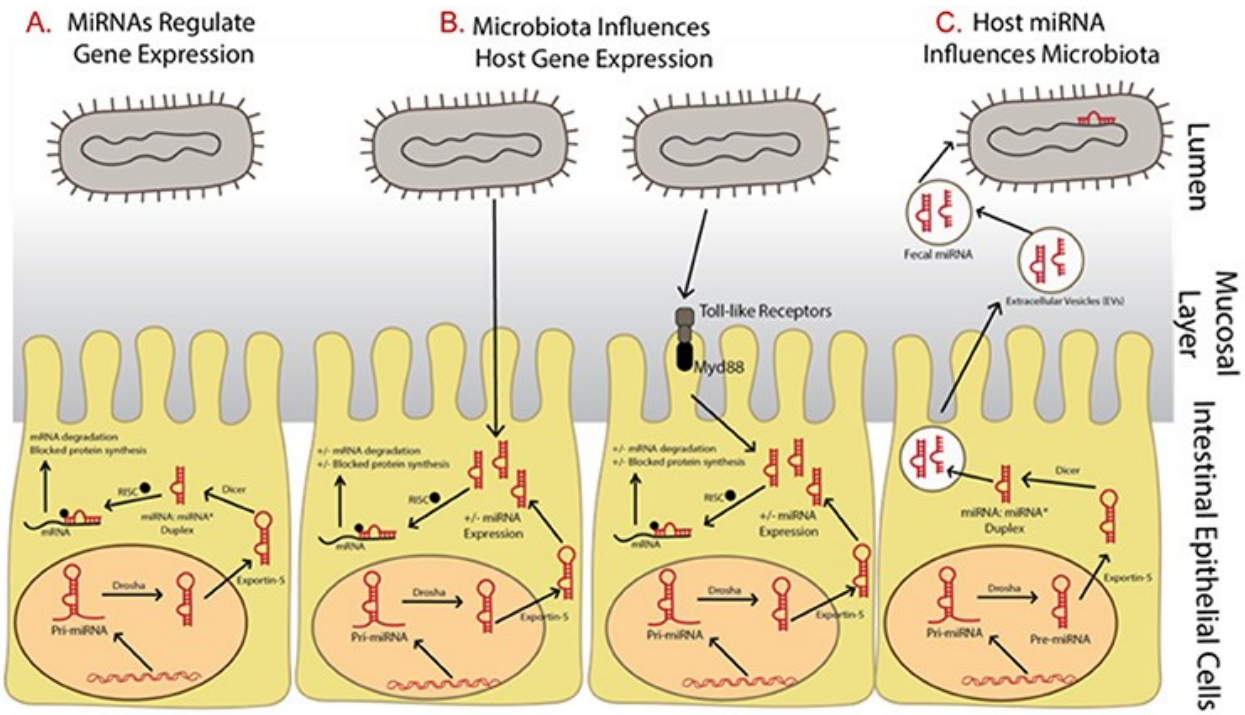


Figure 2.9. Summary of the potential interplay between miRNAs and microbiota and the effects on gene expression regulation in human gut (from *Williams M.R. et al., Front Microbiol, 2017*). (A) After biogenesis, miRNA regulate gene expression; (B) It has been proposed that microbiota may regulate miRNA expression, via the toll-like receptor/Myd88-dependent pathways. (C) The host may affect the gut microbiome though the action of fecal miRNAs, subsequently taken up by bacteria.

Therefore, host miRNAs and the gut microbiota may cooperate with each other and influence their expression and abundance, respectively.

To date, only two studies examined the potential association between bacteria and microRNAs in oral cavity. The first study [279] explored the daily oscillations of salivary miRNA and microbiome in physiological condition, to investigate their potential interactions and the implications. They observed the association among five circadian miRNAs and four circadian microbial RNAs. The second study [268], evaluated the salivary poly-omic RNA measurements in 19–83 months ASD and non-ASD children, aimed to propose a non-invasive approach for the discrimination of ASD. Moderate associations between piR-6463 and Clostridiales, *Pasteurella multocida*, *Leadbetterella byssophilia* and between piR-29114 and Clostridiales were revealed by correlation analysis.

Significance, purposes and introduction of the research projects

Increasing studies are continuously providing new missing pieces of the complex architecture of neurodevelopmental disorders. However, many questions are still unsolved and many related issues have not been yet addressed.

For instance, the choice of experimental models for studying the pathophysiological mechanisms of NDDs has been quite questionable over time for many limitations. Among these, tissue biopsies (e.g., post-mortem brain samples) are far from being easily accessible, contrarily to liquid biopsies which are emerging as a precious source of molecular biomarkers to further unveil new pathological mechanisms of such elusive diseases.

In addition, delineating psychiatric disorders poses significant challenges for diagnosis because of the complexity and heterogeneity of phenotypes. However, clinical diagnosis of neurodevelopmental disorders only relies on subjective behavior-based assessments. Therefore, an unbiased molecular approach is needed to support the current diagnostic practice.

Based on these premises, the two research projects conducted during my PhD program aimed to profile circulating miRNA expression (i) in saliva from ASD patients and (ii) in serum from AC, TS and comorbid ACTS patients, compared to healthy individuals. Moreover, the first project aimed to verify the existence of a molecular correlation between miRNA dysregulation and microbiota alterations, as well as their contribution to ASD pathogenesis.

Many studies proposed the use of circulating miRNAs as promising biomarkers for several pathologies, but very few studies profiled salivary miRNAs in ASD, much fewer ones explored serum miRNAs in TS context, and no one in AC syndrome and in the very rare comorbid condition between AC syndrome and TS (ACTS). In addition, while mounting evidences suggest a key role for the gut microbiota in ASD, the etiopathogenetic contribution of microorganisms living in the oral cavity has not been satisfactorily explored.

The following chapters introduce the two research projects carried out during my PhD program.

The first chapter describes the project findings obtained by (i) a combined approach of miRNA expression profiling by NanoString technology, followed by validation experiments in qPCR, and 16S rRNA microbiome analysis in saliva of ASD compared to neurologically unaffected children; (ii) correlation and negative binomial regression analyses among salivary miRNAs, bacteria and neuropsychological/hematological parameters; (iii) univariate and multivariate ROC curve analysis, which assessed the diagnostic accuracy of salivary miRNAs and microbiome taxa; (iv) functional enrichment analysis, which identified the functional involvement of the salivary miRNAs in molecular signaling pathways related to ASD.

The manuscript of this research project has been published in August 2020, with the title "Potential Associations Among Alteration of Salivary miRNAs, Saliva Microbiome Structure, and Cognitive Impairments in Autistic Children", co-authored by Ragusa M., Santagati M., Mirabella F., Lauretta G., Cirnigliaro M., Brex D., Barbagallo C., Domini C.N., Gulisano M., Barone R., Trovato L., Oliveri S., Mongelli G., Spitale A., Barbagallo D., Di Pietro C., Stefani S., Rizzo R., Purrello M., on the scientific journal titled “International Journal of Molecular Sciences”, as part of the Special Issue Medical Genetics, Genomics and Bioinformatics [280].

The second chapter describes the project findings obtained by (i) miRNA expression profiling by NanoString technology in serum of AC, TS and ACTS patients compared to unaffected controls; (ii) correlation analysis between serum miRNA expression levels and neuropsychological, neuroimaging parameters; (iii) functional enrichment analysis, that identified the functional involvement of the serum miRNAs in molecular signaling pathways related to AC syndrome and TS.

The manuscript of this research project has been published in January 2021, with the title “Enrichment and correlation analysis of serum miRNAs in comorbidity between Arnold-Chiari and Tourette syndrome contribute to clarify their molecular bases”, co-authored by Mirabella F., Gulisano M.,

Capelli M., Lauretta G., Cirnigliaro M., Palmucci S., Stella M., Barbagallo D., Di Pietro C., Purrello M., Ragusa M., Rizzo R., on the scientific journal titled “Frontiers in Molecular Neuroscience” [281].

The realization of these projects has been made possible by the collaboration between the research team led by Professor Michele Purrello (Section of Biology and Genetics G. Sichel, University of Catania), the research team led by Professor Renata Rizzo (Section of Child and Adolescent Psychiatry, University of Catania) and the research team led by Professor Stefania Stefani (Section of Microbiology, University of Catania) for the first project. Professors Michele Purrello, Marco Ragusa, Renata Rizzo conceived the two projects and planned the experiments, together with Professor Stefania Stefani for the ASD project. Professor Renata Rizzo performed clinical diagnosis and, together with her research team, carried out patients’ recruitment.

I would like to express my sincere gratitude to my Supervisor, Professor Michele Purrello, for providing me the opportunity to join his team and conduct the research projects, inspiring and guiding my way through this invaluable PhD journey. I am also profoundly grateful to Professor Marco Ragusa, for his precious guidance and for all the insightful comments which were critical to the implementation of the projects. My sincere thanks also go to all the research team members for their contribution in the research presented here and for supporting me during these three amazing years.

Chapter 3. Potential associations among alteration of salivary miRNAs, saliva microbiome structure and cognitive impairments in autistic children

3.1. Abstract

Saliva represents an important reservoir of biomarkers that range from metabolites, RNAs and proteins, to the microbiota structure. Recent evidences have demonstrated that salivary molecules, such as microRNAs, and bacterial populations can be perturbed by several pathological conditions, including neuropsychiatric diseases. This relationship between brain functionality and saliva composition could be exploited to unveil new pathomechanisms of elusive diseases, such as Autistic Spectrum Disorder (ASD). To investigate salivary ASD-related miRnome and microbiome alterations, we performed a combined approach of miRNA expression profiling by NanoString technology, followed by validation experiments in qPCR, and 16S rRNA microbiome analysis on saliva from 53 ASD and 27 neurologically unaffected control (NUC) children.

MiR-29a-3p and miR-141-3p were upregulated, while miR-16-5p, let-7b-5p, and miR-451a were downregulated in ASD compared to NUCs. Microbiome analysis on the same subjects revealed that *Rothia, Filifactor, Actinobacillus*, *Weeksellaceae, Ralstonia, Pasteurellaceae* and *Aggregatibacter* increased their abundance in ASD patients, while *Tannerella, Moryella* and *TM7-3* decreased. Variations of both miRNAs and microbes were statistically associated to different neuropsychological scores related to anomalies in social interaction and communication. Among miRNA/bacteria associations, the most relevant was the negative correlation between salivary miR-141-3p expression and *Tannerella* abundance.

Univariate ROC curve analysis for miRNAs and bacteria showed moderate but significant Area Under Curves (AUCs) values, while, testing the combination of all miRNAs and bacteria through

multivariate ROC curve analysis, the predictive performance notably increased. In fact, results showed that the combination of the 5 salivary miRNAs and 4 bacterial species (i.e., *Filifactor*, *Moryella*, *Tannerella* and *TM7-3*), in a single multivariate model, significantly represented the best-fit performing model for ASD diagnosis, suggesting a potential application as non-invasive biomarkers. In this study we report that miRNA and microbiome dysregulations found in saliva of ASD children are potentially associated with cognitive impairments of the subjects. These findings could pave the way to new potential valuable tools for molecular diagnosis of ASD. Furthermore, a potential cross-talking between circulating miRNAs and resident bacteria could occur in saliva of ASD.

3.2. Results

3.2.1. Demographic and neuropsychological characteristics

We recruited 115 children from various socio-economic contexts (age range 4 – 8): 76 treatment-naïve patients affected by ASD (M:F 62:15), mean age 7 (SD ± 1.5), which were compared to 39 NUCs (M:F 29:11), mean age 6.75 (SD ± 1.51). Demographic, neuropsychological and hematological characteristics of the clinical sample are shown in table 3.1.

	ASD	NUC
Number of participants	76	39
Sex (M:F)	60:16	28:11
Age (years)	6.9 (± 1.5)	6.9 (± 1.8)
Time of collection (h)	09:58 (± 00:28)	10:03 (± 00:30)
Time since last meal (h)	2.99 (± 0.12)	2.98 (± 0.13)
IQ (score)		
TIQ	69.6 (± 19.3)	96.7 (± 11.6)
VIQ	67.8 (± 19.7)	96.2 (± 13.3)
PIQ	71 (± 21.4)	97.9 (± 12.7)
ADOS (score)		
A	4.6 (± 1.9)	0
B	7.7 (± 2.4)	0
C	2.1 (± 1.2)	0
D	2.6 (± 1.3)	0
ADI-R (score)		
A	10.9 (± 3.7)	0
B	9.4 (± 2.5)	0
C	5.9 (± 2.8)	0
D	3.2 (± 1.3)	0
Prolactine (µUI/mL)	229.8 (± 89.5)	/
Ceruloplasmin (mg/dL)	27.6 (± 5.6)	/
Lactate (mg/dl)	15.3 (± 4.8)	/
Ammonium (µmol/L)	24.7 (± 9.3)	/
TSH (µUI/mL)	2.3 (± 0.9)	/

Table 3.1. Demographic, neuropsychological and hematological characteristics of the clinical samples. Data are shown as means and ± standard deviations between brackets. IQ: Intelligence Quotient; TIQ: Total Intelligence Quotient; VIQ: Verbal Intelligence Quotient; PIQ: Performance Intelligence Quotient; ADOS: Autism Diagnostic Observation Schedule; ADOS-A: Communication; ADOS-B: Social Interaction; ADOS-C: Imagination; ADOS-D: Repetitive and Restricted Behaviour; ADI-R: Autism Diagnostic Interview-Revised; ADI-A: Qualitative anomalies in social interaction; ADI-B: Qualitative anomalies in communication; ADI-C: Repetitive and restricted behaviour; ADI-D: Anomalies in neurodevelopment arisen before 36 months; TSH: Thyroid stimulant hormone. According to two-tailed Mann–Whitney U test, there were no statistically significant differences among groups in sex (p-value = 0.3496), age (p-value = 0.6731), time of sample collection (p-value = 0.2008) and time since last meal (p-value = 0.4456).

3.2.2. Salivary miRNA expression profiling

By using the nCounter NanoString technology, we performed a high-throughput expression analysis of 800 microRNAs in saliva of 23 ASD patients and 12 NUC subjects. We identified 10 miRNAs as significantly differentially expressed in ASD patients compared to negative controls, 6 upregulated (miR-29a-3p, miR-141-3p, miR-146a-5p, miR-200a-3p, miR-200b-3p, miR-4454+miR-7975) and 4

downregulated (miR-16-5p, miR-205-5p, miR-451a, let-7b-5p). The relative expression of DE miRNAs is shown as a heat-map in Figure 3.1.

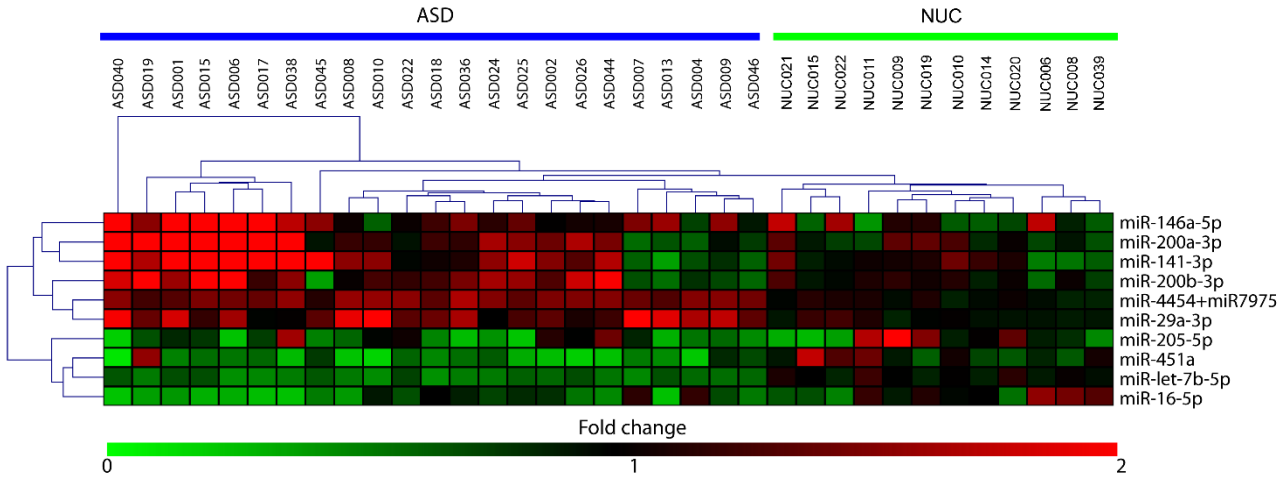


Figure 3.1. Heat-map of DE-miRNAs in saliva of ASD and neurologically unaffected control (NUC) individuals. The values of fold changes for each miRNA are color coded, as shown in the colored bar. The matrix was generated by plotting the fold changes calculated as the ratio between the normalized counts of each sample and the mean of normalized counts of all NUC samples. Sample clustering obtained through hierarchical clustering (Manhattan distance metric) approach is shown.

3.2.3. Salivary miRNA expression validation

To validate these findings, we tested the expression of the 7 most dysregulated miRNAs (miR-16-5p, miR-29a-3p, miR-141-3p, miR-146a-5p, miR-200a-3p, miR-451a, let-7b-5p) through single TaqMan assays in an independent cohort, composed of 53 ASD patients and 27 NUC subjects. RT-PCR results showed that 5 salivary miRNAs were altered in ASD patients compared to NUCs in a statistically significant manner. More specifically, miR-29a-3p (p-value = 0.0123, Cliff's δ= 0.341) and miR-141-3p (p-value = 0.0431, Cliff's δ= 0.277) were upregulated, while miR-16-5p (p-value = 0.0002, Cliff's δ= -0.502), miR-451a (p-value < 0.0001, Cliff's δ= -0.520) and let-7b-5p (p-value = 0.0002, Cliff's δ= -0.499) were downregulated in the validation group, confirming the results obtained by NanoString analysis. Although the expression of miR-146a-5p and miR-200a-3p evaluated by RT-PCR had the same dysregulation trend observed by NanoString profiling, these alterations were not statistically significant. Table 3.2 and Figure 3.2 show the relative expressions of the 5 DE miRNAs.

DE miRNA (ASD vs NUC)	FC	p-values
let-7b-5p	-1.99	0.0002
miR-16-5p	-1.68	0.0002
miR-29a-3p	1.43	0.0123
miR-141-3p	2.93	0.0431
miR-451a	-3.58	< 0.0001

Table 3.2. Validation analysis of miRNA expression in saliva. Salivary miRNA expression validation in ASD and NUC groups. P-values were obtained from Mann-Whitney test. Expression FC (Fold Change) values are shown.

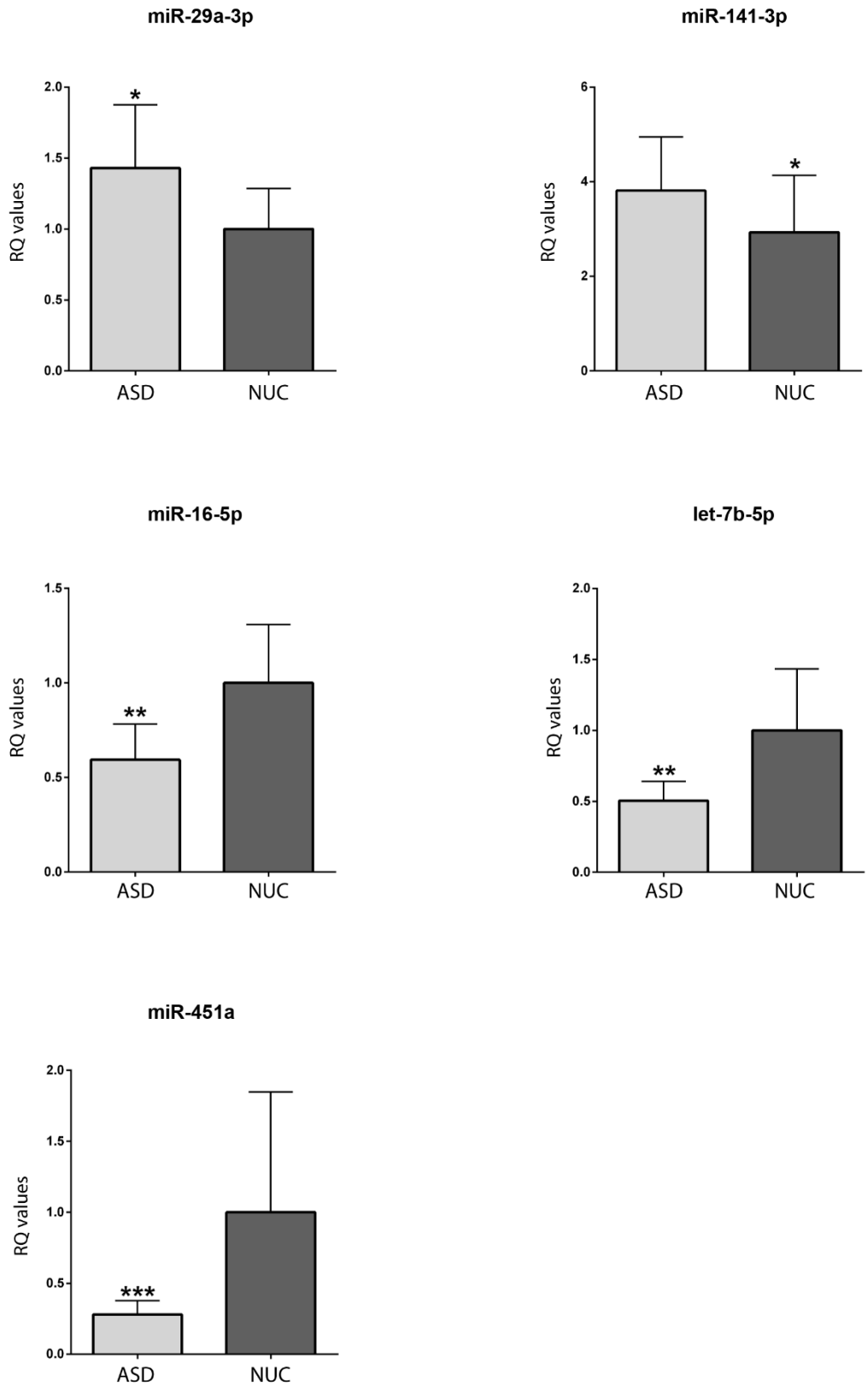


Figure 3.2. Expression validation of the 5 candidate salivary miRNAs. Bar plots of relative expression of the 5 miRNAs which showed a statistically significant dysregulation in the validation group, assessed by Single TaqMan Assays: miR-29a-3p, miR-141-3p, miR-16-5p, let-7b-5p, miR-451a. Data are shown as geometric mean with 95% of CI. Y-axis represents the distribution of RQ values. * p-value ≤ 0.05; ** p-value ≤ 0.0005; *** p-value ≤ 0.0001.

The expression values of ASD and NUC are quite heterogeneous, similar to the results of NanoString profiling. Notwithstanding this endogenous variability, their expression differences resulted statistically significant. Cliff's delta effect size values ranged from -0.5206 to 0.3417, showing small and medium effect sizes according to Cliff's statistics, likely due to the complex heterogeneity of autism and the limited sample size.

3.2.4. Microbial Structure of the Saliva Microbiome in Children with ASD and NUCs

To evaluate changes in the microbial composition of the salivary microbiome, a total of 53 ASD and 27 NUC samples were sequenced using the Illumina MiSeq platform.

A total of 10,919,413 valid reads with an average of 127,464 reads per sample were generated (74,469–1,641,234 range). All sequences were assigned to 341 Operational taxonomic Units (OTUs) with at least 97% similarity level showing 10 phyla, 65 genera, and 86 species. The alpha diversity, Chao1 index (richness), Shannon index (diversity), and Shannon Evenness E index (evenness) revealed no significant differences between ASD and NUC groups (p-value > 0.05) although the Chao1 index showed a slight decrease in the ASD group (Figure 3.3 A). The overall dissimilarities of microbial community structure between the two groups were calculated by using the weighted UniFrac distance. The beta diversity shown by PCoA (principal coordinate analysis) plot on weighted (accounting for the abundance of OTUs) revealed no clustering differentiation of the bacterial communities in the two groups (Figure 3.3B). The composition of the salivary microbiome between ASD and NUC was explored in terms of the relative abundances at different taxonomic levels through White's non-parametric t-test (p-value ≤ 0.05), using STAMP software. Predominant phyla in ASD and NUC groups were Firmicutes (41.1% vs. 42.7%), Bacteroidetes (18.9% vs. 22.3%), Proteobacteria (16.9% vs. 10.8%), Actinobacteria (14.1% vs. 13.3%), and Fusobacteria (7.6% vs. 9.3%) constituting 98.6% of salivary microbes (see Supplementary Figure S3.1). Overall, Proteobacteria were more abundant in ASD patients compared to control participants and a slight

increase was also observed in Actinobacteria, while the other phyla were more abundant in the NUC group.

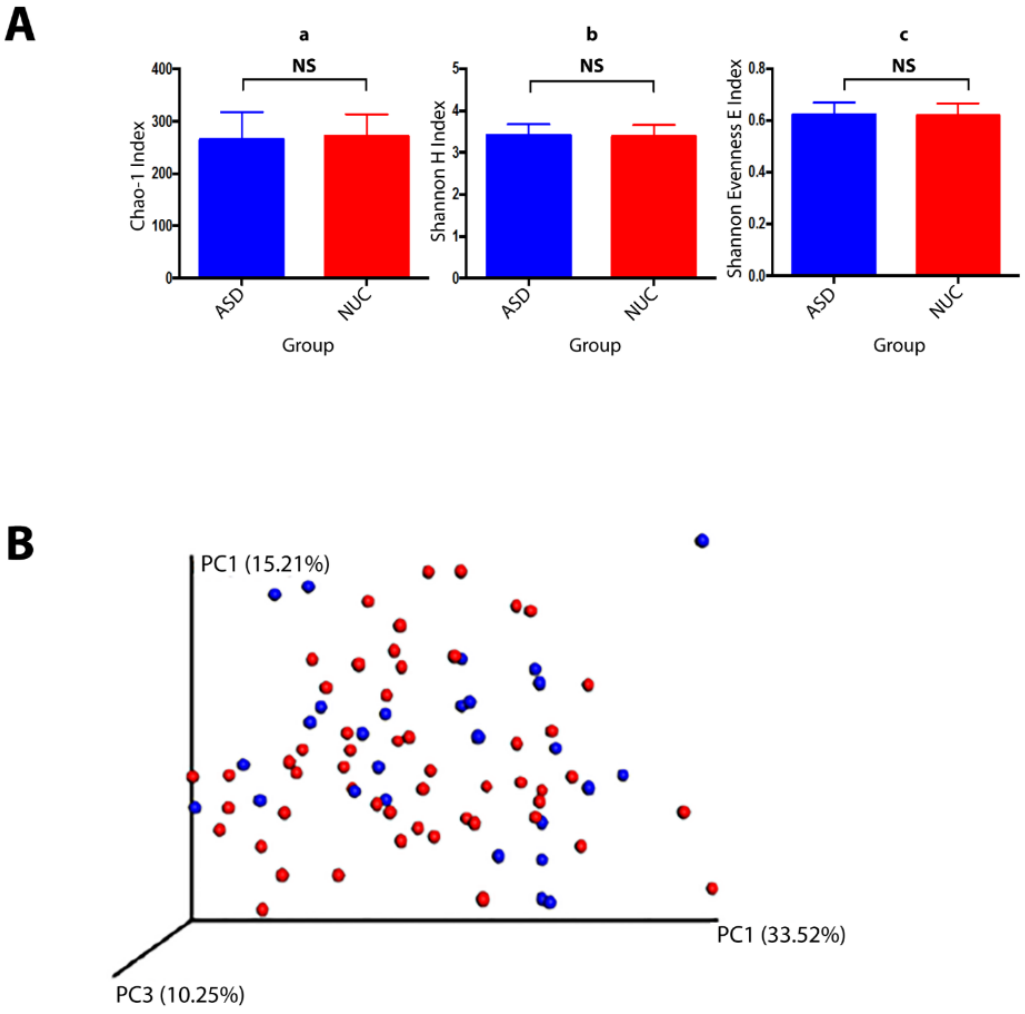


Figure 3.3. Bacterial community structures of the salivary microbiome in children with ASD and NUC groups. (A) Structural comparison of α-diversity of the salivary microbiome. Sequences were randomly sub-sampled at the rarefaction point (74,469) from dataset. Chao-1 index (a, community richness), Shannon H index (b, diversity), Shannon E index (c, evenness) were calculated for saliva samples. The bars depict mean ± SD of relative abundance rates. NS = non-statistically significant (p-value > 0.05). ASD (salivary samples n = 53), NUC (salivary samples collected from healthy controls, n = 27). (B) β-diversity. PCoA plot generated using weighted UniFrac distances show none differences between the two groups (ASD in red and NUC in blue).

A total of 65 genera were detected (supplementary Figure S3.2) and among these, 10 genera showed a statistically significant difference in relative abundance between ASD and NUC children by applying the two-sided White's non-parametric t-test (Figure 3.4A), and also confirmed by applying the Mann–Whitney–Kruskal–Wallis test and t-test/ANOVA (supplementary Table S3.1).

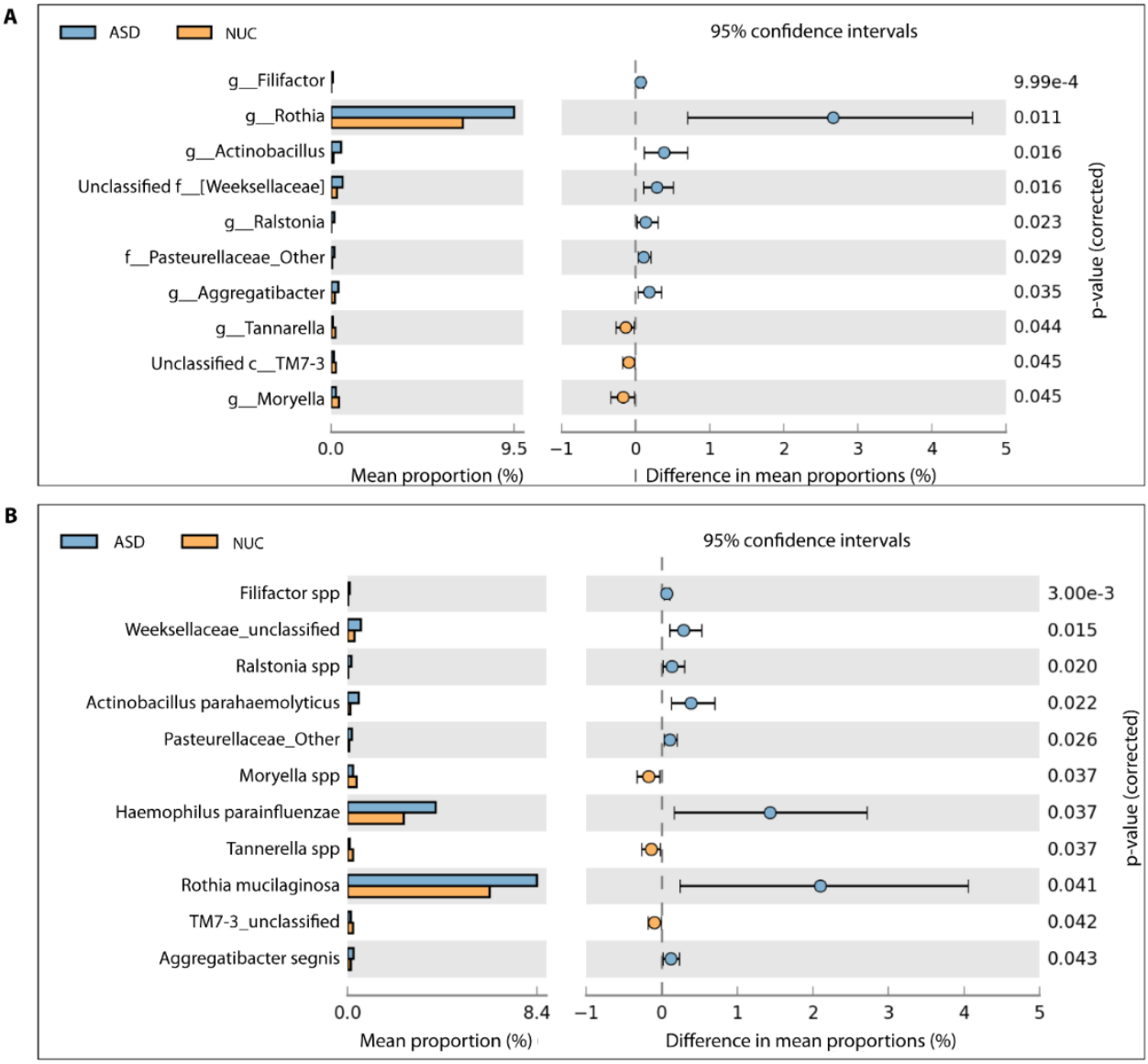


Figure 3.4. Different bacterial abundance in saliva of ASD and NUC groups. Statistical analysis of the bacterial abundance at genus (A) and species level (B) in ASD and NUC groups by applying a two-sided White's non-parametric t-test.

In particular, *Rothia*, *Filifactor*, *Actinobacillus*, *Weeksellaceae*, *Ralstonia*, *Pasteurellaceae*, and *Aggregatibacter* increased their abundance rates in ASD, while *Tannerella*, *Moryella*, and *TM7-3* decreased (Figure 3.4A). At the species level, we found statistically significant differences for 11 taxa out of 86 species (Figure 3.4B and Supplementary Figure S3.3). More specifically, *Filifactor* (Cliff's $\delta = 0.261$), *Weeksellaceae* (Cliff's $\delta = 0.096$), *Ralstonia* (Cliff's $\delta = 0.217$), *Actinobacillus parahaemolyticus* (Cliff's $\delta = 0.077$), *Pasteurellaceae* (Cliff's $\delta = 0.043$), *Haemophilus parainfluenzae* (Cliff's $\delta = 0.220$), *Rothia mucilaginosa* (Cliff's $\delta = 0.078$), and *Aggregatibacter*

segnis (Cliff's δ = 0.032) were more abundant in saliva of ASD patients, in contrast to *Moryella* (Cliff's δ = −0.400), *Tannerella* (Cliff's δ = −0.412), and *TM7-3* (Cliff's δ = −0.455) (Figure 3.4B). Cliff's delta effect size values ranged between −0.4556 and 0.2613 and, according to the Cliff's statistics, could be considered small and mostly moderate.

3.2.5. Correlation and Negative Binomial Regression Analyses Among Salivary miRNAs, Bacteria, and Neuropsychological/Hematological Parameters

Spearman correlation analyses were computed among DE miRNAs, bacteria differentiating ASD and NUCs and neuropsychological/hematological scores (Figure 3.5).

This statistical analysis revealed a negative relationship between let-7b-5p, miR-451a, and ADI-A (Qualitative anomalies in social interaction) (r = −0.37, r = −0.38, respectively to let-7b-5p and miR-451a), ADI-B (Qualitative anomalies in communication) (r = −0.33, r = −0.36, respectively), Autism Diagnostic Observation Schedule (ADOS)-A (Communication) (r = −0.32, r = −0.39, respectively), ADOS-D (Repetitive and Restricted Behavior) (r = −0.38, r = −0.36, respectively). MiR-451a showed a negative correlation with ADI-D (Anomalies in neurodevelopment arising before 36 months) and ADOS-B (Social Interaction) (r = −0.34, r = −0.40, respectively). MiR-16-5p was negatively related to ADOS-C (Imagination) (r = −0.37) and ADOS-D (r = −0.36); while a positive correlation was found between miR-29a-3p and ADI-B (r = 0.37) and ADOS-A (r = 0.33). Finally, miR-141-3p was the only miRNA to show a positive linear correlation with a metabolic parameter (i.e., lactate, r = 0.38) (Figure 3.5A).

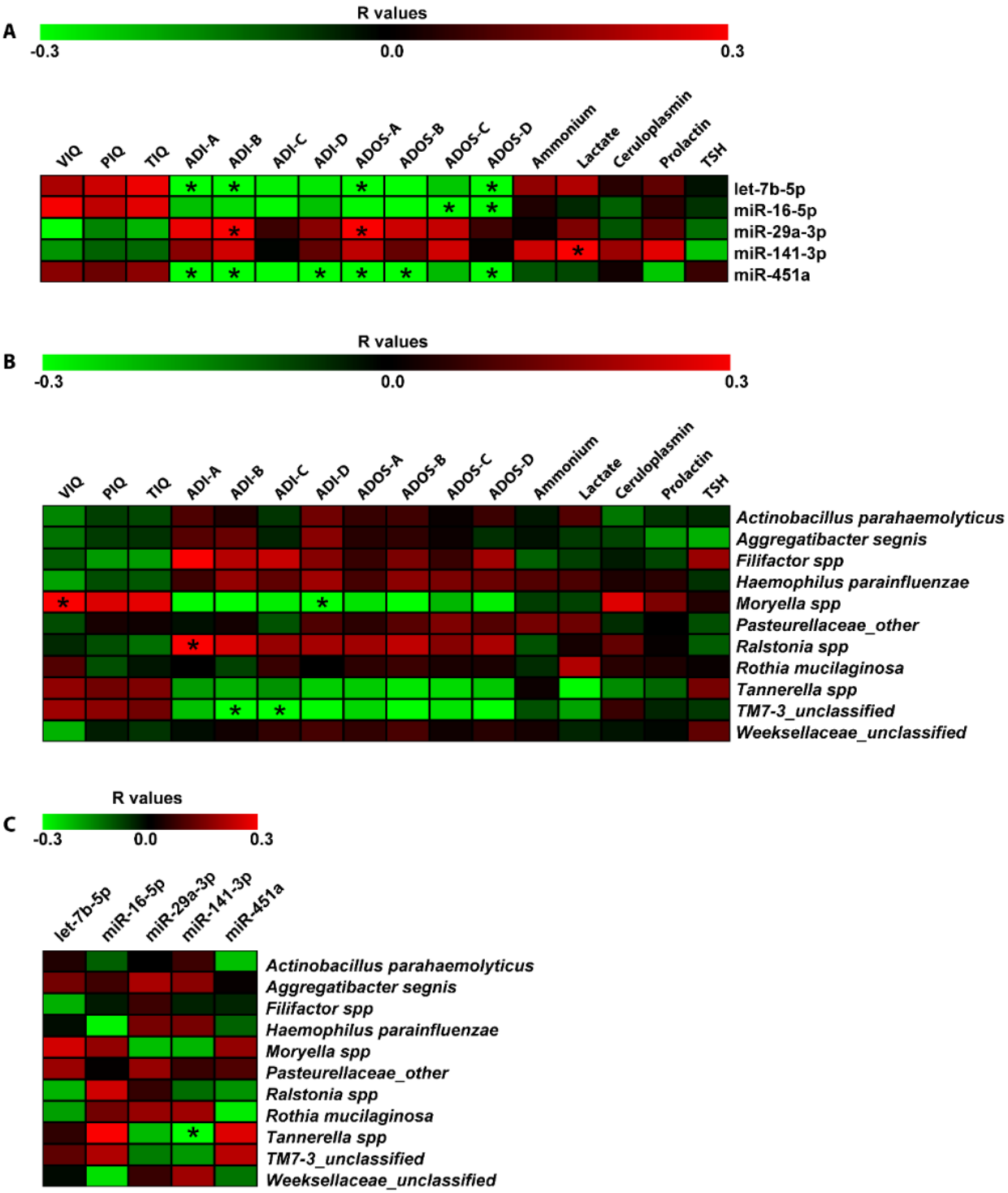


Figure 3.5. Correlation analysis of miRNA expression, microbiome Operational taxonomic Units (OTUs), neuropsychiatric/metabolic parameters. Correlation matrices were built by calculating Spearman correlation coefficients for (A) miRNA expression and neuropsychiatric/metabolic parameters; (B) microbiome OTUs and neuropsychiatric/metabolic parameters; and (C) microbiome OTUs and miRNA expression. The correlation coefficient is indicated by a color gradient from green (negative correlation) to red (positive correlation), as shown in the colored bar. Statistically significant p-values corrected for multiple comparisons by using Bonferroni–Šídák approach are indicated by asterisks. VIQ: Verbal Intelligence Quotient; PIQ: performance Intelligence Quotient, TIQ: Total Intelligence Quotient; ADOS-A: Communication; ADOS-B: Social Interaction; ADOS-C: Imagination; ADOS-D: Repetitive and Restricted Behavior; ADI-A: Qualitative anomalies in social interaction; ADI-B: Qualitative anomalies in communication; ADI-C: Repetitive and restricted behavior; ADI-D: Anomalies in neurodevelopment arisen before 36 months.

The same correlation analysis was also performed on salivary bacteria. We found a positive correlation between *Moryella* and VIQ ($r = 0.32$), and a negative relationship between *Moryella* and ADI-D ($r = -0.34$). *TM7-3* exhibited a negative relationship with ADI-B ($r = -0.37$) and ADI-C ($r = -0.34$); while, *Ralstonia* showed a positive correlation with ADI-A ($r = 0.34$) (Figure 3.5B). Furthermore, computing Spearman correlations between salivary DE miRNAs and bacteria, we detected a negative association between *Tannerella* and miR-141-3p ($r = -0.30$) (Figure 3.5C).

For microbiome data, we also applied the negative binomial regression.

The Negative Binomial (NB) model was positively assessed for seven (*Tannerella*, *Weeksellaceae*, *Moryella*, *Filifactor*, *Ralstonia*, *Pasteurellaceae*, and *Actinobacillus parahaemolyticus*) of the eleven microbiome taxa and revealed many statistically significant regressions shown in Figure 3.6. For each of the predictor variable, supplementary table S3.2 reports a regression coefficient (B) with a p-value (p-value ≤ 0.05) resulting from the Wald Chi-Square test. Effect size measures, reported as Standardized mean difference (SMD) values, showed small and moderate effects (see Supplementary Table S3.2). Negative binomial regression (NBR) results were partially confirmed applying the multiple and simple linear regression models. These additional analyses confirmed the regression between Total Intelligence Quotient (TIQ), ADOS-A, ADOS-B and *Tannerella*; miR-141-3p and *Weeksellaceae*; miR-141-3p, ceruloplasmin and *Moryella*; PIQ and *Filifactor*; miR-141-3p, ADI-C, ADI-D, ammonium and *Actinobacillus parahaemolyticus* (Supplementary Table S3.3).

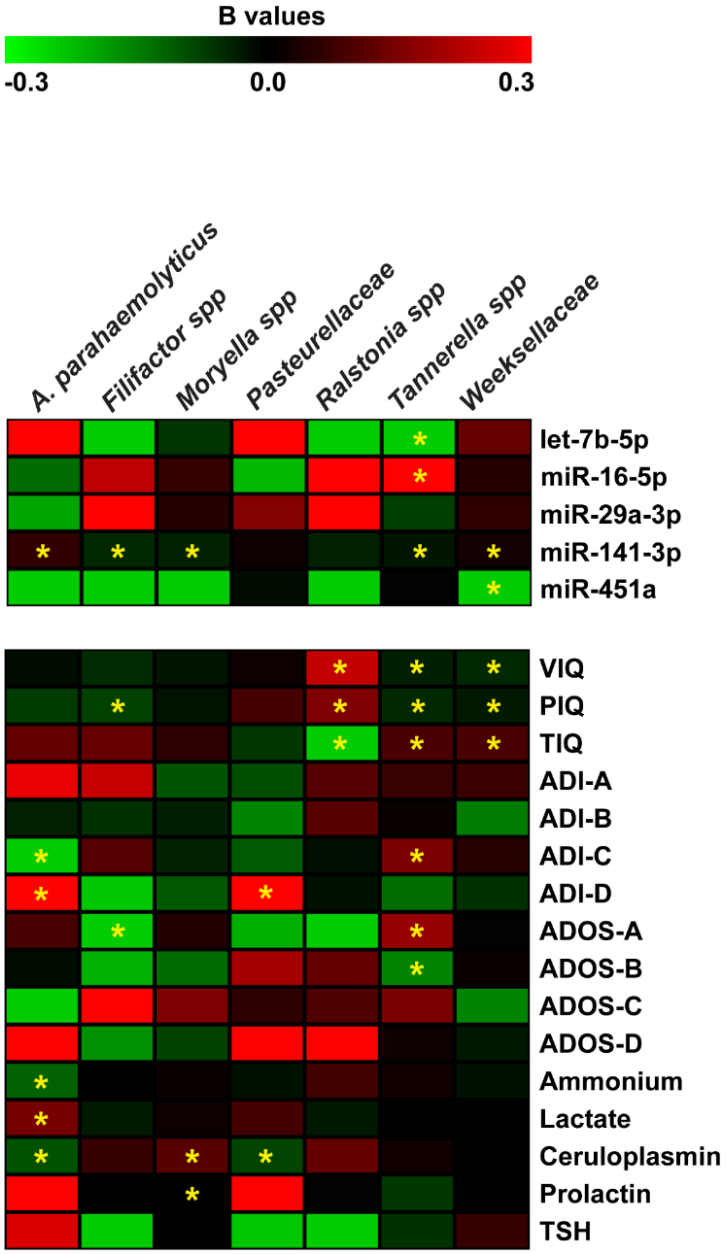


Figure 3.6. Negative binomial regression analysis between miRNA expression, neuropsychiatric/metabolic parameters and microbiome abundance. Coefficient regression matrix from negative binomial regression model predicting abundances of microbiome species. The regression coefficient is indicated by a color gradient from green (negative prediction) to red (positive prediction), as shown in the colored bar. Statistically significant p-values are indicated by asterisks.

3.2.6. Univariate and multivariate ROC curve analysis

Univariate and multivariate ROC curve analyses were performed to investigate the discriminating power of salivary DE miRNAs and bacteria. In table 3.3 the comparison of diagnostic accuracy of miRNA and microbial univariate and multivariate ROC curves is shown.

Markers	AUC	Sens. (%)	Spec. (%)	Acc. (%)	Positive predictive value (%)	Negative predictive value (%)	Positive Likelihood Ratio	Negative Likelihood Ratio	p-values	p-values after permutation
let-7b-5p	0.75	77	63	73	80	59	2.09	0.36	0	0.18
miR-16-5p	0.75	60	89	70	91	53	5.43	0.45	0	0.12
miR-29a-3p	0.67	51	81	61	84	46	2.75	0.6	0.01	0.04
miR-141-3p	0.64	64	67	65	79	49	1.92	0.54	0.04	0.08
miR-451a	0.76	92	63	83	83	81	2.5	0.12	0	<0.01
Filifactor	0.64	40	88	56	88	42	3.43	0.68	0.05	0.62
Moryella	0.69	77	58	71	79	56	1.83	0.39	0.01	0.02
Tannerella	0.7	91	50	77	79	72	1.81	0.19	0	0.02
TM7-3	0.72	77	62	72	80	57	2.01	0.37	0	0.01
5 miRNA model Multivariate curve	0.83	75	78	76	87	62	3.4	0.32	/	<0.01
4 taxa model Multivariate curve	0.82	83	69	78	85	67	2.7	0.25	/	<0.01
5 miRNA + 4 taxa model Multivariate curve	0.84	81	85	82	91	69	5.27	0.22	/	<0.01

Table 3.3. Comparison of diagnostic accuracy of miRNA and microbial univariate and multivariate ROC curves. Sens: sensitivity; Spec: Specificity; Acc: Accuracy; /: value not applicable.

In particular, the table reports: (i) AUC values, a measure for diagnostic accuracy; (ii) specificity and (iii) sensitivity values; (iv) accuracy values, which represent the overall probability that a patient has been correctly classified; (v) positive and negative predictive values, indicating the probability that the disease is present or not, respectively; (vi) positive and negative likelihood ratio, indicating the ratio True positive rate/False positive rate and the ratio False negative rate/True negative rate, respectively. The univariate ROC curve analysis revealed a statistical significance for all miRNAs and four of eleven taxa, but moderate AUC values: let-7b-5p (AUC = 0.75; p-value = 0.0003), miR-16-5p (AUC = 0.75, p-value = 0.0002), miR-29a-3p (AUC = 0.67, p-value = 0.0128), miR-141-3p (AUC = 0.64, p-value = 0.0434), miR-451a (AUC = 0.76, p-value = 0.0001) (Figure 3.7A), *Filifactor* (AUC = 0.64, p-value = 0.0486), *Moryella* (AUC = 0.69, p-value = 0.0067), *Tannerella* (AUC = 0.70, p-value = 0.0044) and *TM7-3* (AUC = 0.72, p-value = 0.0018) (Figure 3.7B).

Permutation testing revealed statistical significance for all aforementioned miRNAs and taxa, except for let-7b-5p, miR-16-5p and *Filifactor* (see Table 3.3).

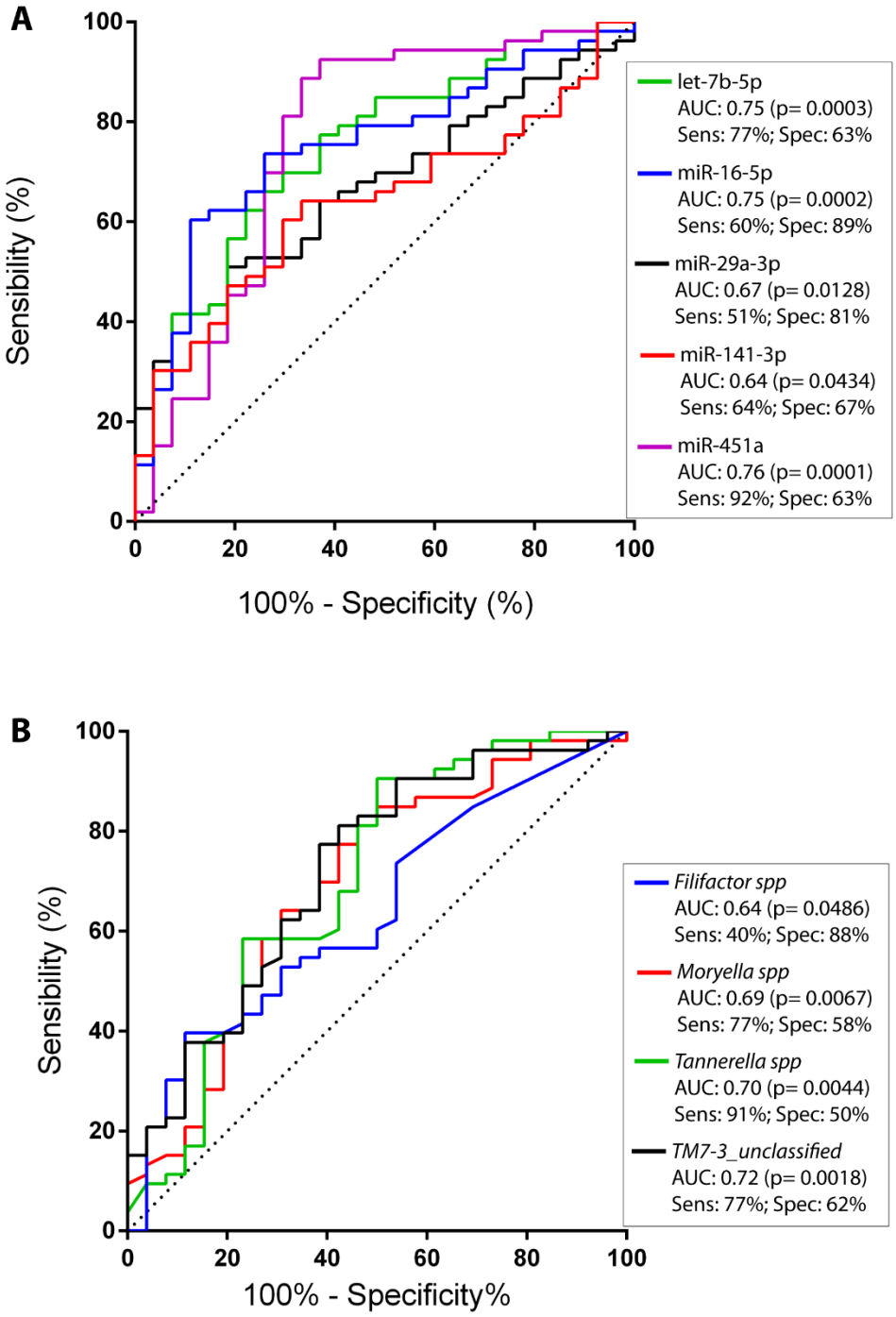


Figure 3.7. Univariate ROC curve analysis for miRNAs and 4 bacterial species. The plot of the true positive rate (y-axis) (Sensitivity) in function of the false positive rate (y-axis) (1-Specificity) is representative of univariate ROC curves of (A) let-7b-5p, miR-16-5p, miR-29a-3p, miR-141-3p, miR-451a and (B) *Filifactor*, *Moryella*, *Tannerella*, *TM7-3* species differentiating ASD patients from NUCs.

Therefore, we evaluated multivariate ROC curves derived from a combination of (i) 2, 3, 4, or 5 miRNAs, (ii) 2, 3, 4 bacteria and (iii) all miRNAs and bacteria.

Interestingly, the combination of 9 features (i.e., miRNAs and bacteria together) notably increased the predictive performance (AUC = 0.84) (Figure 3.10), compared to the single miRNA and taxa univariate models (AUC range = 0.64 to 0.76) (Figure 3.7), as well as slightly improved the test performance compared to the 5 miRNA features-model (Figure 3.8) and the 4 taxa features-model (Figure 3.9) (AUC = 0.83 and 0.82, respectively).

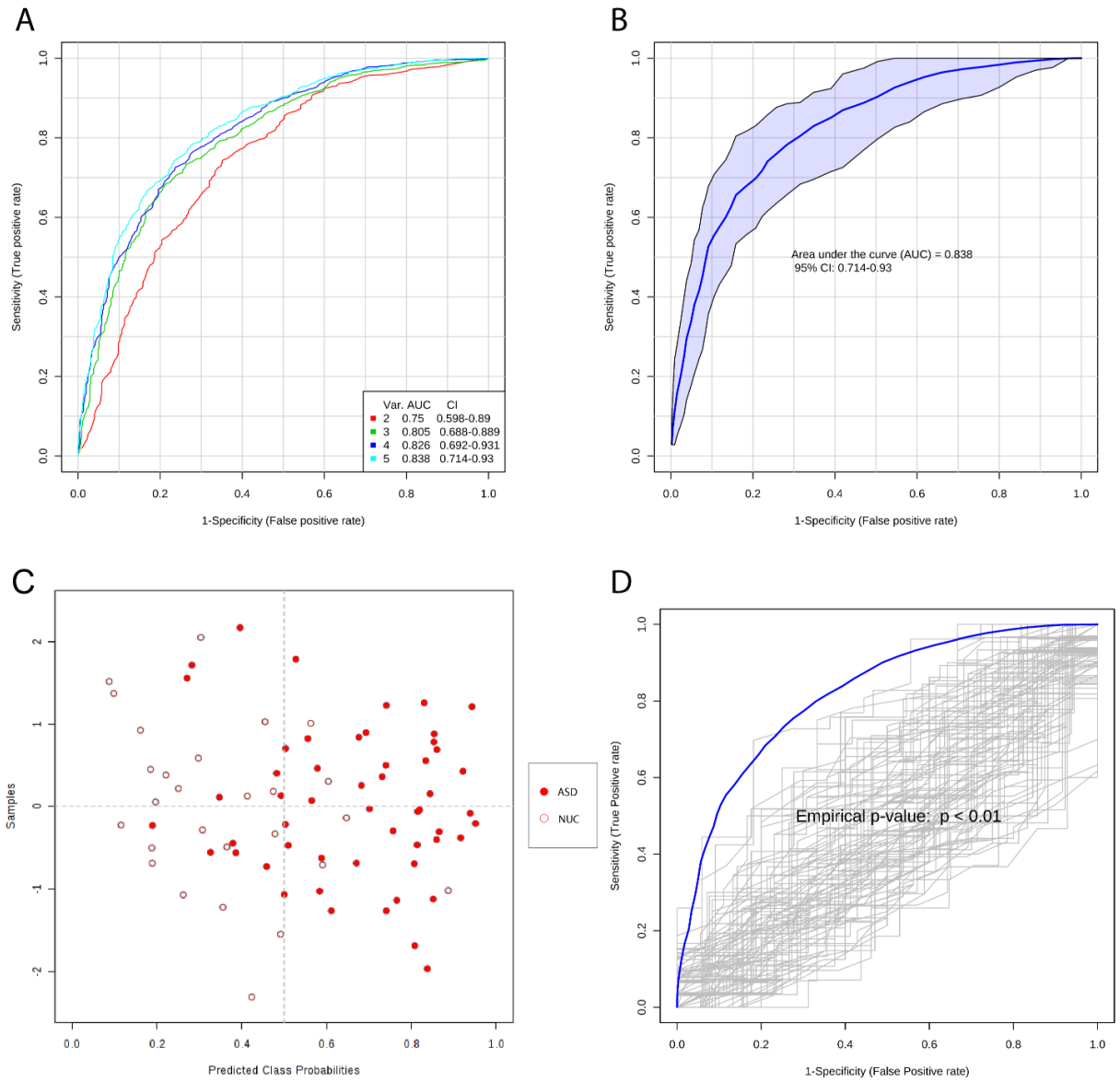


Figure 3.8. Multivariate ROC curve analysis for miRNAs. (A) Four models of multivariate ROC curves, built on a combination of 2, 3, 4 or 5 miRNAs. (B) Five-miRNA model of ROC curve with confidence intervals highlighted in blue. (C) Sample classification plot generated from the 5-miRNA ROC curve model: the average of predicted class probabilities (x-axis) of each sample (y-axis), from the 100 cross validation iterations, is

shown as dots. Since the algorithm uses a balanced sub-sampling approach, the classification boundary is located at the centre of the plot. Probability scores more than 0.5 belong to the ASD group, those less than 0.5 belong to the negative control group. This model accurately assigned 78% of the samples to the correct group. (D) Results from the permutation tests on the performance of 5 features-model.

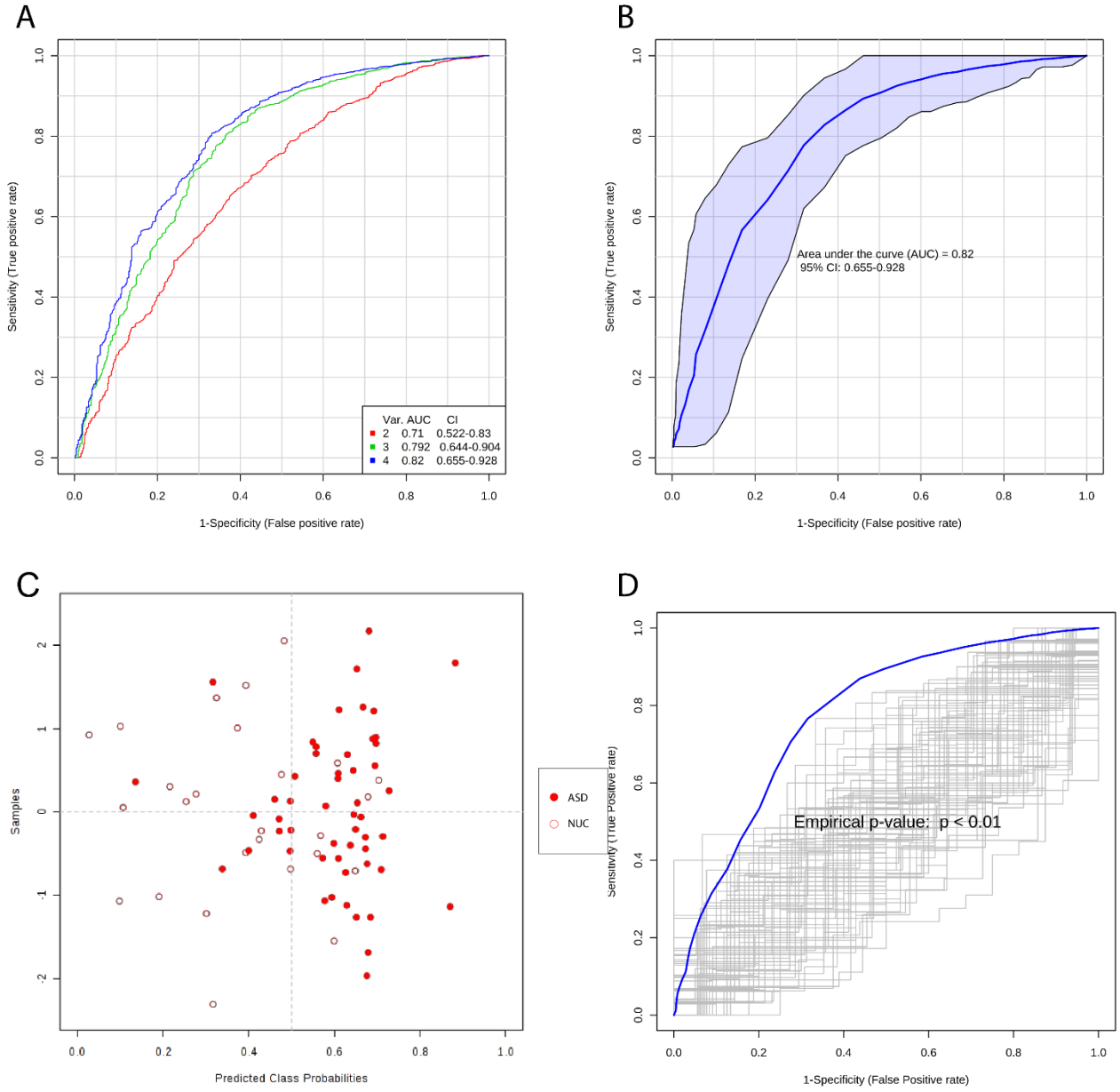


Figure 3.9. Multivariate ROC curve analysis for bacterial species. (A) Three models of multivariate ROC curves, built on a combination of 2, 3, 4 bacterial species. (B) Four-taxa model ROC curve with confidence intervals highlighted in blue. (C) Sample classification plot generated from the 4-taxon ROC curve model: the average of predicted class probabilities (x-axis) of each sample (y-axis), from the 100 cross validation iterations, is shown as dots. Since the algorithm uses a balanced sub-sampling approach, the classification boundary is located at the centre of the plot. Probability scores more than 0.5 belong to the ASD group, those less than 0.5 belong to the negative control group. This model accurately assigned 76% of the samples to the correct group. (D) Results from the permutation tests on the performance of 4 features-model.

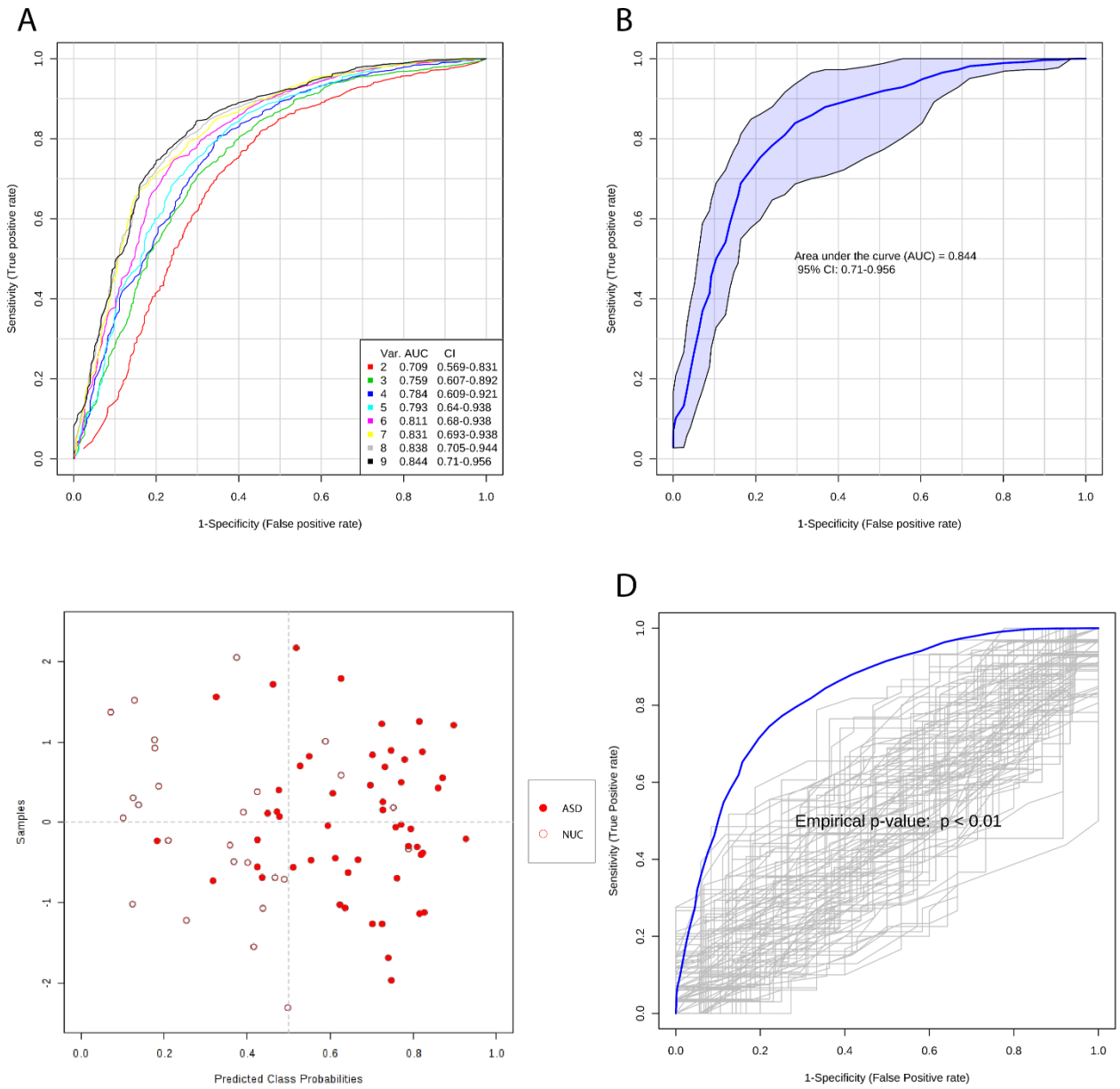


Figure 3.10. Multivariate ROC Curve analysis based on the combination of 5 salivary miRNAs and 4 taxa. (A) Nine models of multivariate ROC curves, built on a combination of 2, 3, 4, 5, 6, 7, 8, 9 salivary miRNAs and species-level bacterial taxa. (B) Nine-miRNA and taxa model ROC curve with confidence intervals highlighted in blue. (C) Sample classification plot generated from the 9-combination ROC curve model: the average of predicted class probabilities (x-axis) of each sample (y-axis), from the 100 cross validation iterations, is shown as dots. Since the algorithm uses a balanced sub-sampling approach, the classification boundary is located at the centre of the plot. Probability scores more than 0.5 belong to the ASD group, those less than 0.5 belong to the negative control group. This model accurately assigned 82% of the samples to the correct group. (D) Results from the permutation tests on the performance of 9 features-model.

In conclusion, results showed that the combination of 5 salivary miRNAs and the 4 bacterial species in a single multivariate model significantly represented the best-fit performing model for ASD diagnosis.

3.2.7. Functional Enrichment Analyses

Pathway enrichment analyses were computed to investigate the potential biological impact associated with the differential expression of the miRNAs reported in this study. We computationally identified a list of statistically over-represented gene ontologies and biological pathways (Figure 3.11) potentially related to ASD, as Pre-NOTCH transcription and Translation (False Discovery Rate (FDR) corrected p-value = 0.0015) and Pre-NOTCH Expression and Processing signaling pathway (FDR corrected p-value = 0.00471), signaling by NOTCH (FDR corrected p-value = 0.0221), signaling by NGF (FDR corrected p-value = 0.0291) through which cell fate decisions in neuronal development are regulated. Moreover, we found Parkinson's disease related pathways (FDR corrected p-value = 0.0002), involved in synaptic and mitochondrial dysfunction and neuroinflammation; Hippo signaling pathway (FDR corrected p-value = 3.991×10^{-6}), FoxO (Forkhead box O) signaling pathway (FDR corrected p-value = 0.0001), PI3K-Akt signaling pathway (FDR corrected p-value = 0.0008), and mTOR signaling pathway (FDR corrected p-value = 0.02), involved in many biological events such as apoptosis, cellular stress and cell-cycle control. In addition, bacterial invasion of epithelial cell (FDR corrected p-value = 1.19×10^{-6}) related pathways were also found. This data would represent an interesting link between the differential expression of miRNAs and the bacterial abundance alterations found in saliva.

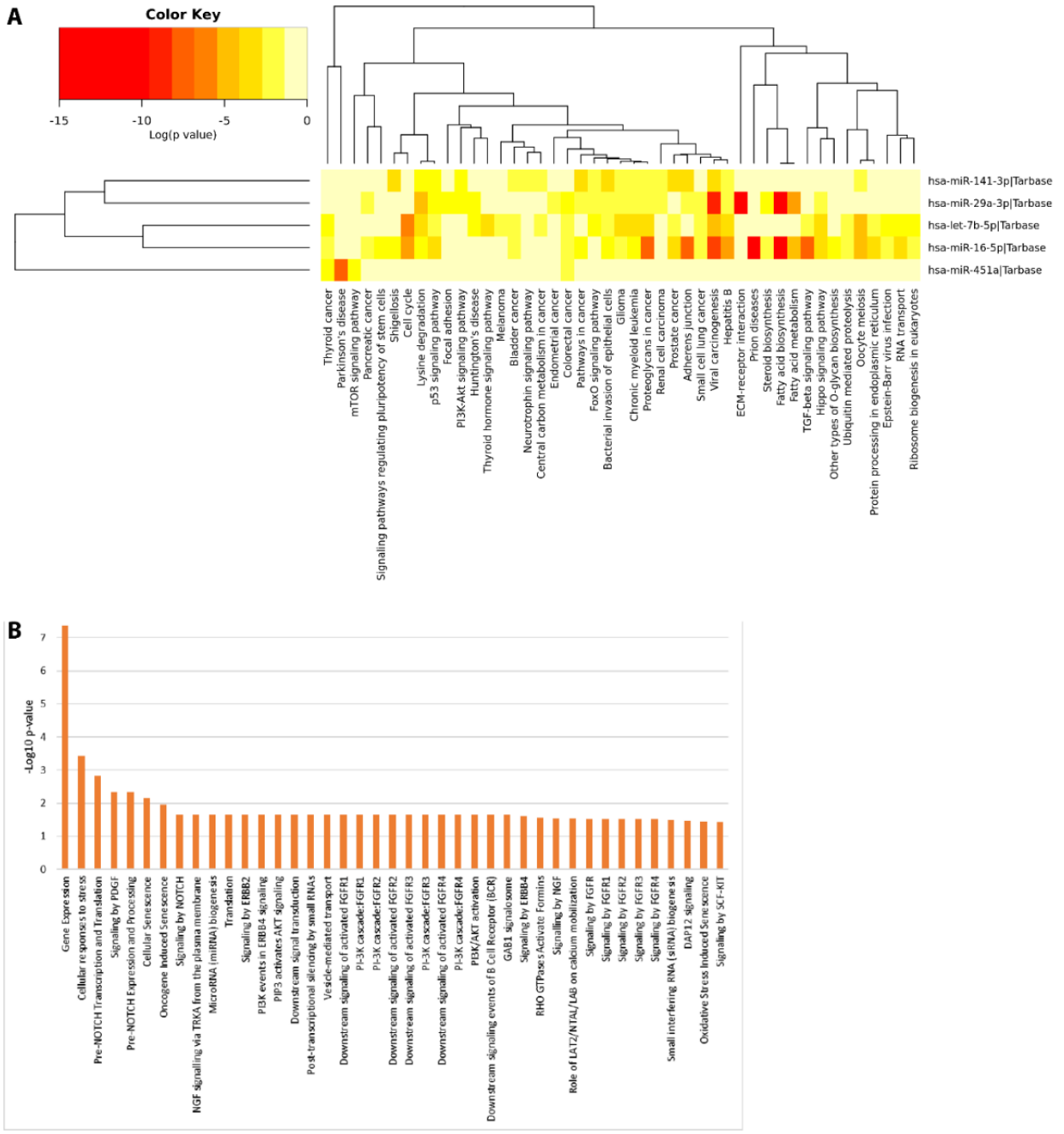


Figure 3.11. Functional Enrichment analysis of DE miRNAs. Functional enrichment analysis of miRNA targets using (A) KEGG pathway (Kyoto Encyclopedia of Genes and Genomes) (hierarchical clustering based on a complete linkage method and the significance levels of the interactions) and (B) Reactome database by DIANA-mirPath v.3 web server and miRNet tool, respectively.

These computational data would suggest a functional involvement of DE salivary miRNAs in molecular signaling pathways related to the development of cognitive functions, often reported to be dysfunctional in ASD. This observation is particularly intriguing because it hints at the possibility that some molecular alterations of ASD neuronal circuits could be mirrored in saliva by means of differential secretion of miRNAs.

3.3. Discussion

3.3.1. Circulating miRNAs and Microbiome Structure are Altered in Saliva of Pediatric ASD Patients

Inside the oral cavity, miRNAs could represent the RNA-based signals that cells exchange with each other by micro- and nano-vesicles. Salivary DNAs can be exploited to analyze the microbes in the mouth. These two types of information may be used to understand whether and how salivary miRNAs and resident bacterial species change in specific diseases and whether a link between these dysregulations could exist.

In fact, perturbations in the normal structure of microbiome have been closely associated to human health and disease [37, 124, 282] and there is mounting evidence that alterations of the GI microbiome may influence neurological disorders and autistic behavior [119, 283, 284]. The shift of microbial communities in the oral cavity in these diseases is being increasingly studied to look at possible early diagnostic markers [127, 128, 285]. Many studies already revealed that miRNAs play very important roles in neural developmental processes [214, 230, 286-288], resulting in neuropsychiatric disorders [261, 271] and neurodegenerative diseases [254, 289], although their exact pathophysiologic role remains unclear.

Based on these premises, the identification of specific patterns of salivary bacterial species and miRNA levels could provide a valuable and innovative approach to further explore the molecular basis of ASD by utilizing an easy liquid biopsy-based method.

In the present study, circulating miRNAs and the oral microbiome were detected in saliva of a group of ASD pediatric patients, compared with neurotypical subjects.

Through 16S rRNA microbiome analysis, at phyla level, we identified a higher relative abundance of *Proteobacteria* and *Actinobacteria* and a lower amount of *Bacteroidetes*, *Firmicutes,* and *Fusobacteria* phylotypes in ASD patients compared to neurologically unaffected controls. At species level, an increased abundance of *Rothia mucilaginosa, Filifactor, Actinobacillus parahaemolyticus*,

Weeksellaceae, Ralstonia, Pasteurellaceae, *Agregatibacter segnis*, and *Haemophilus parainfluenzae,* as well as a reduced amount of *Tannerella, Moryella* and *TM7-3* species were observed.

These findings could contribute to define the dysbiotic signatures of ASD status. Interestingly, a similar trend for *Haemophilus* and *Rothi*a is supported by previous observations [131, 132], as well as, their involvement in several diseases, especially in immunocompromised hosts [290, 291].

Many taxa identified in this study were previously associated with CNS (Central Nervous System) disorders, such as Parkinson's and Alzheimer's disease [292, 293]. It is noteworthy that *Filifactor* and *Tannerella* were previously reported as strong indicators of periodontal diseases [294, 295], often associated to autism.

On the other hand, by NanoString high-throughput analysis and validation experiments by qPCR assays, five circulating miRNAs were identified as dysregulated in ASD saliva: miR-29a-3p, miR-141-3p, miR-16-5p, let-7b-5p and miR-451a.

Interestingly, they were previously associated and proposed as candidate biomarkers for several neurological disorders, including ASD, in both cellular and extra-cellular contexts.

In Alzheimer patients, miR-29a was upregulated in cerebrospinal fluid (CSF) [296], while miR-451a and miR-16-5p were downregulated in exosomes from CSF of young-onset (YOAD) subjects [297]. The expression pattern of miR-29a-3p in whole blood [298] and miR-141-3p in serum of Parkinson patients [299] have been reported as downregulated. Levels of let-7b-5p were decreased in the saliva of children with mild traumatic brain injury [300]. Intriguingly, miR-451a and miR-16-5p have already been associated with ASD: a downregulation of miR-451a and miR-16-5p was identified in peripheral blood of ASD children [266]; miR-451a and miR-16-5p were upregulated and downregulated, respectively, in lymphoblastoid cell lines from autistic monozygotic twins [236]; miR-451 showed an increased abundance in post-mortem ASD brain [238].

These findings suggest that salivary miRNAs and microbial taxa detected in this study have a critical role in the development of cognitive functions and in neuronal physiology, which are both strongly affected in autism. However, the multiple and variable associations found among miRNAs and

microbial species identified in our study and the different pathological conditions might also indicate a limitation in the diagnostic specificity for ASD: further analyses in larger independent cohorts should validate their diagnostic potentiality and address this critical issue.

To date, this is the fourth high-throughput study that profiles circulating miRNAs and the fourth one which evaluates oral microbiome from saliva of ASD patients compared to neurotypical subjects. Other research groups independently reported salivary miRnome [267-270] and oral microbiome dysregulation [131-133] in patients affected by ASD, but there is only a partial concordance with the data presented here. However, it is worth noting that the salivary microbiome structure in human beings is strongly affected by the geographical, climatic and ethnic origin [301-303]. This could be due to the different ratio of dietary protein/carbohydrate intake that, in turn, modulates salivary pH [304]. The biochemical variability, such as the inconstancy of desquamated oral epithelial cells in saliva among people, would lead to a heterogeneous population of miRNAs in saliva among individuals [305].

Functional enrichment analysis highlighted and supported the potential involvement of DE miRNAs in ASD related mechanisms, allowing to further explore the molecular basis of the disorder. In fact, we detected several molecular signaling pathways, many of which have already been associated with ASD pathomechanisms.

For instance, PI3K signaling pathway in neural tissue impacts on neuronal morphology, synaptic transmission and, more rarely, learning and behavioral functions. Also, somatic mutations in p110α, the PI3K catalytic subunit-α, lead to a brain overgrowth, which is one of the hallmarks of ASD [306]. Akt/mammalian target of rapamycin (mTOR) signaling pathway is involved in the memory formation by the process of long-term potentiation (LTP) of synapses and immune function. Higher activity of the extracellular receptor kinase mTOR has been detected in cells from ASD patients [307]. IGF-I/PI3K/AKT/mTOR signaling affects myelination, synaptic plasticity, mechanisms of social interactions, learning and immune functions [74, 88, 307]. The Hippo signaling pathway plays an important role in neural development and neuronal maintenance [308]. Finally, NOTCH, FoxO, and

NGF pathways are crucially involved in the CNS regulating neurogenesis, synaptic plasticity, learning, memory and behavior [309-311]. Overall, the study on the potential intracellular context of DE circulating miRNAs depicts an interesting framework in which miRNAs are functionally and crucially involved in molecular signaling pathways reported as dysregulated in ASD. This observation suggests that salivary miRNAs may reflect the intracellular alterations related to ASD.

3.3.2. Potential applications in ASD diagnosis

In clinical practice, the Diagnostic and Statistical Manual of Mental Disorders-5th edition (DSM-5) criteria represent the current "gold standard" for ASD diagnosis and screening methods, which are behavior-based and rely on a subjective evaluation [312]. However, delineating psychiatric disorders is still challenging because of the complexity and heterogeneity of phenotypes; therefore, an unbiased molecular approach is still needed.

Through ROC curve analyses and performance evaluation of predictive models, we explored the diagnostic accuracy of 5 salivary miRNAs and 11 taxa for the discrimination between ASD and NUC individuals. While each single miRNA and bacterial taxa showed a moderate discriminatory power, the combination of 5 miRNAs (i.e., miR-29a-3p, miR-141-3p, miR-16-5p, let-7b-5p and miR-451a) and 4 bacterial species (i.e., *Filifactor*, *Moryella*, *Tannerella*, *TM7-3*) considerably optimized the predictive performance (see Table 3.3). Therefore, we suggest that the aforementioned miRNAs and taxa could be used as non-invasive biomarkers and an objective support for ASD diagnosis. However, further studies in larger independent cohorts are considerably needed to evaluate and improve the potentiality of this test and its clinical usefulness.

3.3.3. Potential Associations Among Cognitive Impairments, Salivary miRNA Expression and Microbiome Alteration in ASD Children

In this study, all participants were clinically assessed through different instruments, such as ADOS and ADI-R, which evaluated ASD symptoms and severity. Interestingly, through correlation analyses,

anomalies of social interaction and communication have been detected associated with salivary miRNA expression, expecially for let-7b, miR-451a, and miR-29a-3p (Figure 3.5A).

Impairments in social-emotional reciprocity and relationships, verbal and nonverbal communicative behavior, cognitive skills, lead to increase the risk of social isolation and rejection in daily social contexts of ASD children. Some studies already reported a significant association between social isolation, consequent psychological stress and dysregulation of most of miRNAs identified in this study. For instance, miR-141-3p expression progressively increases in a time-dependent manner in mouse models of post-stroke social isolation [313] and members of the miR-29 family increased their expression during the healing process of oral palatal mucosal wounds [314]. Plasmatic let-7b-5p strongly correlated with cognitive impairments in the presence of severe alcohol disorder [315] and it was also related to high stress conditions [316]. A depression-like phenotype is often observed in autistic patients and, interestingly, levels of circulating miR-451a of depressed patients were found reduced and negatively associated with the Hamilton Depression Scale, that is used to rate the severity of depression [317, 318].

Furthermore, in ASD patient's blood, the elevated levels of lactate and its synthesizing enzyme, lactate dehydrogenase (LDH), reflect the mitochondrial energy metabolism dysfunctions [319]. Interestingly, miR-141-3p was found to induce mitochondrial dysfunctions in obese mice by inhibiting PTEN [320]. Furthermore, circulating miR-141-3p positively correlated with LDH levels in rectal cancer [321], as well as, miR-141 positively regulated expression of LDH by inhibiting MAP4K4 in breast cancer [322]. These observations corroborate the positive correlation we found between miR-141 and lactate and suggest that it could represent a combined extracellular phenotype of ASD metabolic abnormalities.

Looking at the correlation analyses involving salivary bacteria, we identified several significant associations with typical clinical signatures of ASD patients.

For example, *Moryella* showed a signature that negatively correlated with anomalies in neurodevelopment arising before the age of 36 months (ADI-D); also, *Moryella* was linked to the

Verbal Intelligence Quotient (VIQ). *Ralstonia* positively correlated with a worst qualitative anomaly in social interaction (ADI-A). Finally, the low abundance of *TM7-3* negatively correlated with the increasing of both qualitative anomalies in communication (ADI-B) and repetitive and restricted behavior (ADI-C) (Figure 3.5B).

Although bivariate correlations provided interesting information on the general association between each pair of variables, they did not reveal more about the nature of these relationships. Therefore, we applied a Negative Binomial regression to explore the potential effects of the clinical parameters and miRNA expression on the microbial abundances.

Negative binomial regression analyses revealed that seven bacterial species (i.e., *Tannerella*, *Weeksellaceae*, *Moryella*, *Filifactor*, *Ralstonia*, *Pasteurellaceae*, *Actinobacillus*) were significantly predicted by individual variations in clinical parameters, especially for *Tannerella* (Figure 3.6). In particular, the rate of occurrence of *Tannerella* resulted to be associated with VIQ, PIQ and TIQ, together with behavior (ADI-C), speech and communication anomalies (ADOS-A). Interestingly, the pathogen *Tannerella* has been considered an indicator of periodontal disease, which, in turn, has been often related to ASD [323]. Therefore, it is not surprising that *Tannerella* has been also associated with cognitive decline [324], disability, speech and communication impairment, low self-esteem and quality of life [325].

Furthermore, the regression analyses uncovered the new significant relationship of the Intelligence Quotients (i.e., VIQ, PIQ, and TIQ) as predictors for *Weeksellaceae* and *Ralstonia* abundances, which were previously undetectable by using linear correlation tests.

Overall, these results suggest that the specific miRNA expression and microbiome structure alterations could support the diagnostic evaluation of ASD and they could be considered as a consequence of ASD symptomatology.

3.3.4. Potential Crosstalk between miRNAs and the Microbiome in Saliva

It is known that microbiota can secrete bioactive compounds able to modify the host epigenome; as well as, miRNAs from the host could selectively regulate the functions of microbiota.

Despite the cross-talk between host miRNAs and the gut microbiota has been largely explored [277, 326-328], to date, only two previous studies examined the potential association between oral bacteria and microRNAs, one of which performed human and microbial salivary poly-omic RNA measurement in ASD framework. The first study [279] explored the daily oscillations of salivary miRNA and microbiome in human health, to investigate their potential interactions and the implications. They observed the association among five circadian miRNAs and four circadian microbial RNAs. The second study [268], evaluated the salivary poly-omic RNA measurements in 19–83 months ASD and non-ASD children, aimed to propose a non-invasive approach for the discrimination of ASD. However, correlation analysis identified only moderate associations which did not include microRNAs. The geographical, climatic and ethnic origin of the study participants, the different ratio of dietary protein/carbohydrate intake, the biochemical variability in saliva among people, as well as the different experimental technique used, would lead to a lack of concordance with our results.

Among them, in our study a negative relationship was identified between salivary miR-141-3p and *Tannerella* through linear correlation analysis (Figure 3.5C). Interestingly, this inverse correlation has already been reported from independent researchers. It has been demonstrated that miR-141-3p was downregulated in the gingiva of patients affected by periodontal disease compared to healthy gingival tissue [329], while, *Tannerella* is one of the major Gram-negative periodontal pathogens and it is already identified as a marker of autism [131, 323].

The negative binomial regression sheds a light to other considerations regarding the potential interaction between: (i) miRNAs and neuropsychological parameters (as predictors) and (ii) microbiome (as outcome variable) in terms of expected change in the outcome variable for a one-unit change in the predictor variable. For example, NB regression revealed new potential interactions

between the miRNAs previously associated with cognitive impairments (based on linear correlation analyses) and specific bacteria: let-7b-5p and miR-16-5p with *Tannerella*; miR-451a and *Weeksellaceae;* miR-141-3p and *Tannerella*, *Weeksellaceae*, *Moryella, Filifactor* (Figure 3.6).

About the potential crosstalk between miRNAs and microbiome, recent reports demonstrated that fecal miRNAs could contribute to shaping the composition of the gut microbiome [276, 277], suggesting a mechanism by which host cells can regulate the microbial community. Interestingly, one of the miRNAs found DE in our study, miR-141-3p, is known to be expressed in intestinal epithelial cells and a potential biomarker for gut dysbiosis [330].

Overall, the significant relationships found in the regression analysis may suggest that the potential crosstalk between oral bacteria and salivary miRNAs could be based on a general response of host to dysbiosis involving several miRNA families, as well as bacterial phyla [331-338].

3.4. Concluding remarks and future perspectives

Inside the salivary environment of autistic children, quantitative differences in miRNA expression and bacteria abundance are present and are potentially associated with anomalies in social interaction and communication. This finding could help to discriminate patients from neurologically unaffected children, and accordingly, miRNAs and bacteria could be exploited as potential salivary markers of ASD. MiRNA dysregulation could be the result of host response to dysbiosis in the oral cavity that, in turn, may be the consequence of the particular dietary behavior of ASD children. To date, the potential reciprocal effects of these miRNAs on the structure of the bacterial population remain unknown.

The limitations of our study could encourage future perspectives. Because of the limited size of the group of patients, further studies in larger independent cohorts with a lower mean age (e.g., 1–2 years) will be needed to validate the diagnostic potentiality and specificity of circulating miRNAs and oral microbiota as non-invasive and unbiased molecular tools for ASD diagnosis. Enlarging the cohort of study would produce a larger amount of data from miRnome and microbiome, which will be more effective to perform multiparametric regression tests in order to infer the potential cross-talk between

salivary bacteria and miRNAs. Secondarily, it would be possible to investigate their hypothetical synergic non-linear contribution to the cognitive impairments in ASD patients.

Moreover, it is important to consider that the results of this study may have been influenced by geographical and ethnic origin of the enrolled patients as a consequence of its epigenetic nature: this may explain the reproducibility issues reported.

Molecular and cellular characterization of *in vitro* and *in vivo* cross-talking between host and microbiota will contribute to further clarify the heterogeneous molecular basis of ASD.

3.5. Materials and Methods

3.5.1. Ethics Approval and Consent to Participate

All experiments were approved by the local Ethics Committee, Comitato Etico Catania 1, University of Catania (ID: 002430-36) prior to sample collection. Written, informed consent was obtained from all parents and each participant who was able to give the informed assent.

All experimental methods were in accordance with Helsinki Declaration.

3.5.2. Participant Selection

From a database of more than 2000 patients, 76 treatment-naïve patients affected by ASD were recruited and studied from January to October 2018 in the outpatient service of the Child and Adolescent Psychiatry Unit (Department of Clinical and Experimental Medicine, University Hospital of Catania).

The inclusion criteria were clinical diagnosis of ASD, according to the criteria of DSM-5 and the absence of other medical, neurological, genetic or metabolic conditions such as epilepsy, cytogenetically visible chromosomal abnormalities, copy number variants (CNVs) or single-gene disorders. They were compared to 39 NUCs, without any history of ASD and who suffered from neither chronic neurological, metabolic or genetic diseases nor psychiatric disorders. All study

participants were Caucasians from Sicily, randomly recruited from various socio-economic contexts. The study was controlled for many confounding factors as soon as possible in both phases of participant recruitment and sample collection, and, additionally, we evaluated more deeply the potential influence of gender and age by quantifying group differences between ASD and NUC subjects.

The entire cohort was split into 2 sets of samples: (a) discovery set, composed by 23 ASD and 12 NUC; (b) validation set, made by 53 ASD and 27 NUC. The discovery set was used to perform expression profiling by NanoString technology, while the validation set was analyzed by real-time PCR single assays and 16S rRNA microbiome screening.

3.5.3. Assessment

All participants were assessed by a child and adolescent neuropsychiatrist expert in ASD (RR) with the following instruments: WISC-III (Wechsler Intelligence Scale for Children, III edition) [339] or WPSSI (Wechsler Preschool and Primary Scale of Intelligence) [340] as an evaluation of both IQ (Intelligence Quotient) and cognitive functioning, ADOS (Autism Diagnostic Observation Schedule) and ADI-R (Autism Diagnostic Interview–Revised) to evaluate ASD symptoms.

Based on these scales and schedules, the assessment procedure was carefully conducted assigning specific scores which provided a measure of autism severity. Neuropsychological features of participants are summarized in Table 3.1.

3.5.4. Sample Collection

All participants were instructed to refrain from eating or drinking for at least 3 hours prior to saliva collection. Saliva samples were collected in a good status of oral hygiene (i.e., brushing teeth once/twice daily), without any tooth decay. Saliva collection was always performed between 8:30 and 10:30 a.m. to avoid any potential microbiome and miRnome oscillation due to the circadian rhythms. From a minimum of 800 μL to a maximum of 4 mL, non-stimulated and naturally outflowed saliva

was collected into 50 mL conical centrifuge tubes. Saliva samples were centrifuged at 10,000 rpm for 15 min at 4°C to separate the pellet and supernatant for microbiological analysis and miRNA expression assays, respectively. The pellets were immediately processed, while supernatants were aliquoted into 2 mL RNase-free tubes and stored at −80°C until analysis.

To test the hematological parameters of each study participant, all participants were instructed to refrain from eating or drinking for at least 3 hours prior to blood collection, which was performed between 8:30 and 10:30 a.m. Peripheral blood samples were collected through a butterfly device into a 5 mL collection tube. Collection tubes were treated according to current and standard procedures for clinical samples.

3.5.5. RNA Extraction

Extraction of total RNA was carried out from 800 µL of saliva samples using Qiagen miRNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany), according to Qiagen Supplementary Protocol for purification of RNA (including small RNAs) from serum or plasma [341, 342]. RNA was eluted in 200 µL RNAse-free water and then precipitated by adding 20 µg glycogen, 0.1 volumes 3 M sodium acetate and 2.5 volumes ice-cold 100% ethanol. After incubation at −80°C overnight, RNA was centrifuged and washed twice in ice-cold 75% ethanol and resuspended in 7 µL RNAse-free water. The yield and quality of the RNA samples were assessed by using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

3.5.6. MiRNA Profiling by NanoString Technology

To profile the expression of circulating miRNAs from saliva, NanoString nCounter system assays were performed using the NanoString platform and nCounter Human v3 miRNA Expression Assay Kits (NanoString Technologies, Seattle, WA, USA), according to the manufacturer's instructions. MiRNA profiling was performed on 23 ASD patients and 12 NUCs, starting from 3 µL of isolated RNA (approximately 150 ng). Samples were processed using the automated nCounter Prep Station;

after the hybridization step, they were purified and immobilized on a sample cartridge for quantification and data collection by using the nCounter Digital Analyzer. The nSolver 3.0 software was used for data analysis. The endogenous control was selected from the arrays in a similar way to the GMN (global median normalization) method [261, 343]. By this approach, we identified miR-21-5p as the best endogenous control for our experimental model.

3.5.7. MiRNA Data Validation by Single TaqMan Assays

MiRNAs found differentially expressed by NanoString analysis were assessed in a larger independent cohort of 53 ASD patients and 27 NUCs. Validation analysis was performed on 20 ng of salivary RNA by using single TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA, Life Technologies) in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies), according to the manufacturer's instructions. Data analysis was computed on SDS v2.4 and RQ Manager 1.2.1 software (Applied Biosystems, Life Technologies). Expression fold change values are shown as geometric means of relative quantification (RQ) values obtained by applying the $2^{-\Delta\Delta Ct}$ method [344].

3.5.8. DNA Extraction, 16S rRNA Gene Library Preparation, and Sequencing

DNA from salivary samples was extracted with the PureLink Genomic DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Extracted DNA was checked for quality and quantity by NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, USA). All genomic DNA was frozen at −80°C until sequencing. Extracted DNA (approximately 10 ng) was prepared for 16S amplicon sequencing by the MiSeq platform using the Illumina protocol (Part # 15044223, Rev. B) with modifications to ensure sufficient amplification of low amounts of DNA. The V3-V4 region of the 16S ribosomal RNA gene was amplified using the primers forward (5′-CCTACGGGNGGCWGCAG-3′) and reverse (5′-GACTACHVGGGTATCTAATCC-3′) [345]. All PCR products were purified by Agencourt AMPure XP magnetic beads (Beckman Coulter). The

samples and mock community aliquots were then barcoded by Illumina's dual indexing strategy (Nextera XT Index Kit v2, Sets A and B, Illumina) by using the default barcode layout from the Illumina Experiment Manager software v1.13.1, as described in the Illumina protocol. The quality of PCR products was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The amplicon libraries underwent further purification and quality checking, followed by dilution and equimolar pooling. Finally, 12 pM of the library mixtures, spiked with 20% PhiX control, were paired-end (2 × 300) sequenced using the MiSeq platform (Illumina, San Diego, CA, USA) at the Centro Servizi - B.R.I.T. (University of Catania).

3.5.9. Processing and Analyses of Sequencing Data

QIIME pipeline (Quantitative Insights into Microbial Ecology) v.1.9.1 was used to process the generated raw FASTQ files [346]. V3-V4 16S rRNA FASTQ were de-multiplexed using the barcodes. The paired-end sequences were assembled to form a single read using FLASH [347] and quality-filtered ≥80% bases in a read above Q30 (see Supplementary Table S3.4). Merged reads were length-filtered based on 445 bp (the expected length). The ends of retained (not-merged) forward reads were clipped to a total read length of 270 bp to remove low quality bases. The high-quality reads were clustered against a reference sequence collection with QIIME. To focus only on the prominent taxa, a filtering step of 0.01% at the Operational taxonomic Unit (OTU) level was performed by running a workflow on QIIME (filter_otus_from_otu_table.py). The taxonomy of each 16S rRNA gene sequence was collapsed to OTUs using the open reference-based OTU picking method against Greengenes database at 97% of sequence similarity [348]. Chimeras were identified and removed by Chimera Slayer and the UCHIME algorithm. Any reads that did not match the reference sequence collection were subsequently clustered de novo. To avoid sample size biases in downstream analyses, rarefaction curves were generated with QIIME (alpha_rarefaction.py workflow) and calculated by applying Explicet and a maximum depth of 74.469 sequences/sample [349]. The OTU tables were used for assessing α-diversity indices (Chao-1, Shannon and Shannon evenness) calculated from the

taxonomic profiles and compared across the ASD and NUC groups by QIIME algorithms. Independent Student's t-test and Mann–Whitney U test were used to evaluate α-diversity among the taxonomic profiles and compared across the ASD and NUC groups. β-diversity between ASD and NUC groups was analyzed by weighted and unweighted UniFrac distance matrices (beta_diversity.py workflow) and visualized through tridimensional PCoA plot by using EMPeror (http://boocore.github.io/emperor/). The differentially abundant OTUs across two sample categories were identified by QIIME scripts (differential_abundance.py). The core microbiome was determined by QIIME algorithms (compute_core_microbiome.py) and the diversity analysis was performed with the script core_diversity_analyses.py.

DNA sequences were deposited in the Sequence Read Archive under BioProjects PRJNA518756 and PRJNA518760.

3.5.10. Correlation and Negative Binomial Regression Analyses

To investigate whether a linear relationship exists between the differential expression of salivary miRNAs, microbiome taxa and the neuropsychological and hematological parameters for each selected participant, we performed linear correlation analyses.

Additionally, we applied a Negative Binomial (NB) regression to explore the potential effects of the clinical parameters and the miRNA expression (potential predictors) on the relative microbial abundances (outcome variables).

3.5.11. ROC Curve Analysis and performance evaluation

Classical univariate ROC (Receiver Operator Characteristic) curve analyses were performed to calculate the performance prediction of each salivary DE miRNA and bacterial species as ASD biomarkers.

The univariate ROC curve provides a predictive performance of the model, based on a binary outcome and continuous predictor. Since this analysis is idealized and potentially preliminary, the performance

power was independently evaluated in a correspondent model testing. Therefore, using the server MetaboAnalyst 4.0 [350], logistic regression models were carried out and tested by Monte-Carlo cross validation (MCCV) and permutation testing. Cross validation was used for evaluating the potential of a specific model to predict the correct assignment of new samples. Successively, permutation test on the performance measure AUC was used to measure the statistical significance of the model. Furthermore, multivariate ROC curve analyses were computed to evaluate if the combination of (i) 2, 3, 4, 5 miRNAs; (ii) 2, 3, 4 bacteria and (iii) the overall model (4 miRNAs + 5 bacteria), could provide a significant and higher discriminating power. MCCV and permutation test were applied to evaluate the classification performance of multivariate models.

3.5.12. Computational Enrichment Analysis

To investigate the functional involvement of the identified DE miRNAs, DIANA-mirPath v.3 web server [351] and miRNet tool [352] were used for pathway enrichment analysis from KEGG (Kyoto Encyclopedia of Genes and Genomes) and Reactome gene annotation databases.

3.5.13. Statistical Approach

Although we attempted to making the dataset uniform as soon as possible, based on important demographic factors (e.g., gender and age), the ASD group had a larger proportion of boys (76 of 115; 66%) than girls (39 of 115; 34%). For evaluating the statistical significance and quantifying group differences in gender and age between ASD and NUC subjects, we applied the non-parametric two-tailed Mann–Whitney U test, since data were not normally distributed according to the Shapiro–Wilk test. We also explored the necessary conditions for variables in order to be considered as potential confounders through (i) a binomial logistic regression to assess the potential effects of gender and age (continuous independent variables) on the likelihood that participants are affected by ASD (dichotomous dependent variable) and (ii) Spearman correlation test to assess any linear association between the salivary miRNA expression, microbiome abundance and demographic characteristics.

Same statistical tests were applied to evaluate food intake, in terms of timing since last meal, as well as the time of collection as potential confounding factors for our analysis on oral microbiota.

For miRNA profiling analysis, SAM (Significance of Microarrays Analysis) statistical tests were computed by using MeV (Multi experiment viewer v4.8.1) statistical analysis software. We computed a two-class unpaired test, based on 100 permutations. Fold change values were obtained by calculating the ratio between the normalized count mean of each group. MeV was also used to generate a heat-map of identified DE-miRNAs.

Concerning microbiome analyses, OTU frequencies across sample groups were performed by the Kruskal–Wallis test. Statistical analysis of taxonomic profiles was performed using STAMP (Software Testing AMPlification) [353-355] by the two-sided White's non-parametric t-test. Extended error bar plots were produced by STAMP (White's non-parametric t-test and p-value ≤ 0.05) showing the bacterial taxa with a significant difference (p-value ≤ 0.05). Mann–Whitney–Kruskal–Wallis, t-test/ANOVA were used for confirmation (Supplementary Table S3.1).

The correction for multiple tests of high throughput analyses was performed by applying Benjamini–Hochberg FDR (False Discovery Rate).

The Mann–Whitney U test was applied to evaluate the differential expression of the selected miRNAs between the two groups in the validation analysis (p-value ≤ 0.05). Cliff's delta statistics [356, 357] was used to estimate the non-parametric effect sizes and were calculated by using the Cliff's Delta Calculator by Macbeth et al. [358].

Since data were not distributed normally, correlation analyses were performed by applying the Spearman test with two-sided p-values corrected for multiple comparisons by using the Bonferroni-Šídák approach. Statistical analyses were computed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). To determine the strength of associations between variables, the correlation coefficients themselves were interpreted as index of effect size.

ROC curve analyses were performed through GraphPad Prism software. Using RQ values and microbiome OTUs as input, for miRNAs and bacteria respectively, the maximum Youden index J

(max [(sensitivity + specificity)−1]) was calculated, which identified a RQ/OTU value as a cut-off point maximizing both sensitivity and specificity. The validity of diagnostic tests towards the ability to discriminate between ASD and NUC conditions was evaluated by calculating the area under the curve (AUC), specificity, sensitivity, accuracy, positive predictive, negative predictive values, together with positive and negative likelihood ratio. By using MetaboAnalyst 4.0 server, we applied Cross-Validation. The full data set was randomly subdivided into a training set (2/3 of samples) used to perform the model, and a test set (1/3 of samples) used as a validation set, for assessing the model performance. 100-fold CVs were performed to gain a realistic estimate of the model performance and the AUC values are then averaged. Permutation test applied the 100 times repeated procedure provided for: (i) randomly re-assigning a clinical outcome to each sample; (ii) random sub-sampling CV; (iii) comparing the AUC performance measure of the models obtained [359]. Statistical significance was established at a p-value ≤ 0.05.

The classification performance of multivariate models was evaluated through MCCV, using the Random Forest statistical algorithm, and further validated using the permutation test.

NB regression analysis was performed by IBM SPSS Statistics software and according to IBM manual on generalized linear models (GLM). In particular, relationships were evaluated assuming a negative binomial distribution and log link function, since our variable response is represented by over-dispersed count data (that is, the variance of the count outcome is greater than the mean) which didn't have an excessive number of zeros [360].

As numerous studies reported [361-363], microbiome count data are often overdispersed and heavily right-skewed, since the distribution among samples is highly variable. Moreover, the negative binomial regression has already been applied to microbiome data [353, 364].

In our study, the first step was the assessment of the model fit evaluating the results of the Likelihood ratio (LR) chi-square test, which provides a test of the overall model by comparing the full model against a null (intercept-only) model. Only when statistically significant (p-value ≤ 0.05), we proceeded with the evaluation of the regression coefficients. The table on goodness of the model fit

provided us some information regarding the nature of the count data, by evaluating the ratio of the deviance or Pearson chi-square to the degree of freedom (df): for all species assessed, such Ratio was greater than 1, providing indication of the presence of overdispersion. Wald test was applied to estimate regression coefficients (B), and accordingly also the statistically significant effect of each predictor in the model. The effect sizes were provided by RcountD (https://stefany.shinyapps.io/RcountD).

Multiple and simple linear regression analyses were performed to validate the robustness of the results from NB analysis, although we believe that the NB model is the most appropriate for our study, for the reasons aforementioned (i.e., over-dispersion of microbiome counts, as well as, addressed issues on normality of residuals and the risk of multicollinearity among independent variables).

Performing a multiple linear regression, we analyzed the relationship between miRNA expression, neuropsychological and hematological parameters (independent variables) and relative abundances (proportions) of each microbial taxon (dependent variable). The first step was the assessment of the model fit evaluating the results of the coefficients of determination (R^2 values) and the statistical significance of overall regression model (F-ratio and corresponding p-values). Only when the regression models were statistically significant (p-value ≤ 0.05), we proceeded in the evaluation of the regression coefficients estimated. Although some multiple regression models did not reach the overall statistical significance (p-value for the F-test > 0.05), their regression coefficients were statistically significant (p-value for the t-test ≤ 0.05), confirming the previous results obtained by negative binomial regression. Therefore, we wondered whether the independent variables separately produced significant relationships with such dependent variables, and, accordingly, we evaluated the simple linear regression models. Multiple and simple linear regression analyses were performed by IBM SPSS Statistics software and according to IBM manual on GLM.

For functional enrichment analysis, Fisher's exact t-test (p-value ≤ 0.05) was applied.

3.6. Supplementary Tables

	White's non parametric t-test	**Mann-Whitney-Kruskal-Wallis test**	**T-test/Anova**
	P-values		
g_Filifactor	9.99×10^{-4}	0.029812	0.0016537
g_Rothia	0.011	0.030216	0.012415
g_Actinobacillus	0.016	0.41668	0.016316
f_weeksellaceae	0.016	0.038872	0.011177
g_Ralstonia	0.023	0.046734	0.078283
f_ Pasteurellaceae	0.029	0.021476	0.122916
g Aggregatibacter	0.035	0.16022	0.020473
g_Tannerella	0.044	0.038368	0.041844
c_TM7	0.045	0.027238	0.015501
g_Moryella	0.045	0.027239	0.033352

Table S3.1. Statistical analysis of the bacterial abundance at the genus level in ASD and NUC groups. Statistical results by applying a two-sided White's non-parametric t-test were confirmed by Mann-Whitney-Kruskal-Wallis and T-test/Anova; statistical significance was achieved at p-value ≤ 0.05.

Species	***Actinobacillus parahaemolyticus***				
Model results	**B**	**Lower 95% CL**	**Upper 95% CL**	**p-value**	**SMD effect size**
let-7b-5p	1.050	-0.673	2.774	0.232	1.387
miR-451a	-0.851	-1.866	0.165	0.101	-0.428
miR-29a-3p	-0.244	-0.824	0.335	0.409	-0.162
miR-16-5p	-0.16	-0.856	0.535	0.651	-0.110
miR-141-3p	0.059	0.028	0.09	**0.000**	0.045
VIQ	-0.02	-0.109	0.069	0.659	-0.015
PIQ	-0.09	-0.194	0.013	0.088	-0.064
TIQ	0.119	-0.063	0.301	0.200	0.094
ADI-A	0.278	-0.015	0.571	0.063	0.239
ADI-B	-0.049	-0.446	0.349	0.81	-0.036
ADI-C	-0.473	-0.706	-0.24	**0.000**	-0.281
ADI-D	0.498	0.12	0.877	**0.010**	0.482
ADOS-A	0.086	-0.297	0.469	0.66	0.067
ADOS-B	-0.023	-0.298	0.253	0.870	-0.017
ADOS-C	-0.602	-1.222	0.017	0.057	-0.338
ADOS-D	0.577	-0.093	1.247	0.091	0.583
Ammonium	-0.145	-0.216	-0.074	**0.000**	-0.101
Lactate	0.142	0.035	0.249	**0.009**	0.114
Ceruloplasmin	-0.121	-0.224	-0.019	**0.021**	-0.085
Prolactin	0.000	-0.005	0.005	0.904	0.000
TSH	0.262	-0.388	0.912	0.430	0.224

Species	***Filifactor spp***				
Model results	**B**	**Lower 95% CL**	**Upper 95% CL**	**p-value**	**SMD effect size**
let-7b-5p	-0.886	0.7233	-2.303	0.221	-0.429
miR-451a	-0.512	0.5175	-1.527	0.322	-0.293
miR-29a-3p	0.455	0.2417	-0.019	0.06	0.421
miR-16-5p	0.227	0.3344	-0.428	0.497	0.186
miR-141-3p	-0.061	0.0157	-0.092	**0.000**	-0.043
VIQ	-0.064	0.0472	-0.156	0.177	-0.045
PIQ	-0.098	0.0482	-0.193	**0.042**	-0.068
TIQ	0.123	0.082	-0.038	0.133	0.096
ADI-A	0.236	0.1223	-0.003	0.053	0.194
ADI-B	-0.072	0.2043	-0.473	0.723	-0.051
ADI-C	0.101	0.122	-0.139	0.410	0.078
ADI-D	-0.295	0.2066	-0.700	0.153	-0.187
ADOS-A	-0.605	0.2051	-1.007	**0.003**	-0.332
ADOS-B	-0.263	0.1434	-0.544	0.067	-0.169
ADOS-C	0.390	0.228	-0.057	0.087	0.349

ADOS-D	-0.215	0.219	-0.645	0.326	-0.141
Species	***Moryella spp***				
Model results	**B**	**Lower 95% CL**	**Upper 95% CL**	**p-value**	**SMD effect size**
let-7b-5p	-0.079	-0.875	0.717	0.846	-0.090
miR-451a	-0.450	-0.952	0.052	0.079	-0.439
miR-29a-3p	0.045	-0.22	0.311	0.739	0.056
miR-16-5p	0.064	-0.257	0.384	0.697	0.08
miR-141-3p	-0.051	-0.069	-0.033	**0.000**	-0.06
VIQ	-0.034	-0.079	0.011	0.141	-0.041
PIQ	-0.031	-0.077	0.015	0.185	-0.037
TIQ	0.055	-0.02	0.13	0.148	0.069
ADI-A	-0.128	-0.329	0.074	0.215	-0.146
ADI-B	-0.047	-0.293	0.199	0.71	-0.056
ADI-C	-0.051	-0.177	0.075	0.429	-0.06
ADI-D	-0.130	-0.367	0.106	0.281	0.148
ADOS-A	0.044	-0.128	0.216	0.616	0.055
ADOS-B	-0.158	-0.331	0.014	0.072	-0.177
ADOS-C	0.152	-0.158	0.462	0.335	0.199
ADOS-D	-0.098	-0.372	0.176	0.482	-0.113
Ammonium	0.013	-0.024	0.049	0.496	0.016
Lactate	0.020	-0.036	0.075	0.492	0.024
Ceruloplasmin	0.106	0.035	0.178	**0.003**	0.136
Prolactin	0.003	0.000	0.006	**0.022**	0.004
TSH	0.003	-0.391	0.397	0.988	0.004

Species	***Pasteurellaceae***				
Model results	**B**	**Lower 95% CL**	**Upper 95% CL**	**p-value**	**SMD effect size**
let-7b-5p	0.734	-0.599	2.066	0.281	0.939
miR-451a	-0.023	-0.686	0.639	0.945	-0.02
miR-29a-3p	0.159	-0.228	0.547	0.421	0.149
miR-16-5p	-0.272	-0.844	0.300	0.351	-0.206
miR-141-3p	0.021	-0.009	0.052	0.175	0.018
VIQ	0.020	-0.060	0.100	0.617	0.018
PIQ	0.082	-0.005	0.170	0.064	0.074
TIQ	-0.082	-0.233	0.068	0.285	-0.068
ADI-A	-0.117	-0.421	0.188	0.454	-0.096
ADI-B	-0.197	-0.584	0.191	0.32	-0.155
ADI-C	-0.134	-0.337	0.068	0.194	-0.109
ADI-D	0.406	0.008	0.804	**0.046**	0.434
ADOS-A	-0.258	-0.552	0.037	0.087	-0.197
ADOS-B	0.199	-0.049	0.448	0.116	0.191
ADOS-C	0.055	-0.406	0.517	0.814	0.049
ADOS-D	0.400	-0.099	0.899	0.116	0.426

Ammonium	-0.029	-0.089	0.030	0.334	-0.025
Lactate	0.082	-0.002	0.167	0.057	0.074
Ceruloplasmin	-0.100	-0.184	-0.017	**0.019**	-0.082
Prolactin	0.000	-0.005	0.004	0.866	0.000
TSH	-0.294	-0.884	0.296	0.329	-0.221

Species	***Ralstonia spp***				
Model results	**B**	**Lower 95% CL**	**Upper 95% CL**	**p-value**	**SMD effect size**
let-7b-5p	-1.763	-4.475	0.950	0.203	-0.284
miR-451a	-2.747	-6.132	0.637	0.112	-0.321
miR-29a-3p	0.419	-0.130	0.967	0.135	0.178
miR-16-5p	1174	-0.067	2.416	0.064	0.766
miR-141-3p	-0.049	-0.102	0.003	0.063	-0.016
VIQ	0.233	0.085	0.380	**0.002**	0.090
PIQ	0.151	0.014	0.288	**0.031**	0.056
TIQ	-0.350	-0.592	-0.107	**0.005**	-0.101
ADI-A	0.102	-0.424	0.627	0.704	0.037
ADI-B	0.107	-0.592	0.805	0.765	0.039
ADI-C	-0.024	-0.368	0.321	0.893	-0.008
ADI-D	-0.027	-0.748	0.694	0.942	-0.009
ADOS-A	-0.372	-1.045	0.301	0.278	-0.106
ADOS-B	0.120	-0.383	0.622	0.64	0.044
ADOS-C	0.095	-0.767	0.958	0.828	0.034
ADOS-D	0.38	-0.446	1.206	0.368	0.158
Ammonium	0.077	-0.028	0.183	0.152	0.027
Lactate	-0.036	-0.275	0.203	0.767	-0.012
Ceruloplasmin	0.119	-0.055	0.292	0.180	0.043
Prolactin	-0.009	-0.019	0.001	0.066	-0.003
TSH	-0.679	-1.985	0.627	0.308	-0.169

Species	***Tannerella spp***				
Model results	**B**	**Lower 95% CL**	**Upper 95% CL**	**p-value**	**SMD effect size**
let-7b-5p	-1.130	-1.868	-0.405	**0.002**	-0.875
miR-451a	-0.008	-0.603	0.588	0.980	-0.010
miR-29a-3p	-0.095	-0.360	0.170	0.482	-0.117
miR-16-5p	0.429	0.148	0.709	**0.003**	0.690
miR-141-3p	-0.035	-0.052	-0.018	**0.000**	-0.044
VIQ	-0.048	-0.093	-0.002	**0.040**	-0.060
PIQ	-0.059	-0.103	-0.015	**0.008**	-0.074
TIQ	0.089	0.011	0.167	**0.025**	0.12
ADI-A	0.069	-0.073	0.212	0.339	0.092
ADI-B	0.014	-0.205	0.232	0.903	0.018

ADI-C	0.146	0.016	0.275	**0.028**	0.203
ADI-D	-0.162	-0.379	0.056	0.145	-0.193
ADOS-A	0.181	0.005	0.357	**0.044**	0.256
ADOS-B	-0.195	-0.342	-0.049	**0.009**	-0.228
ADOS-C	0.146	-0.138	0.430	0.313	0.203
ADOS-D	0.017	-0.217	0.251	0.888	0.022
Ammonium	0.024	-0.011	0.059	0.183	0.031
Lactate	-0.001	-0.055	0.052	0.957	-0.001
Ceruloplasmin	0.024	-0.039	0.087	0.447	0.031
Prolactin	3.000	-0.003	0.003	0.955	-0.104
TSH	-0.073	-0.461	0.315	0.711	-0.091

Species	***Weeksellaceae***				
Model results	**B**	**Lower 95% CL**	**Upper 95% CL**	**p-value**	**SMD effect size**
let-7b-5p	0.123	-0.529	0.774	0.712	0.190
miR-451a	-0.580	-0.941	-0.219	**0.002**	-0.638
miR-29a-3p	0.06	-0.167	0.286	0.606	0.09
miR-16-5p	0.046	-0.218	0.309	0.734	0.068
miR-141-3p	0.026	0.011	0.040	**0.001**	0.038
VIQ	-0.061	-0.099	-0.022	**0.002**	-0.086
PIQ	-0.038	-0.077	0.000	**0.049**	-0.054
TIQ	0.086	0.02	0.153	**0.0110**	0.13
ADI-A	0.071	-0.053	0.195	0.263	0.107
ADI-B	-0.183	-0.374	0.009	0.062	-0.242
ADI-C	0.050	-0.050	0.149	0.326	0.074
ADI-D	-0.070	-0.274	0.134	0.501	-0.098
ADOS-A	-0.008	-0.163	0.148	0.924	-0.012
ADOS-B	0.016	-0.115	0.146	0.813	0.023
ADOS-C	-0.196	-0.394	0.002	0.052	-0.051
ADOS-D	-0.036	-0.268	0.196	0.762	-0.051
Ammonium	-0.028	-0.058	0.001	0.061	-0.04
Lactate	0.004	-0.041	0.049	0.862	0.006
Ceruloplasmin	-0.003	-0.050	0.045	0.918	-0.004
Prolactin	0.001	-0.002	0.003	0.557	0.001
TSH	0.063	-0.261	0.387	0.702	0.094

Table S3.2. Parameter estimates from the negative binomial regression model predicting abundances of microbiome species. Regression coefficient (B); lower and upper limits of the 95% Wald confidence interval; p-values calculated by Wald Chi-Square test; Standardized mean difference (SMD) effect size. Statistically significant p-values are indicated in bold.

Species	***Actinobacillus parahaemolyticus***					
Type of model	**NBR**		**MLR**		**SLR**	
Model results	**B**	**p-value**	**B**	**p-value**	**B**	**p-value**
let-7b-5p	1.050	0.232	1.094	**0.006**	-0.007	0.726
miR-451a	-0.851	0.101	-0.217	0.323	-0.025	0.355
miR-29a-3p	-0.244	0.409	-0.178	0.151	-0.016	0.656
miR-16-5p	-0.160	0.651	-0.318	**0.041**	-0.003	0.696
miR-141-3p	0.059	**0.000**	0.030	**0.001**	0.027	**0.000**
VIQ	-0.020	0.659	0.002	0.927	-0.005	0.270
PIQ	-0.090	0.088	-0.046	**0.043**	-0.006	0.186
TIQ	0.119	0.200	0.048	0.201	-0.004	0.421
ADI-A	0.278	0.063	-0.010	0.891	0.032	0.110
ADI-B	-0.049	0.810	0.192	0.108	0.042	0.070
ADI-C	-0.473	**0.000**	-0.174	**0.006**	0.021	0.472
ADI-D	0.498	**0.010**	0.103	0.379	0.117	**0.032**
ADOS-A	0.086	0.660	0.006	0.949	0.065	0.118
ADOS-B	-0.023	0.870	0.014	0.859	0.049	**0.043**
ADOS-C	-0.602	0.057	-0.191	0.152	0.093	0.185
ADOS-D	0.577	0.091	0.163	0.211	0.118	0.052
Ammonium	-0.145	**0.000**	-0.035	**0.044**	0.002	0.901
Lactate	0.142	**0.009**	0.034	0.253	0.033	0.286
Ceruloplasmin	-0.121	**0.021**	-0.023	0.427	-0.006	0.827
Prolactin	0.000	0.904	0.000	0.900	0.001	0.535
TSH	0.262	0.430	0.333	0.082	0.063	0.726

Species	***Filifactor spp***					
Type of model	**NBR**		**MLR**		**SLR**	
Model results	**B**	**p-value**	**B**	**p-value**	**B**	**p-value**
let-7b-5p	-0.886	0.221	*-0.045*	*0.423*	-0.002	0.384
miR-451a	-0.512	0.322	*0.035*	*0.281*	-0.002	0.543
miR-29a-3p	0.455	0.060	*0.039*	***0.040***	0.002	0.664
miR-16-5p	0.227	0.497	*0.000*	*0.997*	-0.001	0.622
miR-141-3p	-0.061	**0.000**	*-0.003*	***0.035***	-0.001	0.335
VIQ	-0.064	0.177	*-0.004*	*0.255*	-0.001	**0.049**
PIQ	-0.098	**0.042**	*-0.008*	***0.014***	-0.002	**0.001**
TIQ	0.123	0.133	*0.010*	*0.068*	-0.002	**0.004**
ADI-A	0.236	0.053	*0.022*	*0.055*	0.007	**0.007**
ADI-B	-0.072	0.723	*-0.022*	*0.211*	0.007	**0.034**
ADI-C	0.101	0.410	*-0.005*	*0.611*	0.008	**0.040**
ADI-D	-0.295	0.153	*-0.009*	*0.623*	0.015	**0.044**
ADOS-A	-0.605	**0.003**	*-0.015*	*0.297*	0.006	0.269
ADOS-B	-0.263	0.067	*-0.020*	*0.103*	0.005	0.141
ADOS-C	0.390	0.087	*0.003*	*0.862*	0.013	0.177
ADOS-D	-0.215	0.326	*0.008*	*0.671*	0.015	0.059

Ammonium	0.002	0.950	*-0.001*	*0.572*	-0.003	0.190
Lactate	-0.041	0.467	*-0.005*	*0.274*	-0.007	0.060
Ceruloplasmin	0.068	0.336	*0.001*	*0.869*	-0.002	0.510
Prolactin	0.003	0.296	*0.000*	*0.373*	0.000	0.763
TSH	-0.326	0.429	*0.006*	*0.841*	0.030	0.196

Species	***Moryella spp***					
Type of model	**NBR**		**MLR**		**SLR**	
Model results	**B**	**p-value**	**B**	**p-value**	**B**	**p-value**
let-7b-5p	-0.079	0.846	*-0.063*	*0.623*	-0.001	0.845
miR-451a	-0.450	0.079	*0.009*	*0.900*	-0.006	0.528
miR-29a-3p	0.045	0.739	*-0.016*	*0.695*	-0.019	0.141
miR-16-5p	0.064	0.697	*0.020*	*0.705*	-0.002	0.418
miR-141-3p	-0.051	**0.000**	*-0.006*	***0.049***	-0.005	**0.029**
VIQ	-0.034	0.141	*-0.006*	*0.398*	0.005	**0.001**
PIQ	-0.031	0.185	*-0.003*	*0.738*	0.005	**0.002**
TIQ	0.055	0.148	*0.009*	*0.465*	0.005	**0.001**
ADI-A	-0.128	0.215	*-0.019*	*0.458*	-0.018	**0.010**
ADI-B	-0.047	0.710	*0.021*	*0.599*	-0.019	**0.020**
ADI-C	-0.051	0.429	*0.006*	*0.786*	-0.023	**0.027**
ADI-D	-0.130	0.281	*-0.073*	*0.077*	-0.059	**0.002**
ADOS-A	0.044	0.616	*0.031*	*0.354*	-0.025	0.093
ADOS-B	-0.158	0.072	*-0.009*	*0.741*	-0.018	**0.041**
ADOS-C	0.152	0.335	*0.032*	*0.480*	-0.038	0.132
ADOS-D	-0.098	0.482	*-0.017*	*0.697*	-0.038	0.083
Ammonium	0.013	0.496	*0.009*	*0.134*	0.002	0.650
Lactate	0.020	0.492	*-0.013*	*0.196*	-0.011	0.174
Ceruloplasmin	0.106	**0.003**	*0.021*	***0.039***	0.017	**0.026**
Prolactin	0.003	**0.022**	*0.000*	*0.752*	0.000	0.901
TSH	0.003	0.988	*0.022*	*0.730*	-0.008	0.868

Species	***Pasteurellaceae***					
Type of model	**NBR**		**MLR**		**SLR**	
Model results	**B**	**p-value**	**B**	**p-value**	**B**	**p-value**
let-7b-5p	0.734	0.281	*0.069*	*0.664*	0.000	0.976
miR-451a	-0.023	0.945	*0.036*	*0.694*	-0.003	0.699
miR-29a-3p	0.159	0.421	*0.046*	*0.380*	0.009	0.461
miR-16-5p	-0.272	0.351	*-0.040*	*0.535*	0.000	0.873
miR-141-3p	0.021	0.175	*0.004*	*0.264*	0.005	**0.015**
VIQ	0.020	0.617	*0.003*	*0.737*	-0.002	0.077
PIQ	0.082	0.064	*0.006*	*0.508*	-0.002	0.154
TIQ	-0.082	0.285	*-0.006*	*0.692*	-0.002	0.137
ADI-A	-0.117	0.454	*-0.019*	*0.557*	0.006	0.360
ADI-B	-0.197	0.320	*-0.032*	*0.516*	0.010	0.183

ADI-C	-0.134	0.194	*-0.006*	*0.803*	0.009	0.354
ADI-D	0.406	**0.046**	*0.037*	*0.456*	0.030	0.085
ADOS-A	-0.258	0.087	*-0.027*	*0.509*	0.020	0.118
ADOS-B	0.199	0.116	*0.024*	*0.481*	0.016	**0.031**
ADOS-C	0.055	0.814	*0.044*	*0.428*	0.059	**0.006**
ADOS-D	0.400	0.116	*0.070*	*0.207*	0.045	**0.017**
Ammonium	-0.029	0.334	*-0.002*	*0.810*	0.002	0.783
Lactate	0.082	0.057	*0.006*	*0.615*	0.012	0.208
Ceruloplasmin	-0.100	**0.019**	*-0.026*	***0.040***	-0.011	0.212
Prolactin	0.000	0.866	*0.000*	*0.780*	0.000	0.908
TSH	-0.294	0.329	*-0.051*	*0.520*	-0.016	0.773

Species	***Ralstonia spp***					
Type of model	**NBR**		**MLR**		**SLR**	
Model results	**B**	**p-value**	**B**	**p-value**	**B**	**p-value**
let-7b-5p	-1.763	0.203	*-0.168*	*0.547*	0.007	0.513
miR-451a	-2.747	0.112	*-0.065*	*0.692*	0.003	0.831
miR-29a-3p	0.419	0.135	*0.062*	*0.500*	0.023	0.221
miR-16-5p	1.174	0.064	*0.066*	*0.558*	0.004	0.358
miR-141-3p	-0.049	0.063	*-0.003*	*0.575*	-0.002	0.501
VIQ	0.233	**0.002**	*0.001*	*0.965*	-0.003	0.211
PIQ	0.151	**0.031**	*-0.001*	*0.948*	-0.004	0.095
TIQ	-0.350	**0.005**	*-0.005*	*0.865*	-0.004	0.085
ADI-A	0.102	0.704	*0.019*	*0.737*	0.014	0.164
ADI-B	0.107	0.765	*-0.049*	*0.580*	0.014	0.230
ADI-C	-0.024	0.893	*0.017*	*0.702*	0.012	0.414
ADI-D	-0.027	0.942	*-0.006*	*0.941*	0.036	0.199
ADOS-A	-0.372	0.278	*0.058*	*0.419*	0.040	0.053
ADOS-B	0.120	0.640	*-0.051*	*0.392*	0.015	0.211
ADOS-C	0.095	0.828	*-0.017*	*0.862*	0.055	0.118
ADOS-D	0.380	0.368	*0.066*	*0.496*	0.045	0.143
Ammonium	0.077	0.152	*0.005*	*0.692*	-0.007	0.445
Lactate	-0.036	0.767	*-0.010*	*0.656*	-0.024	0.117
Ceruloplasmin	0.119	0.180	*0.036*	*0.098*	0.025	0.075
Prolactin	-0.009	0.066	*0.000*	*0.928*	0.000	0.764
TSH	-0.679	0.308	*-0.077*	*0.582*	-0.105	0.245

Species	***Tannerella spp***					
Type of model	**NBR**		**MLR**		**SLR**	
Model results	**B**	**p-value**	**B**	**p-value**	**B**	**p-value**
let-7b-5p	-1.130	**0.002**	*-0.076*	*0.215*	0.005	0.344
miR-451a	-0.008	0.980	*0.027*	*0.442*	0.003	0.652
miR-29a-3p	-0.095	0.482	*-0.010*	*0.632*	0.001	0.931
miR-16-5p	0.429	**0.003**	*0.024*	*0.326*	0.001	0.490

miR-141-3p	-0.035	**0.000**	*-0.003*	*0.055*	-0.003	0.083
VIQ	-0.048	**0.040**	*-0.006*	*0.067*	0.002	0.093
PIQ	-0.059	**0.008**	*-0.008*	***0.025***	0.002	0.064
TIQ	0.089	**0.025**	*0.013*	***0.040***	0.002	**0.049**
ADI-A	0.069	0.339	*0.017*	*0.167*	-0.010	**0.038**
ADI-B	0.014	0.903	*0.003*	*0.896*	-0.015	**0.011**
ADI-C	0.146	**0.028**	*0.009*	*0.382*	-0.013	0.080
ADI-D	-0.162	0.145	*-0.018*	*0.339*	-0.029	**0.036**
ADOS-A	0.181	**0.044**	*0.009*	*0.543*	-0.025	**0.015**
ADOS-B	-0.195	**0.009**	*-0.027*	***0.047***	-0.017	**0.006**
ADOS-C	0.146	0.313	*0.004*	*0.859*	-0.037	**0.031**
ADOS-D	0.017	0.888	*0.004*	*0.858*	-0.034	**0.025**
Ammonium	0.024	0.183	*0.002*	*0.439*	0.000	0.868
Lactate	-0.001	0.957	*-0.001*	*0.890*	-0.005	0.223
Ceruloplasmin	0.024	0.447	*0.005*	*0.302*	-0.001	0.731
Prolactin	-0.084	0.955	*0.000*	*0.981*	0.000	0.600
TSH	-0.073	0.711	*-0.004*	*0.883*	0.017	0.453

Species	***Weeksellaceae***					
Type of model	**NBR**		**MLR**		**SLR**	
Model results	**B**	**p-value**	**B**	**p-value**	**B**	**p-value**
let-7b-5p	0.123	0.712	0.325	0.192	-0.006	0.653
miR-451a	-0.580	**0.002**	-0.280	0.058	-0.013	0.468
miR-29a-3p	0.060	0.606	0.036	0.657	0.003	0.914
miR-16-5p	0.046	0.734	-0.071	0.474	-0.002	0.703
miR-141-3p	0.026	**0.001**	0.025	**0.000**	0.022	**0.000**
VIQ	-0.061	**0.002**	-0.026	0.059	-0.004	0.131
PIQ	-0.038	**0.049**	-0.025	0.089	-0.002	0.521
TIQ	0.086	**0.011**	0.047	0.058	-0.002	0.528
ADI-A	0.071	0.263	0.038	0.443	0.018	0.160
ADI-B	-0.183	0.062	-0.027	0.729	0.024	0.120
ADI-C	0.050	0.326	0.027	0.500	0.026	0.187
ADI-D	-0.070	0.501	-0.104	0.180	0.031	0.393
ADOS-A	-0.008	0.924	0.009	0.891	0.035	0.205
ADOS-B	0.016	0.813	0.000	0.999	0.020	0.219
ADOS-C	-0.196	0.052	-0.169	0.057	0.000	0.994
ADOS-D	-0.036	0.762	0.003	0.968	0.023	0.576
Ammonium	-0.028	0.061	-0.015	0.168	0.003	0.815
Lactate	0.004	0.862	0.008	0.667	0.021	0.287
Ceruloplasmin	-0.003	0.918	0.005	0.772	-0.001	0.940
Prolactin	0.001	0.557	0.000	0.624	0.000	0.954
TSH	0.063	0.702	0.030	0.806	-0.043	0.712

Table S3.3. Comparison between parameters estimated from the negative binomial regression (NBR), multiple linear regression (MLR) and simple linear regression (SLR) models predicting abundances of microbiome species. Regression coefficient (B); statistical significance of the independent variables (p-values).

Data for those MLR which statistical significance of overall model was not reached (p-value > 0.05) are shown in italic. Statistically significant p-values are indicated in bold (p-value ≤ 0.05).

No	Sample	Read Pairs	Yield (Kbp)*	** %Q30	Mean Q†
1	ASD003	148,566	84,534	87.77	35.14
2	ASD005	130,436	74,218	87.94	35.16
3	ASD011	144,814	82,399	87.01	34.97
4	ASD014	125,809	71,585	87.02	34.96
5	ASD016	137,756	78,383	86.81	34.92
6	ASD020	124,683	70,944	87.93	35.17
7	ASD021	146,143	83,155	88.11	35.22
8	ASD023	108,866	61,944	88.40	35.29
9	ASD027	99,82	56,797	88.52	35.30
10	ASD028	99,622	56,684	87.50	35.08
11	ASD029	96,647	54,992	87.12	34.99
12	ASD030	97,502	55,478	87.82	35.15
13	ASD031	104,632	59,535	87.58	35.08
14	ASD032	87,086	49,551	87.58	35.09
15	ASD033	108,507	61,74	83.82	34.22
16	ASD034	108,913	61,971	88.17	35.23
17	ASD035	108,323	61,635	88.18	35.22
18	ASD037	167,607	95,368	87.49	35.07
19	ASD039	123,64	70,351	87.54	35.07
20	ASD041	117,894	67,081	87.03	34.97
21	ASD042	115,575	65,762	86.91	34.94
22	ASD043	120,787	68,727	86.92	34.95
23	ASD047	110,398	62,816	88.17	35.23
24	ASD048	125,261	71,273	87.60	35.10
25	ASD049	109,87	62,516	87.85	35.16
26	ASD050	169,879	96,661	87.61	35.10
27	ASD051	116,122	66,073	87.41	35.06
28	ASD052	91,188	51,885	87.59	35.11
29	ASD053	115,546	65,745	87.38	35.05
30	ASD054	118,956	67,685	88.43	35.29
31	ASD055	105,393	59,968	87.81	35.14
32	ASD056	99,169	56,427	88.40	35.28
33	ASD057	93,034	52,936	88.29	35.25
34	ASD058	103,7	59,005	87.49	35.08
35	ASD059	93,37	53,127	87.24	35.01
36	ASD060	91,262	51,928	86.53	34.86
37	ASD061	90,346	51,406	87.92	35.15
38	ASD062	90,172	51,307	87.98	35.18
39	ASD063	103,342	58,801	88.00	35.19
40	ASD064	139,269	79,244	87.71	35.13
41	ASD065	150,987	85,911	87.43	35.06
42	ASD066	145,56	82,823	87.24	35.02

43	ASD067	136,286	77,546	86.62	34.88
44	ASD068	145,483	82,779	87.64	35.10
45	ASD069	144,006	81,939	87.20	35.01
46	ASD070	145,072	82,545	87.54	35.09
47	ASD071	131,541	74,846	87.57	35.10
48	ASD072	169,413	96,395	87.81	35.15
49	ASD073	180,706	102,821	88.21	35.24
50	ASD074	151,876	86,417	87.32	35.04
51	ASD075	126,173	71,792	87.00	34.96
52	ASD076	120,554	68,595	86.88	34.95
53	ASD077	143,931	81,896	87.77	35.13
54	NUC001	149,7	85,179	87.96	35.18
55	NUC002	131,637	74,901	88.69	35.35
56	NUC003	100,436	57,148	86.95	34.96
57	NUC005	151,361	86,124	87.56	35.09

Table S3.4. Various statistics describing the sorted reads of 80 saliva samples. All reads are passed filter, i.e., reads have passed the default Illumina filter procedure (chastity filter); *Yield (Kbp): number of bases called in kilobases; **%Q30: percentage of bases with a quality score of at least 30 (inferred base call accuracy of 99.9%); † Mean Q: Q-score is a prediction of the probability of a wrong base call.

3.7. Supplementary Figures

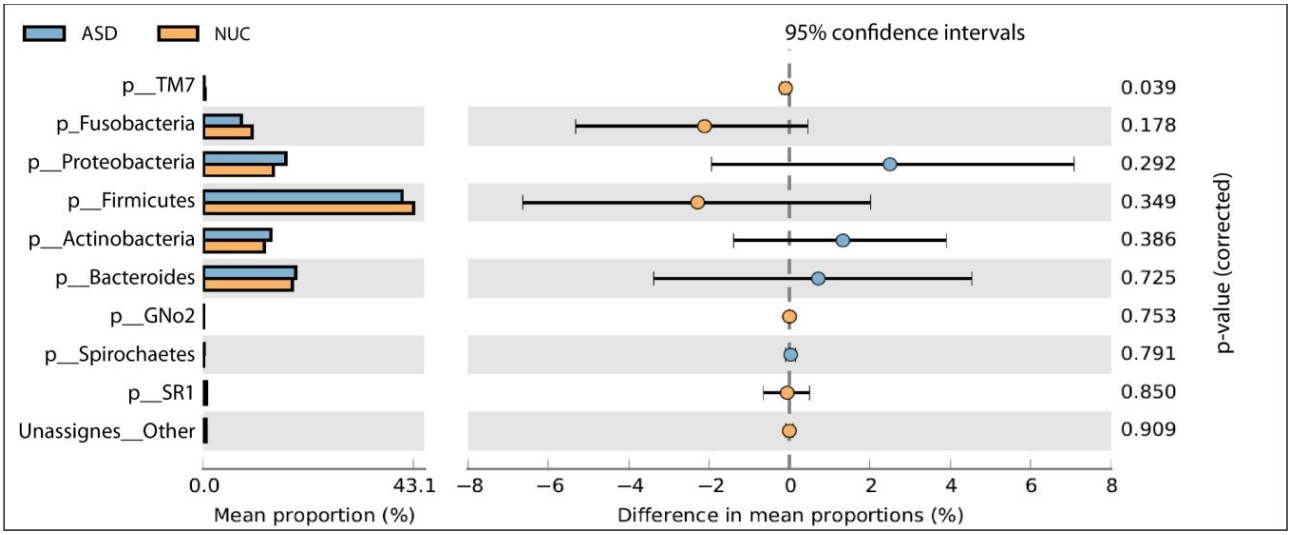


Figure S3.1. The bacterial taxonomic abundance of all phyla in ASD and NUC samples. The composition of salivary microbiome between ASD and NUC was explored in terms of the relative abundances through White's non-parametric t-test (p-value ≤ 0.05), using STAMP software.

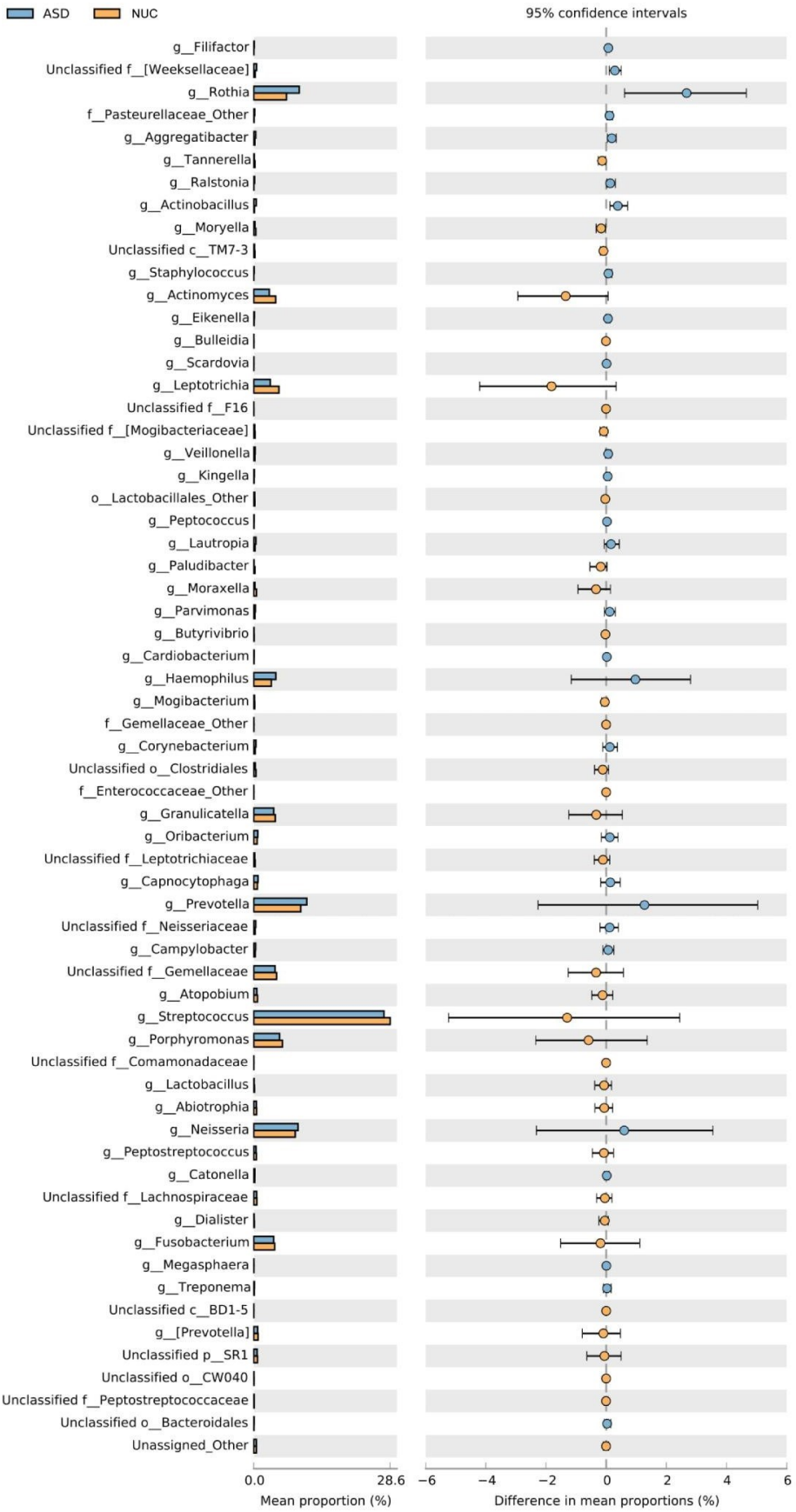


Figure S3.2. The bacterial taxonomic abundance of all genera in ASD and NUC samples. The composition of salivary microbiome between ASD and NUC was explored in terms of the relative abundances through White's non-parametric t-test (p-value ≤ 0.05), using STAMP software.

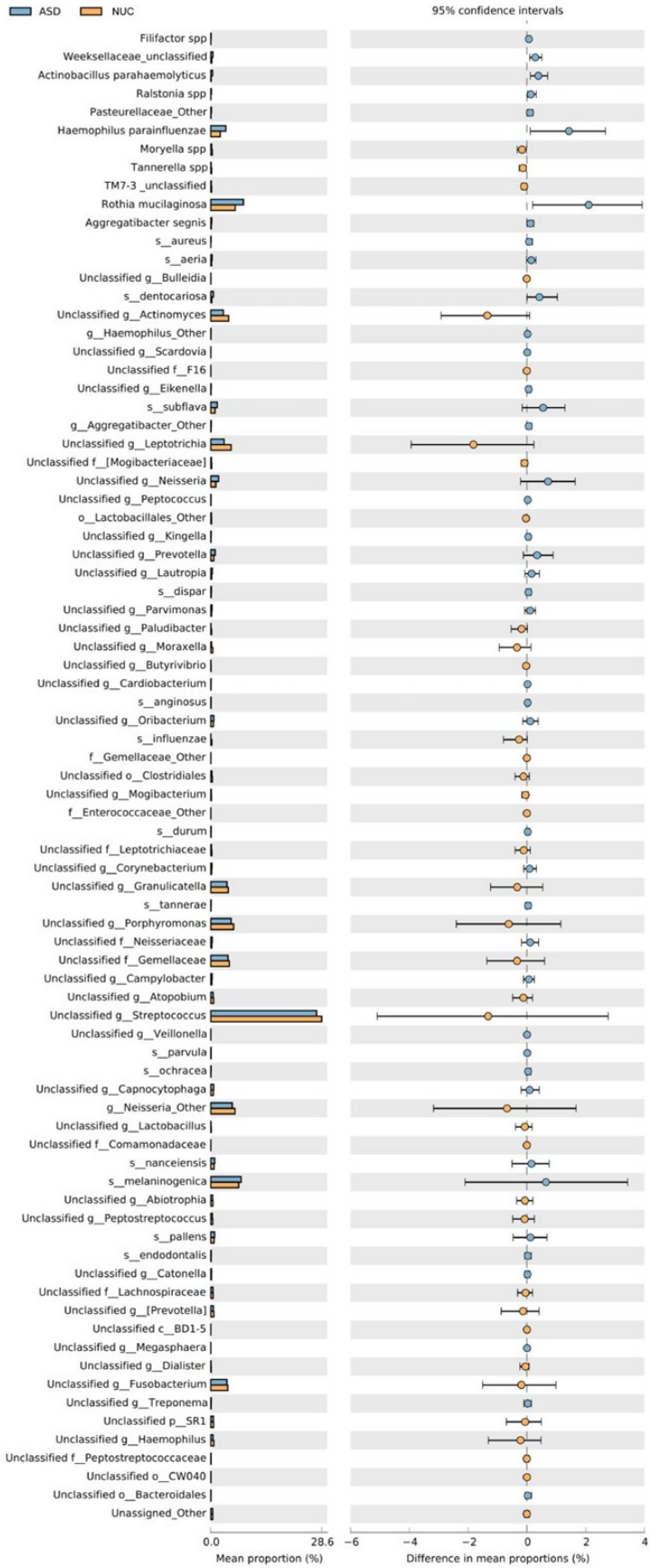


Figure S3.3. The bacterial taxonomic abundance of all species in ASD and NUC samples. The composition of salivary microbiome between ASD and NUC was explored in terms of the relative abundances through White's non-parametric t-test (p-value ≤ 0.05), using STAMP software.

Chapter 4. Enrichment and correlation analysis of serum miRNAs in comorbidity between Arnold-Chiari and Tourette syndrome

4.1. Abstract

The molecular basis of Arnold-Chiari (AC) syndrome remains almost totally unknown, due to the multifactorial and heterogeneous nature of the disorder, coupled with its rarity. The relationship with neuropsychiatric disorders, including Tourette syndrome (TS), is also undetermined. The very rare co-occurrence of both disorders (ACTS) complicates the framework of clinical diagnosis and makes it vulnerable to bias. Moreover, the molecular basis of the comorbid condition has not been explored. Discovering molecular signatures of the disorders may help the diagnostic process to discriminate patients among the different pathophysiological conditions.

In this exploratory study, we aimed to identify serum microRNA expression profiles as molecular fingerprints for AC, TS and ACTS, by using NanoString technology. For this aim, 10 AC patients, 11 ACTS patients, 6 TS patients, and 8 unaffected controls (NC) were recruited. Nine miRNAs resulted differentially expressed: let-7b-5p (upregulated in ACTS vs TS); miR-21-5p (upregulated in ACTS vs AC; downregulated in AC vs TS); miR-23a-3p (upregulated in TS vs NC; downregulated in AC vs TS); miR-25-3p (upregulated in AC vs TS and NC; downregulated in ACTS vs AC); miR-93-5p (upregulated in AC vs TS); miR-130a-3p (downregulated in ACTS and TS vs NC); miR-144-3p (downregulated in ACTS vs AC; upregulated in AC vs TS); miR-222-3p (upregulated in ACTS vs NC); miR-451a (upregulated in AC vs TS and NC; in ACTS vs NC). MiRNA expression was statistically correlated to neuroimaging and neuropsychological anomalies. Furthermore, computational analyses indicated that DE miRNAs are involved in AC and TS pathomechanisms.

Finally, we propose the miRNA set as a potential molecular tool for supporting the current diagnosis of AC, TS and ACTS by using liquid biopsies, in an unbiased and non-invasive way.

4.2. Results

4.2.1. Demographics

We recruited a total of 35 children from various socio-economic contexts: 10 affected by Arnold Chiari Syndrome (AC) (M: F= 6:4), mean age 13.1 (±3.1) (age range 9-17); 11 affected by Arnold Chiari Syndrome and comorbid Tourette Syndrome (ACTS) (M: F=9:2), mean age 12.3 (±2.7) (age range 8-16); 6 affected by Tourette Syndrome (TS) (M: F= 6:0), mean age 13.8 (±2) (age range 11-16); 8 unaffected controls (NC) (M: F=8:0), mean age 12.6 (±2.6) (age range 9-16), recruited from local schools. Demographic and neuropsychological characteristics of the clinical sample are shown in table 4.1.

	ACTS	**AC**	**TS**	**NC**
Number of participants (36)	11	10	6	8
Sex (M:F)	9:2	6:4	6:0	7:1
Mean age (years)	12.3 (±2.7)	13.1 (±3.1)	13.8 (±2)	12.6 (±2.6)
IQ				
TIQ	79.8 (±23.3)	74.3 (±16.3)	96.3 (±11.2)	88.6 (±9.4)
VIQ	79.9 (±22.5)	74.9 (±17.2)	95.3 (±11.3)	86.2 (±9.3)
PIQ	86.7 (±21.6)	77.8 (±17.4)	98.6 (±16.4)	93.1 (±8.5)
YGTSS				
Total	19.9 (±8.3)	0	22.5 (±7.2)	0
Motor	12 (±4.1)	0	13.6 (±4.7)	0
Fonic	7.9 (±5.4)	0	8.8 (±2.9)	0
CYBOCS				
Total	14.4 (±5.4)	8.2 (±2.8)	18 (±9.7)	7.2 (±3.1)
Obsessions	6.5 (±2.2)	4 (±2.2)	9.3 (±4.8)	4 (±2.7)
Compulsions	7.9 (±3.4)	4.2 (±1.2)	8.6 (±5.3)	6.6 (±9.5)
CBCL				
Total	54.3 (±34.4)	26.8 (±12.1)	48.1 (±15.3)	24.9 (±5.8)
Internalizing	16.6 (±11.3)	8.7 (±3.7)	11 (±3.4)	9 (±4.9)
Externalizing	21.4 (±11.9)	10.9 (±6.2)	18.5 (±6.6)	6.4 (±2.6)
Conners	29.5 (±22.3)	25.1 (±14.1)	48.1 (±15.3)	11.2 (±4.5)

Table 4.1. Demographic and neuropsychological characteristics of the clinical samples. Data are shown as means and ± standard deviations between brackets. ACTS: Arnold Chiari + Tourette Syndrome; AC: Arnold Chiari; TS: Tourette Syndrome; NC: Negative Control; TIQ: Total intelligence quotient; VIQ: Verbal intelligence quotient; PIQ: performance intelligence quotient; YGTSS: Yale Global Tic Severity Scale; CYBOCS Tot: Children Yale Brown Obsessive Compulsive Scale total score; CBCL-Tot: Child Behavior

Check List. According to two-tailed Mann–Whitney U test, there were no statistically significant sex differences between groups in the comparisons between TS and AC (p-value = 0.2335), TS and ACTS (p-value = 0.2952), TS and NC (p-value > 0.999), NC and AC (p-value = 0.313), NC and ACTS (p-value > 0.999), AC and ACTS (p-value = 0.292) groups.

4.2.2. Neuropsychological findings

(i) Intelligent quotient (Total, Verbal, and Performance) assessed by using the Wechsler scale: the TS group showed the highest mean value (Table 4.1); no statistically significant differences were found in the comparison between clinical groups, except for AC versus TS (p-value = 0.005).

(ii) Tic evaluation assessed by using the YGTSS instrument: TS presented higher scores compared to ACTS group, but these differences were not statistically significant in any subscale (motor, vocal, total). We found scores equal to zero in the AC group and in the unaffected control group: this could be explained by the exclusion of patients with a tic who did not fulfill the criteria for TS to avoid confounding factors (see inclusion section).

(iii) Evaluation of obsessions and compulsions, assessed by using the CYBOCS instrument: AC and NC groups presented statistically significant lower scores compared to TS and ACTS. These scores are not able to identify, by themselves, an obsessive-compulsive disorder, but could be the expression of obsessive and/or compulsive behavior that does not interfere with the normal life of the patient. Comparing TS and ACTS groups, TS presented the higher scores that are identifiable with OCD disorder. No statistically significant differences were found, even if the disorder in ACTS is less severe than TS.

(iv) Evaluation of behavioral problems, assessed by using CBCL and Conners' scales: ACTS group presented higher scores (total, internalizing and externalizing CBCL), but no statistically significant differences were found compared to the scores of TS group.

Compared to the other clinical groups, the scores presented in the AC and NC groups showed a lower statistical significance level.

Finally, measuring with the Conners' scale, the TS group presented the highest statistically significant score compared with the other clinical groups and the NC group. In the comparison between AC and ACTS, no statistically significant differences were found; compared to the three clinical groups, the score of the NC group showed a lower statistical significance level.

4.2.3. Neuroimaging measurement

Patients and unaffected controls underwent MRI and all the measurements described in methods section were performed. All measurement details are reported in table 4.2.

	ACTS	**AC**	**TS**	**NC**
Protrusion right tonsilla (mm)	4.8 (±1.3)	5.9 (±2)	not applicable	not applicable
Protrusion left tonsilla (mm)	4.5 (±2.6.)	5.8 (±2.5)	not applicable	not applicable
Upper posterior cranial fossa area	1129.6 (±193.4)	1099.2 (±128.9)	1197.2 (±231.6)	1142.9 (±159.9)
Lower posterior cranial fossa area	1971.5 (±305)	1717.7 (±557.8)	1982.6 (±257)	1997.8 (±431.4)
Total posterior cranial fossa area	3101.1 (±323.3)	2738.7 (±487.7)	3265.6 (±288.7)	3362.7 (±274.2)
Basal angle	135.2 (±4.1)	136.4 (±2.7)	131.7 (±6)	131.7 (±6)
Boogaard angle	118.7 (±7.4)	124.7 (±6.3)	120.3 (±,7)	118.1 (±4.7)
Occipital angle	137.5 (±7.2)	132.3 (±7.4)	137.1 (±8.8)	137.2 (±7.8)
Tentorial angle	77.7 (±6.8)	79.8 (±10.8)	82.5 (±8.2)	83.9 (±8.4)

Table 4.2. Neuroimaging measurements. Data are shown as means and ± standard deviations between brackets.

By applying 2-tailed Student t tests, statistically significant differences were found in the areas of total PCF values between ACTS and NC (p-value = 0.0053); AC and TS (p-value = 0.0316); AC and NC (p-value = 0.0053) groups. No statistically significant differences were found in all the other comparisons.

4.2.4. Circulating miRNA expression profiling

By using nCounter NanoString technology, we profiled serum expression of 800 microRNAs in a sample set of 10 AC, 11 ACTS, 6 TS patients and 8 NC subjects.

We identified 9 miRNAs as significantly differentially expressed between the different groups (FDR < 0.05 in each pairwise comparison) (Table 4.3).

DE miRNA	AC vs NC	ACTS vs NC	ACTS vs AC	TS vs NC	AC vs TS	ACTS vs TS
let-7b-5p	-	-	-	-	-	2
miR-21-5p	-	-	1.38	-	-1.62	-
miR-23a-3p	-	-	-	1.67	-2	-
miR-25-3p	2.43	-	-2.65	-	2.44	-
miR-93-5p	-	-	-	-	1.75	-
miR-130a-3p	-	-1.56	-	-1.61	-	-
miR-144-3p	-	-	-2.09	-	2.15	-
miR-222-3p	-	1.95	-	-	-	-
miR-451a	2.73	1.58	-	-	2.53	-

Table 4.3. Dysregulation of 9 miRNAs in different pairwise comparisons. Expression FC values of each DE miRNA are reported for different comparisons, only when statistically significant (FDR < 0.05); -: value not reported because FDR > 0.05.

More specifically, we observed the following results: (1) let-7b-5p upregulated in ACTS compared to TS patients; (2) miR-21-5p upregulated in ACTS compared to AC patients and downregulated in AC compared to TS patients; (3) miR-23a-3p upregulated in TS patients compared to NCs and downregulated in AC compared to TS patients; (4) miR-25-3p upregulated in AC compared to TS patients as well as NCs and downregulated in ACTS compared to AC patients; (5) miR-93-5p upregulated in AC compared to TS patients; (6) miR-130a-3p downregulated in ACTS and TS patients compared to NCs; (7) miR-144-3p downregulated in ACTS compared to AC patients and upregulated in AC compared to TS patients; (8) miR-222-3p upregulated in ACTS compared to NCs; (9) miR-451a upregulated in AC compared to TS patients and NCs, and in ACTS patients compared to NCs. The relative expression of DE miRNAs is shown in Figure 4.1.

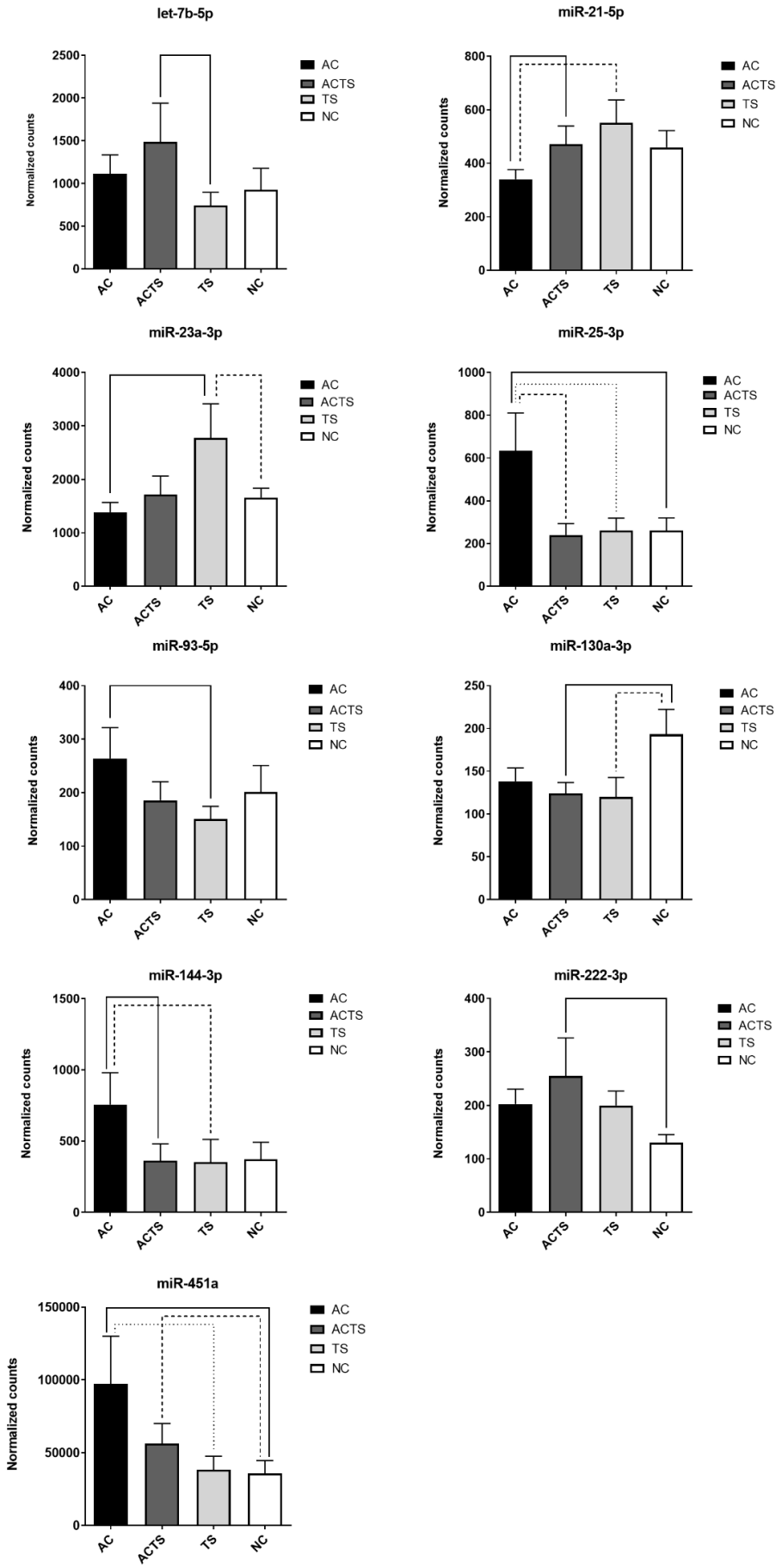

let-7b-5p
Normalized counts
2500
2000
1500
1000
500
0
AC
ACTS
TS
NC
miR-21-5p
Normalized counts
800
600
400
200
0
AC
ACTS
TS
NC
miR-23a-3p
Normalized counts
4000
3000
2000
1000
0
AC
ACTS
TS
NC
miR-25-3p
Normalized counts
1000
800
600
400
200
0
AC
ACTS
TS
NC
miR-93-5p
Normalized counts
400
300
200
100
0
AC
ACTS
TS
NC
miR-130a-3p
Normalized counts
250
200
150
100
50
0
AC
ACTS
TS
NC
miR-144-3p
Normalized counts
1500
1000
500
0
AC
ACTS
TS
NC
miR-222-3p
Normalized counts
400
300
200
100
0
AC
ACTS
TS
NC
miR-451a
Normalized counts
150000
100000
50000
0
AC
ACTS
TS
NC

Figure 4.1. MiRNA expression in the serum of AC, TS, ACTS and NC subjects. Bar plots of the relative expression of the 9 miRNAs for each pairwise comparison: let-7b-5p, miR-21-5p, miR-23a-3p, miR-25-3p, miR-93-5p, miR-130a-3p, miR-144-3p, miR-222-3p, and miR-451a. Each pairwise whose miRNA dysregulation was found statistically significant (FDR< 0.05) is linked by a continuous or dotted line. Data are shown as means with SEM. Y-axis represents the distribution of normalized counts.

4.2.5. Correlation analysis between serum miRNA expression levels and neuropsychological, neurological parameters

Using the Spearman correlation test and applying two-sided p-values, the normalized counts of serum miRNAs were correlated with the neuropsychological and neurological scores for each comparison (Figure 4.2).

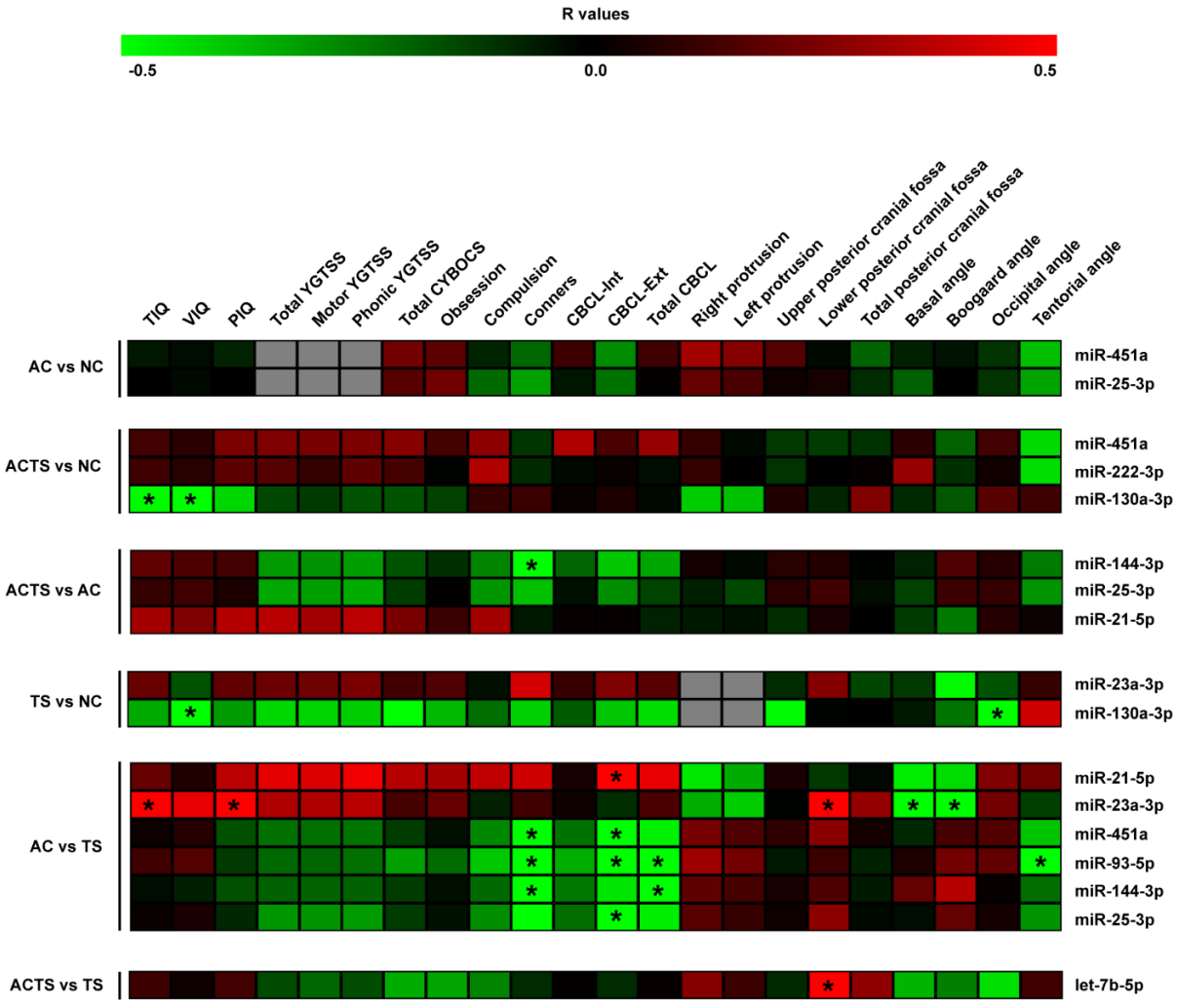


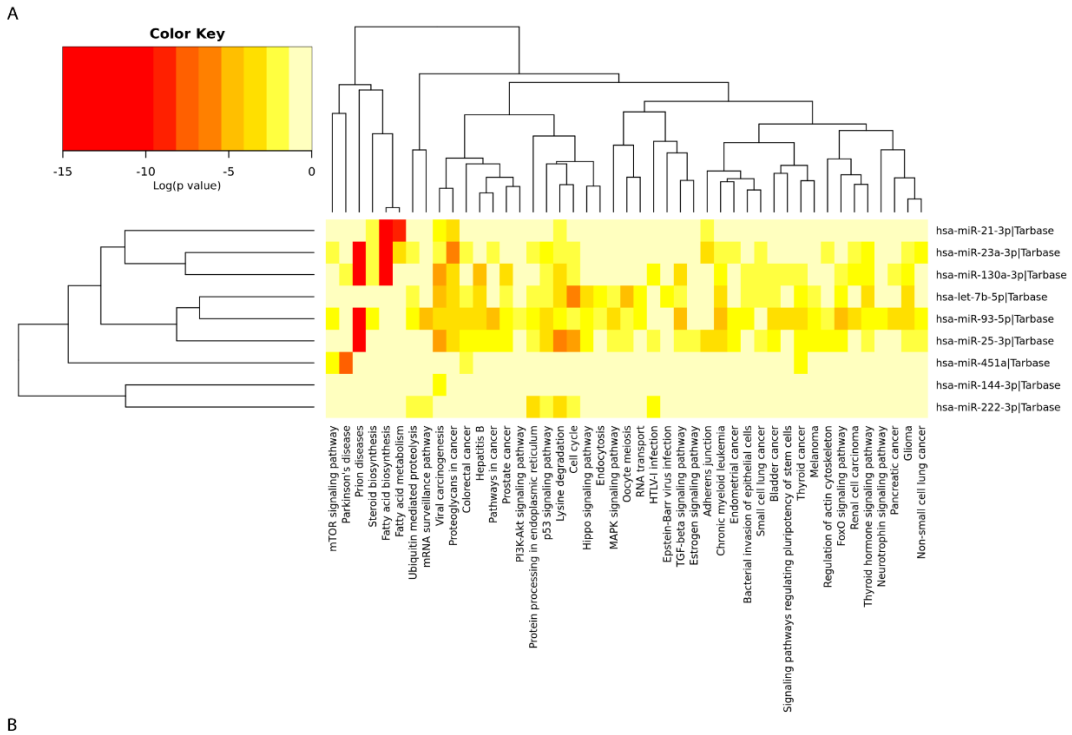
Figure 4.2. Correlation analysis of serum miRNA expression, cognitive impairments and neuroimaging measurements. Heat-maps of the correlations obtained by calculating Spearman correlation coefficients for miRNA expression, cognitive impairments and neuroimaging measurements of the assessed participants and for each group of comparison. The correlation coefficient is indicated by a color gradient from green (negative correlation) to red (positive correlation), as shown in the colored bar. Statistically significant p-values (p-value ≤ 0.05) are indicated by asterisks.

By comparing ACTS and NC groups, we found a negative relationship between intelligent quotient scores (e.g., TIQ and VIQ) and miR-130a-3p (r = -0.49, r =-0.53, respectively), as well as between VIQ and miR-130a-3p (r = -0.65) by comparing TS and NC groups and a positive correlation between TIQ, PIQ and miR-23a-3p (r = 0.57, r = 0.54, respectively), in AC vs TS comparison. We also found a negative relationship between Conners' scale and (i) miR-144-3p (r = -0.5) by comparing ACTS and AC groups and (ii) miR-451a (r = -0.8), miR-93-5p (r = -0.79), miR-144-3p (r = -0.79) comparing AC and TS groups. CBCL-ext scores were found positively correlated with miR-21-5p levels (r = 0.51) and negatively correlated with miR-451a (r = -0.52), miR-93-5p (r = -0.66), miR-25-3p (r = -0.50) in AC vs TS comparison. From the same comparison, total CBCL scores were found negatively correlated with miR-93-5p (r = -0.67) and miR-144-3p (r = -0.50). On the other hand, neuroimaging measurements (e.g., lower posterior cranial fossa) were found positively correlated with miR-23a-3p (r = 0.63) by comparing AC and TS groups and let-7b-p (r = 0.53) by comparing ACTS vs TS groups. From the same comparison, we found a negative correlation between Basal and Boogard angle measurements and miR-23a-3p (r = -0.66, r = -0.64, respectively). Finally, we found negative correlations between occipital angle measurements and miR-130a-3p (r = -0.56) by comparing TS vs NC groups; tentorial angle and miR-93-5p (r = -0.63) by comparing AC vs TS groups. These findings suggest that a link potentially exists between the expression of some of the nine serum miRNAs and numerous clinical parameters.

4.2.6. Functional enrichment analyses

In order to evaluate the potential biological impact related to the differential expression of all the miRNAs identified in this study, we computed pathway enrichment analyses. This analysis showed a list of statistically over-represented biological pathways (Figure 4.3), potentially related to the different pathological conditions. Supplementary Figure S4.1, S4.2, S4.3 report all the data from the

class-specific functional enrichment analysis, including those miRNAs found DE for each pathological condition with respect to the NC group.



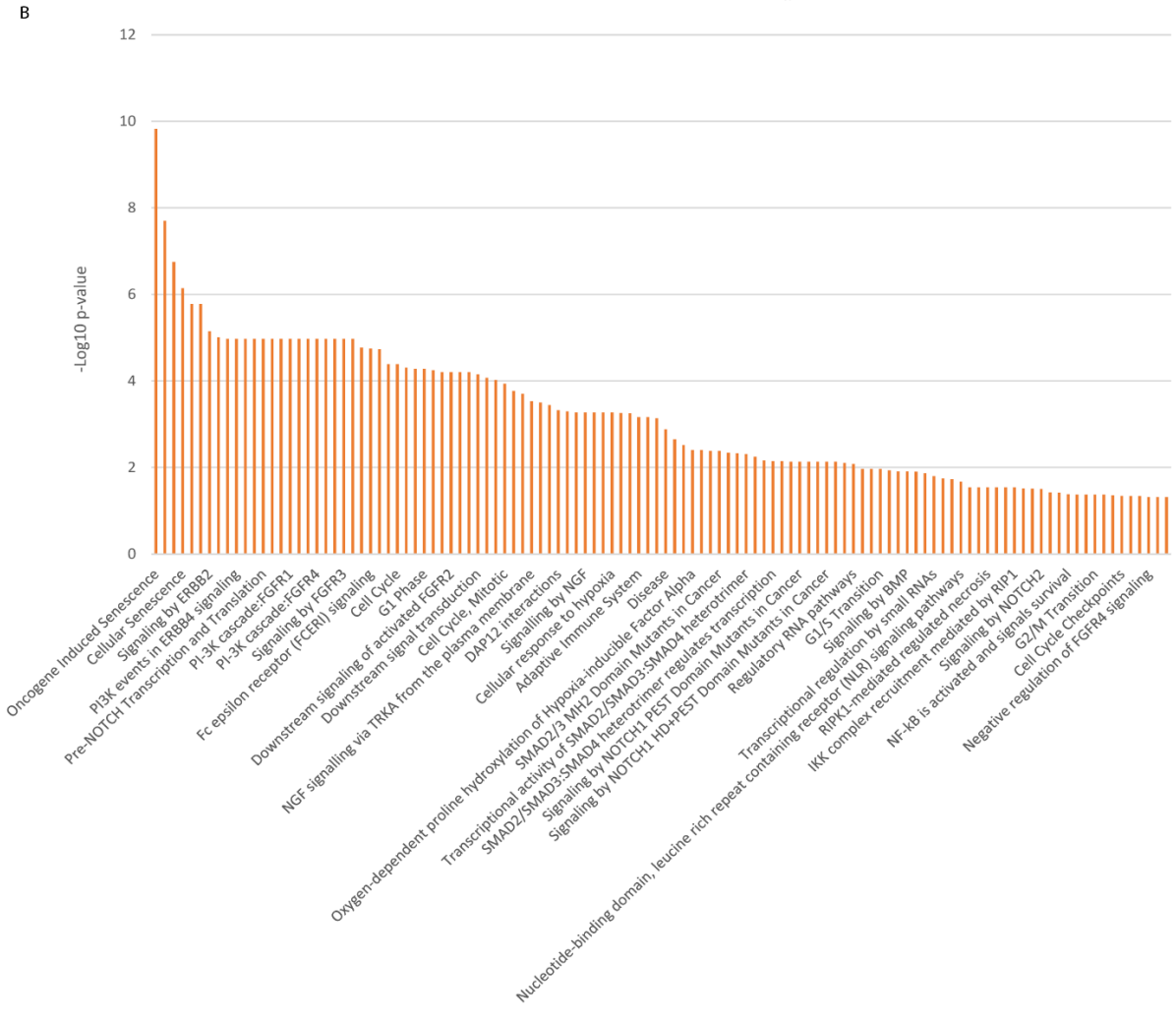


Figure 4.3. Functional Enrichment analysis of DE miRNAs. Functional enrichment analysis of DE miRNA targets using (A) KEGG pathway (hierarchical clustering based on a complete linkage method and the significance levels of the interactions) and (B) Reactome database by DIANA-mirPath v.3 web server and miRNet tool, respectively.

Among the most interesting terms, we found signaling by NOTCH (p-value = 0.00000166), pre-NOTCH expression and processing signaling pathway (p-value = 0.0000106), NGF signaling via TRKA from the plasma membrane (p-value = 0.000293), signaling by NGF (p-value = 0.00053), NOTCH1 intracellular domain regulated transcription (p-value = 0.000551), signaling by NOTCH1 (p-value = 0.00391), constitutive signaling by NOTCH1 HD+PEST Domain Mutants (p-value = 0.00729), signaling by NOTCH2 (p-value = 0.0315), and signaling by Wnt (p-value = 0.0108), through which neuronal functions and development are regulated. Moreover, we found pathways that mediate fear conditioning and behavior signaling by ERBB2 (p-value = 0.00000711) and ERBB4 (p-value = 0.0000106), also involved in the development of neurodegenerative diseases. Additionally, the PI3K/AKT activation pathway (p-value = 0.0000169) was also pinpointed, which is a major regulator of neuron survival, as well as FGFR1/2/3/4 (p-value = 0.0000106), signaling by FGFR1/2/3/4 (p-value = 0.0000106), downstream signaling of activated FGFR1/2/3/4 (p-value = 0.0000624), FoxO signaling pathway (p-value = 3.22^{-07}), Hippo signaling pathway (p-value = 1.58^{-05}), neurotrophin signaling pathway (p-value = 0.04106071) and recycling pathway of L1 (p-value = 0.0187), involved in neural development, including axon outgrowth and neuronal migration. Other molecular signaling pathways were revealed, such as signaling by BMP (p-value = 0.0123), $Ca2^{+}$ pathway (p-value = 0.0438), TGF-beta receptor signaling activates SMADs (p-value = 0.00053) and transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer (p-value = 0.00491). These pathways are intriguing since they are related to disorders in bone tissue, muscle contraction, embryonic skeletal development, postnatal bone homeostasis dorsoventral polarity, which analyzed together suggest that the differential expression of these miRNAs could cause the developmental impairments leading to AC pathogenesis.

4.3. Discussion

The complexity of phenotypes and the clinical variability of AC syndrome and TS increase in comorbid condition with other disturbances: it is still unclear whether or not they both contribute to the genetic expression, or whether some etiological pathway components may be shared, as well as how the comorbid disorders influence each other [365]. To date, a coexistence of both AC syndrome and TS in the same individual (i.e., ACTS) has not been reported. For this reason, in our study we designed to select patients suffering from AC syndrome, TS and comorbid AC syndrome with TS, evaluated through standardized approaches used for neuropsychological and neuroimaging examinations. Furthermore, it is worth noting that delineating neuropsychiatric disorders, such as TS, is still challenging and there is an urgent need for potential molecular biomarkers, considering the etiological and clinical heterogeneity, the lack of an unbiased diagnostic test and no existing associations with any neuroimaging abnormality [365, 366]. Circulating miRNAs may objectively support the current diagnosis strategies, unveiling evasive cases that are otherwise not easily detectable. Therefore, we hypothesized that serum profile of circulating miRNAs may contain some specific molecular fingerprints for AC, TS and ACTS, which could support the clinical discrimination process of patients in an unbiased way. Analyzing miRNA expression profiles in body fluids could reflect the real pathophysiologic processes of patients through very simple and non-invasive approaches, since brain tissue is not easily accessible to researchers [367].

Despite many studies investigated miRNA expression profile in neurological and psychiatric disorders [368-371], only a few included Tourette syndrome [261, 271] and none is focused on AC and ACTS.

Through a high-throughput approach, we investigated circulating miRNA expression in serum of 10 AC, 11 ACTS, 6 TS and 8 NC subjects. Results led to the identification of 9 serum miRNAs as differentially expressed in different groups of comparison (Figure 4.1). Interestingly, we observed that some DE miRNAs identified (i.e., miR-451a, miR-21-5p, miR-25-3p, miR-144-3p) mostly showed a marked differential expression trend between AC and ACTS groups, rather than in the

comparison between ACTS and TS groups (Figure 4.1). This observation may suggest that, when in comorbid condition, miRNA expression could be mostly affected by TS etiopathogenetic contribution: further investigations are needed to evaluate this hypothesis.

The nine DE miRNAs detected in this study had been previously identified as dysregulated in cellular and extra-cellular frameworks of other neurological and/or bone disorders [238, 267, 372-377]. For instance, in the context of ASD, it has been reported that miR-21-5p can target OXTR (Oxytocin Receptor) gene and, modulating its expression, may regulate social behavior [238]. Serum miR-25-3p was proposed as non-invasive biomarker for tumor monitoring and prognostic prediction of osteosarcoma [372]. Circulating miR-93-5p was detected as dysregulated in blood of patients with TIONFH (Trauma-Induced Osteonecrosis of the Femoral Head) and was suggested to inhibit osteogenic differentiation by targeting BMP-2 (Bone Morphogenetic Protein-2) [374], as well as Smad5 (Small mother against decapentaplegic 5), as reported from studies in mouse mesenchymal stem cells [375].

These findings suggest a critical role of miRNAs identified in our study in developing cognitive functions and bone morphogenesis, which are crucial steps for the pathophysiology of TS and AC syndrome. However, the multiple associations found among miRNAs identified in our study and the different pathological conditions might also indicate a limitation in the diagnostic specificity for AC syndrome and TS: further analyses in larger independent cohorts should validate their diagnostic potentiality and address this critical issue.

Molecular pathways identified in our functional enrichment analysis would support the involvement of these miRNAs in AC and TS mechanisms (Figure 4.3). Among the most interesting, Wnt pathway could be associated with AC pathogenesis by affecting the bone homeostasis and bone remodeling process [378]. Wnt pathway is also involved in a crosstalk with Bone Morphogenetic Protein (BMP) and TGF-β signaling during osteoblast and chondrocyte differentiation [379]. As key regulators of TGFβ-induced chondrogenesis of human mesenchymal stem cells (BMSCs), activated SMAD2 and SMAD3 proteins form complexes with co-factor SMAD4 to regulate gene

transcription [380, 381]. Notch signaling, in bone marrow, plays an important role determining the maintenance of a pool of mesenchymal progenitors by suppressing osteoblast differentiation [382]. Furthermore, alterations in signaling by Notch and NGF could have important effects on neurogenesis and neurodevelopmental processes [309, 311], along with Hippo signaling [383], PI3K/AKT activation [384], FoxO signaling [385] and signaling by ERBB [386], which overall control crucial biological processes such as neuronal maintenance, brain functions, behavioral manifestation. All these findings reveal that the miRNAs identified in this study could indirectly act as regulators of crucial mechanisms and biological pathways related to AC and TS pathophysiology.

Finally, we evaluated whether a relationship could exist between miRNA expression and TS and AC clinical parameters, commonly used for the diagnostic evaluation.

We found several linear correlations between miRNA serum expression levels, intelligent quotient scores, cranial disproportions and behavioral impairments (Figure 4.2).

Among the most interesting associations, the lower concentrations of miR-144-3p in ACTS group, and miR-451a, -93-5p, -144-3p in TS group, both compared to the AC group, have been found associated to higher Conners' scale scores, used to measure TS-related behavioral problems. Actually, the double correlation found between lower serum levels of miR-144-3p in ACTS and TS vs AC comparison and the higher scores regarding behavioral problems, may support the hypothesis that TS phenotype could more strongly impact on miRNA expression compared to AC phenotype. Furthermore, we found significant correlations between the levels of miR-21-5p, -451a, -93-5p, -144-3p, -25-3p in TS, compared to AC group, and higher CBCL scores, which assessed behavior impairments in children.

Overall, the multiplicity of such correlations strongly suggests that the miRNA differential abundance in serum is potentially associated with the occurrence and worsening of common behavioral impairments related to TS psychopathology.

Other interesting associations between some DE miRNAs and neuroimaging measurements have been revealed. Among these, by comparing the AC vs TS groups, we found a relevant association between lower serum concentrations of miR-23a-3p and smaller area measures of the lower PCF in AC group, which is a clinical hallmark of AC patients [191, 387].

Taken together, these associations might indicate that serum miRNA expression is consistent with clinical findings and it could potentially support the current diagnostic evaluation.

The main limitation of our study regards the small sample size. In fact, due to its rarity, the availability of ACTS samples was numerically very limited and did not allow us to design further validation experiments on profiling data by TaqMan assay in an independent validation sample set. In fact, our experimental plan established only a discovery sample set composed by 35 samples altogether employed for miRNA profiling. Biological materials from the discovery set were used up for NanoString analysis and to optimize protocols of RNA extraction from serum. For this technical reason, we were not able to reanalyze the discovery sample set by Real time PCR and to test the diagnostic accuracy of miRNAs as potential biomarkers through ROC curve analysis and performance evaluations.

Despite the exploratory nature, our study offers new insights into the molecular basis of AC, TS, and ACTS diseases. These preliminary results strongly encourage further investigations in independent cohorts that could validate the diagnostic potential of the circulating miRNAs identified in this study as appropriate non-invasive biomarkers.

4.4. Concluding remarks and future perspectives

Though a high-throughput approach, we detected specific microRNA expression profiles in sera of patients affected by Arnold-Chiari, Tourette syndromes and both disorders in comorbid status, compared to neurologically unaffected controls. We propose specific molecular signatures as a potential molecular tool for supporting the current diagnosis in a simple, fast, unbiased and non-

invasive way. Because of the limited size of the group of patients, further studies in larger independent cohorts will be strongly needed to validate the diagnostic potentiality and specificity. In this pilot study, to test this hypothesis, we investigated the associations of microRNAs with the neuropsychological and neuroimaging parameters for each participant and their potential involvement in pathological pathways. TS-related cognitive impairments and AC typical cranial disproportions were found associated with most of the miRNAs identified in this study. MiRNAs could discriminate patients from neurologically unaffected children, and, accordingly, be exploited as potential serum markers. Our enrichment analysis contributes to support the hypothesis, since it revealed that DE miRNAs are involved in many biological pathways potentially dysregulated in AC syndrome and TS.

Molecular and cellular characterization of the 9 miRNAs identified for each pairwise comparison could clarify the molecular basis of TS and AC syndrome. Extending the study to larger independent cohorts is necessary to validate our findings.

4.5. Materials and Methods

4.5.1. Ethics approval and consent to participate

All experiments were approved by the local ethical committee "Comitato Etico Catania 1" (ID: 0024-36-19) prior to sample collection, in accordance with the Helsinki Declaration and its later amendments or comparable ethical standards. Written, informed consent was obtained from parents of all minor age participants (age range 12-13).

4.5.2. Patient Selection

Twenty-seven Caucasian patients were consecutively recruited from July 2017 to December 2018 at the Section of Child and Adolescent Psychiatry (Department of Clinical and Experimental Medicine, University of Catania).

Patients were affected by AC syndrome (n = 10), ACTS (n = 11), and TS (n = 6). They were studied and compared to NCs recruited from local schools (n = 8).

4.5.3. Inclusion criteria

Patients who were included in the study fulfilled the following criteria for each clinical group:

(i) AC group: presence of Arnold Chiari malformation type I, defined by a cerebellar tonsillar position (TP) greater than 3-5 mm below the FM; absence of other neurological or metabolic conditions; absence of movement disorders and comorbid conditions such as obsessive-compulsive disorder (OCD) and/or attention deficit/hyperactivity disorder.

(ii) ACTS group: clinical diagnosis of Tourette Syndrome, according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5, APA 2013); the presence of Arnold Chiari type I malformation is based on brain magnetic resonance imaging (MRI), which measures the cranial malformation by cerebellar TP greater than 3-5 mm below the FM.

(iii) TS group: clinical diagnosis of Tourette Syndrome, according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5, APA 2013); the absence of other neurological or metabolic conditions such as AC syndrome.

Unaffected controls were considered neurologically intact children and adolescents, without any history of movement disorder, normal cerebellar tonsillar position and without chronic neurological, psychiatric, metabolic or genetic diseases. To avoid confounding factors, we did not include children with minor neuropsychiatric diseases (e.g., language delay/disorders or transient tic disorders that did not fulfill criteria for TS) or with minor neurological signs and we attempted in making the dataset uniform as soon as possible, based on important demographic factors (e.g., gender and age). Since the TS group was composed only by males, due to the disproportional frequency of young males affected by TS, we evaluated the gender differences as potential

confounding factor. With this aim, we quantified group differences in gender between the groups of comparison by applying the non-parametric two-tailed Mann–Whitney U test. Moreover, we explored the necessary conditions in order to consider gender as a potential confounding factor (i) through a binomial logistic regression test, to assess the potential effects of gender (continuous independent variable) on the likelihood that participants are affected by AC syndrome, TS, ACTS (dichotomous dependent variables), and (ii) by applying the Spearman correlation test, to evaluate the potential associations between the miRNA counts and the gender of each study participant.

4.5.4. Clinical and neuroimaging assessment

A medical history was obtained from all participants and their parents, with a focus on neurological and psychiatric conditions. Moreover, all participants underwent a physical and neurological examination and weight, height, head circumference were measured. Venous blood samples were collected. Patients and controls underwent a brain MRI with measurements of posterior fossa to evaluate the position of cerebellar tonsils.

T1 weighted sagittal brain MRI images were used. All measurements were taken for right and left herniation. Particular attention was paid to the measurements of the following areas: upper posterior cranial fossa, lower posterior cranial fossa and total posterior cranial fossa; finally, basal, Boogaard, occipital and tentorial angles were measured [168].

4.5.5. Neuropsychological assessment

All participants (AC, ACTS, TS patients and NCs) were assessed by child and adolescent neurologists and psychiatrists (RR; MG) with the following instruments:

(I) Wechsler scale (WISC-III), applied to evaluate Intelligence Quotient (IQ), is a test used to evaluate the intellectual ability of children with age range 6-16. To this aim, the

assessment provides full-scale IQ scores, as well as Performance IQ and Verbal IQ, two secondary scores [339].

(II) The Yale Global Tic Severity Scale (YGTSS) is an 11-item clinician-rated interview, which is able to evaluate motor and phonic tic severity, considering not only the number and frequency, but also the impairment that tics provoke in the patient. The score of the YGTSS has a range of 0-100, including the impairment section. Higher scores indicate higher severity of symptoms and impairment [142].

(III) The Children Yale Brown Obsessive Compulsive Scale (CY-BOCS) is a semi-structured interview, conducted principally with parents, even if patients are encouraged to participate. This interview is able to assess the severity of obsessive-compulsive symptoms in children. The total score of CY-BOCS ranges between 0 and 40. It is possible to evaluate an obsession and a compulsion score separately. Again, higher scores indicate higher severity of symptoms and impairments [388].

(IV) The Conners' Comprehensive Behavior Rating Scales (Conners) is a questionnaire that provides an overview of child and adolescent impairments. Conners is a multi-informant assessment of children and adolescents across multiple settings, with rating forms for parents, teachers, and patients. It is a validated, self- and proxy-rated (parent, teacher) scale used with 12-18-year-olds. It is used to diagnose ADHD and can allow discrimination between subtypes (e.g., predominantly inattentive/ hyperactive-impulsive) [144].

(V) The Child Behavior Checklist (CBCL) is a report form, which evaluates behavioral problems in children. The CBCL is a validated, parent-rated scale assessing the frequency and intensity of behavioral and emotional difficulties shown by a child over the preceding 6 months. It contains 8 syndrome scales (withdrawn, somatic complaints, anxious/depressed, social problems, thought problems, attention problems, delinquent

behavior, and aggressive behavior) and 2 composite scales (externalizing and internalizing problems) [389].

4.5.6. Sample collection and processing

Peripheral blood samples from all participants were collected in the morning through a butterfly device, inserted into serum separation collection tubes with Clot activator and gel for serum separation (BD Biosciences). Collection tubes were treated according to current procedures for clinical samples. Tubes were rotated end-over-end at 20°C for 30' to separate serum from blood cells. Subsequently, they were centrifuged at 3,500 rpm at 4°C for 15′ in a Beckman J-6M/E, supernatants were distributed into 1.5 ml RNase-free tubes, and finally stored at −80°C until analysis [271].

4.5.7. RNA extraction

Extraction of RNA was carried out from 800 µl of serum samples by using a Qiagen miRNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany), according to Qiagen Supplementary Protocol for purification of total RNA (including small RNAs) from serum and plasma [271]. RNA was eluted in 200 µl RNAse-free water and then precipitated by adding 20 µg glycogen, 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold 100% ethanol. After incubation at -80°C overnight, RNA was centrifuged, washed twice in ice-cold 75% ethanol and resuspended in 7 µl RNAse-free water. The yield and quality of the RNA samples were assessed by using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

4.5.8. MiRNA Profiling

Circulating miRNA expression profiling from serum was performed through the NanoString nCounter system assays by using nCounter Human v3 miRNA Expression Assay Kits (NanoString Technologies, Seattle, USA) and the NanoString platform, according to the manufacturer's

instructions. MiRNA profiling was performed on 3 µl (approximately 150 ng) of isolated RNA of 10 AC, 11 ACTS, 6 TS patients and 8 NCs. RNA samples were processed and immobilized in a sample cartridge for quantification and data collection by using the nCounter Prep Station and Digital Analyzer, respectively. Data analysis was performed using nSolver 3.0 software. Quality of raw data was assessed through an evaluation of imaging, binding density, positive control linearity and positive control limit of detection parameters. A code-set normalization was applied to minimize technical noise (variations in purification, binding, hybridization efficiency), according to the nSolver analysis software protocol. For each comparison, SAM (Significance of Microarrays Analysis) statistical analyses were computed with MeV (Multi experiment viewer v4.8.1) statistical analysis software. A two-class unpaired test, based on 100 permutations and with a FDR < 0.05, was computed. We obtained fold change (FC) values, calculating the ratio between the normalized count mean of each group. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [390] and are accessible through GEO Series accession number GSE146509 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE146509).

4.5.9. Correlation analysis

In order to investigate whether linear relationships could exist, correlation analyses were computed between the normalized counts of serum miRNAs and the neuropsychological and neurological scores of the study participants for each comparison.

The neuropsychological parameters chosen for this analysis were: TIQ; VIQ; PIQ; Total YGTSS; motor and phonic Yale Global Tic Severity Scale separately; Total CYBOCS; Children's Yale Brown Obsessive Compulsive Scale for obsession and compulsion, separately; Conners scale; Child Behavior Check list internalizing (CBCL-Int); Child Behavior Check list externalizing (CBCL-Ext); total Child Behavior Check list (Total CBCL).

Neurological parameters referred to measurements of the right and left protrusions (mm), upper posterior cranial fossa area, lower posterior cranial fossa area, total posterior cranial fossa and basal area, Boogaard, occipital and tentorial angles.

Correlation analyses were performed by GraphPad Prism for Windows v8.01 (GraphPad Software, La Jolla California USA). Since the data were not distributed normally, the Spearman correlation test was used, applying two-sided p-values. Statistical significance was established at $p \leq 0.05$.

4.5.10. Computational Analysis

To investigate the biological functions of all the DE miRNAs and their potential etiological involvement in AC syndrome and TS, the DIANA-mirPath v.3 web server [351] and miRNet tool [391] were used for functional enrichment analyses from KEGG (Kyoto Encyclopedia of Genes and Genomes) and Reactome gene annotation databases. A class-specific functional enrichment analysis was performed to separately consider miRNAs that were found DE for each pathological condition with respect to the NC group. Fisher's exact t-test with FDR correction and Hypergeometric test ($p \leq 0.05$) were used for enrichment analysis.

4.6. Supplementary Figures

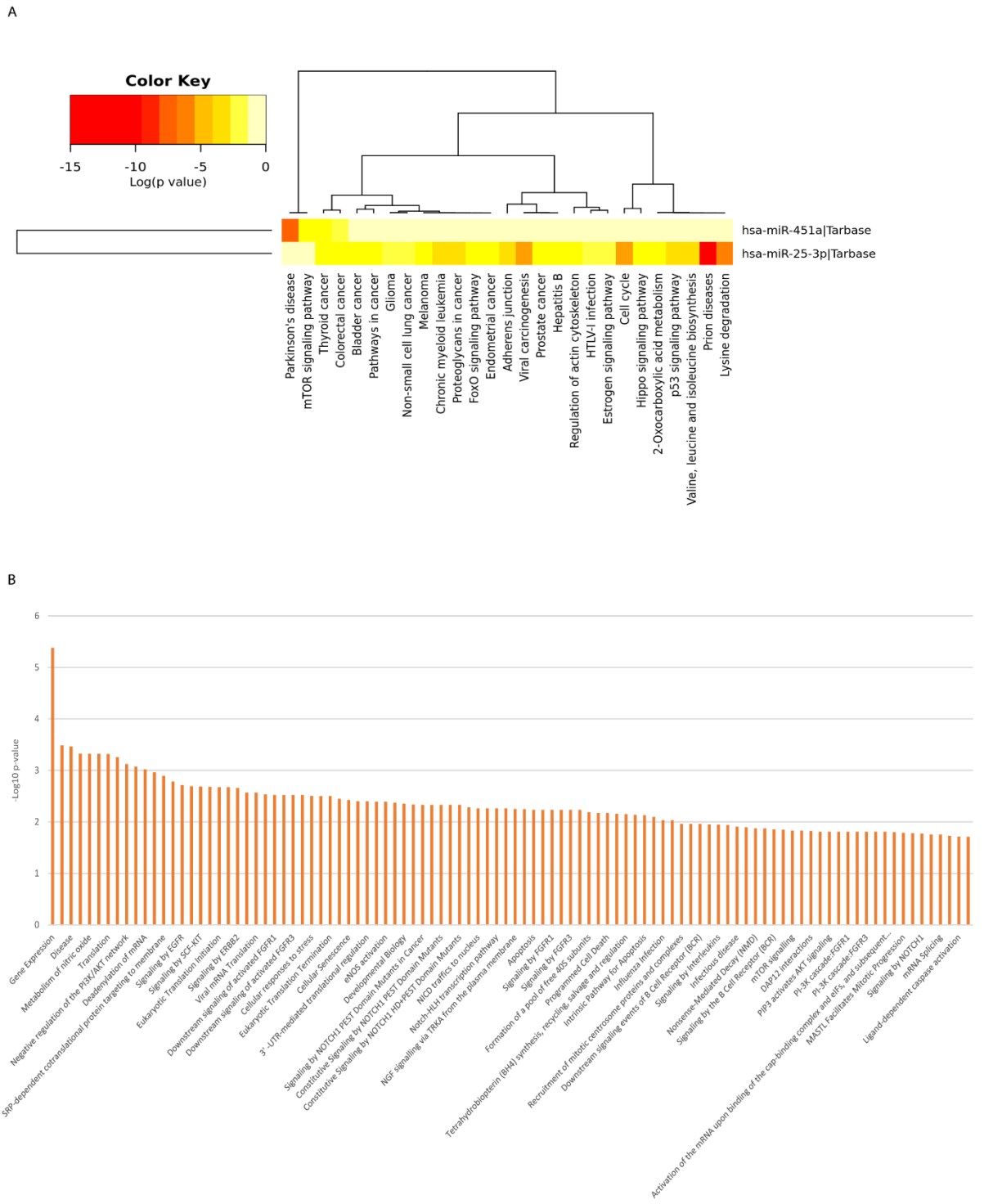


Figure S4.1. Functional Enrichment analysis of DE miRNAs in comparison among AC and NC groups. Functional enrichment analysis of DE miRNA targets using KEGG pathway (hierarchical clustering based on a complete linkage method and the significance levels of the interactions) (A) and Reactome databases (B) by DIANA-mirPath v.3 web server and the miRNet tool, respectively.

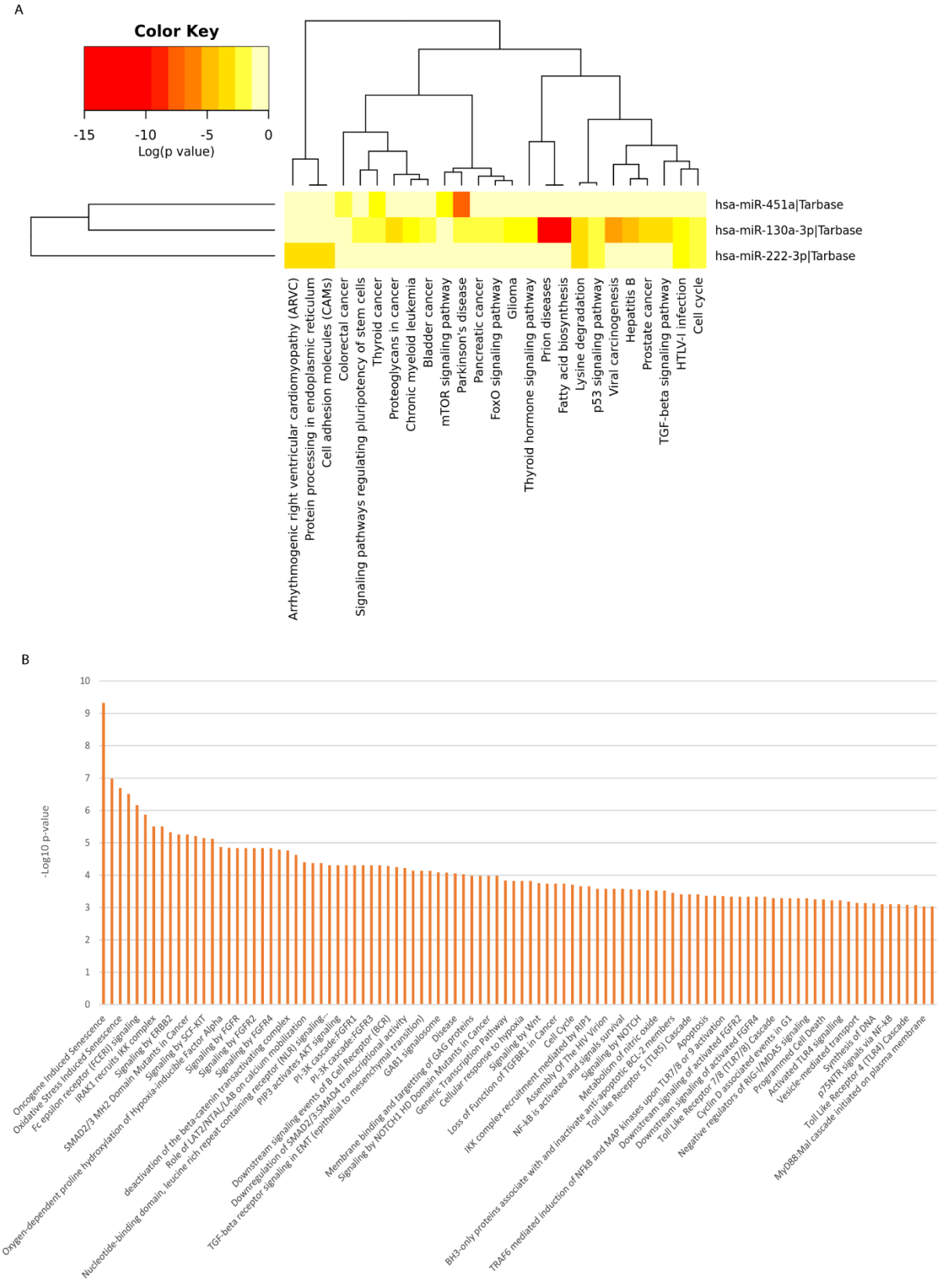


Figure S4.2. Functional Enrichment analysis of DE miRNAs in comparison among ACTS and NC groups. Functional enrichment analysis of DE miRNA targets using KEGG pathway (hierarchical clustering based on a complete linkage method and the significance levels of the interactions) (A) and Reactome databases (B) by DIANA-mirPath v.3 web server and the miRNet tool, respectively.

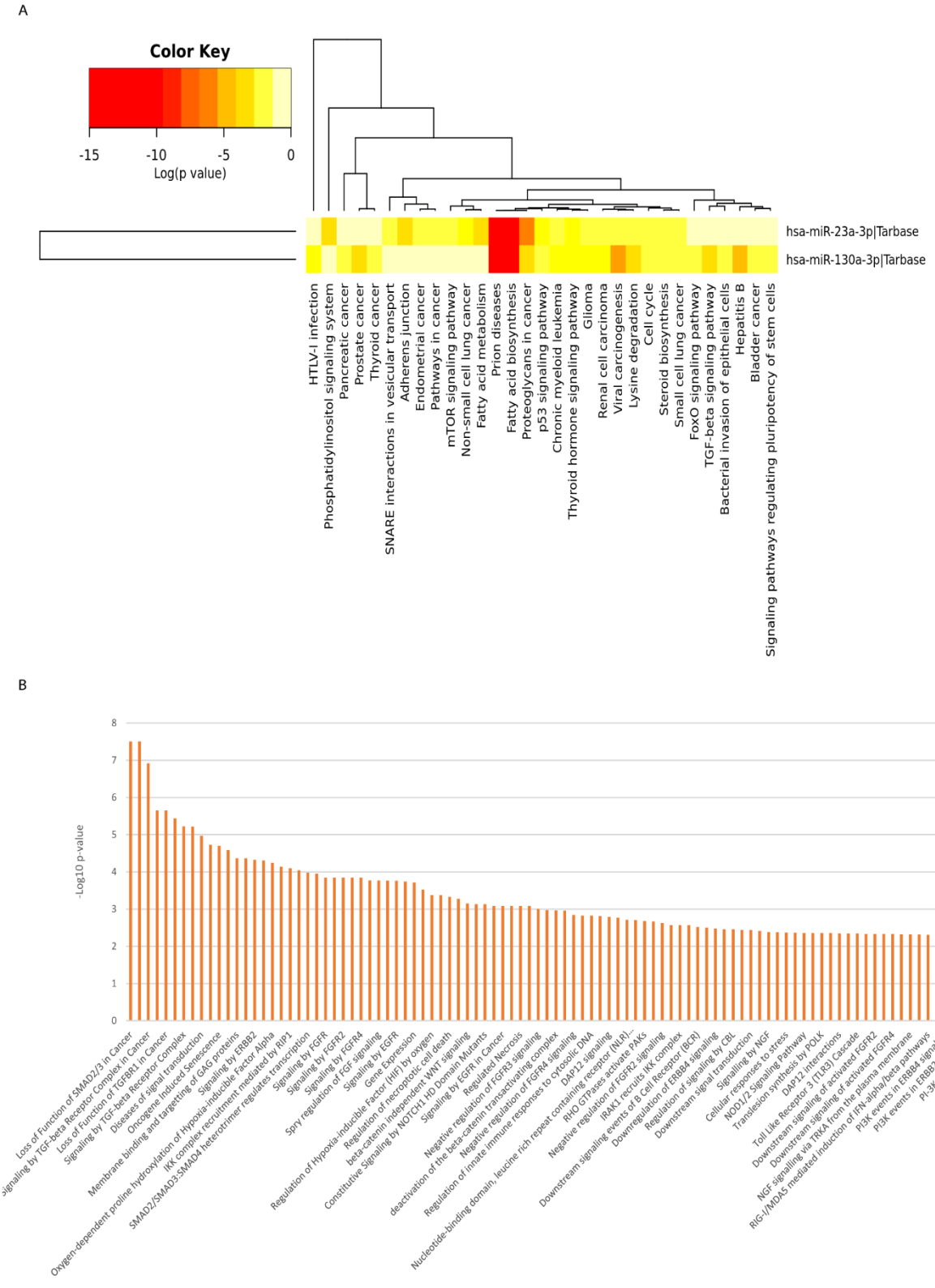


Figure S4.3. Functional Enrichment analysis of DE miRNAs in comparison among TS and NC groups. Functional enrichment analysis of DE miRNA targets using KEGG pathway (hierarchical clustering based on a complete linkage method and the significance levels of the interactions) (A) and Reactome databases (B) by DIANA-mirPath v.3 web server and the miRNet tool, respectively.

Chapter 5. Concluding remarks

The two research projects conducted during my PhD program profiled circulating miRNA expression (i) in saliva of ASD patients and (ii) in serum of AC syndrome, TS, and comorbid ACTS patients, compared to neurologically unaffected individuals. In our analyses, we chose biofluids as precious source of molecular biomarkers to further unveil new pathological mechanisms of neurodevelopmental disorders, such as ASD and TS, and neurological malformations, such as AC syndrome. Furthermore, the use of liquid biopsies could be extremely important to strengthen the clinical diagnostic processes, when tissue biopsies are not easily accessible and when delineating disorders is particularly challenging because of the complexity and heterogeneity of phenotypes. Saliva represents an important reservoir of molecules, including proteins and microRNAs, associated with the pathological development of neurological diseases and brain dysfunctions. The oral cavity is considered an important source of alterations that manifest at distant body sites, including the neural system.

Exploring the salivary environment, we were able to (i) identify dysregulated patterns of oral microbiome and miRnome in ASD children, (ii) test and propose the miRNAs and microbiome taxa identified as potential biomarkers to objectively support ASD diagnosis, (iii) assess the existence of molecular correlations between miRNAs, bacterial species and the typical cognitive impairments of ASD, as well as their involvement in ASD pathomechanisms.

To date, our ASD saliva project is the fourth high-throughput study that profiles circulating miRNAs and the fourth study which evaluates oral microbiome in saliva of ASD patients compared to neurotypical subjects.

The combined approach of miRNA expression profiling using NanoString technology, followed by validation experiments by TaqMan Single assays, and the 16S rRNA microbiome analysis, revealed 5 salivary miRNAs (miR-29a-3p, miR-141-3p, miR-16-5p, let-7b-5p and miR-451a) and 11 salivary bacterial species (*Rothia mucilaginosa, Filifactor, Actinobacillus parahaemolyticus*,

Weeksellaceae, Ralstonia, Pasteurellaceae, *Agregatibacter segnis*, *Haemophilus parainfluenzae, Tannerella, Moryella* and *TM7-3*) as potential molecular fingerprints for ASD.

Our experimental plan established a "discovery set" for NanoString analysis and an independent and larger "validation set" for the confirmatory study by single TaqMan assays, based on DE miRNAs previously identified. The validation step is crucial to overcome technical bias of high-throughput analysis and specifically detects the expression of selected miRNAs. However, we were unable to perform the same steps for the serum study, in which we designed only a "discovery set". In fact, due to its rarity, ACTS samples were numerically very limited and they were altogether employed for miRNA profiling. To date, a coexistence of both syndromes in the same individual (i.e., ACTS) has not been reported. For this reason, in our study we designed to select patients suffering from AC syndrome, TS and AC comorbid with TS, assessed by standardized approaches used for neuropsychological and neuroimaging examinations.

Through NanoString analysis, nine serum miRNAs resulted significantly differentially expressed (let-7b-5p, miR-21-5p, miR-23a-3p, miR-25-3p, miR-93-5p, miR-130a-3p, miR-144-3p, miR-222-3p and miR-451a) for each pairwise comparison among AC, TS, ACTS patients and neurologically unaffected individuals.

Validating the results of both studies in a larger independent cohort could confirm the diagnostic applicability and specificity of miRNAs as molecular biomarkers.

About the ASD study, ROC curve analysis and biomarker performance evaluation allowed to propose salivary miRNAs and microbial taxa as promising ASD biomarkers. In particular, the combination of all miRNAs and bacteria, significantly represented the best-fit performing model for ASD diagnosis. Correlational analysis results indicated that neuropsychological scores related to typical ASD cognitive impairments, especially for anomalies in social interaction and communication, were statistically associated to variations of salivary miRNAs.

Overall, it is possible to infer that salivary miRNA expression, in accordance with the clinical diagnostic evaluation, could support the standard diagnostic process of ASD.

In AC/TS study, TS-related cognitive impairments and AC typical cranial disproportions were found associated with most of miRNAs identified, suggesting that they may support the clinical diagnostic process, and, accordingly, be exploited as potential serum markers.

The functional enrichment analyses provided insight into converging molecular pathways and also confirmed the potential involvement of miRNAs in several biological processes related to ASD, AC syndrome and TS.

Finally, linear correlation analysis performed in ASD study led to the identification of a negative relationship between miR-141-3p and *Tannerella.* The significant relationship may suggest that the potential crosstalk between bacteria, resident in saliva, and miRNAs, secreted in the salivary microenvironment, could be based on a general response of host to dysbiosis involving several miRNA families, as well as bacterial phyla.

To date, only two studies examined the potential association between bacteria and microRNAs in oral cavity, one of which performed measurements of human and microbial salivary poly-omic RNA in the ASD framework. However, correlation analysis, performed in this study, identified only moderate associations which did not include microRNAs. The geographical, climatic and ethnic origin of samples, the different ratio of dietary protein/carbohydrate intake, the biochemical variability in saliva among people, as well as the different experimental technique used, would lead to a lack of concordance with our results.

Enlarging the cohort of ASD study would produce a larger amount of data from miRnome and microbiome, which will be more effective to gain further insight into the unveiled relationships between salivary bacteria and miRNAs. In addition, molecular and cellular characterization of miRNAs identified and *in vitro*, *in vivo* cross-talking between host and microbiota will contribute to clarify the heterogeneous molecular basis of ASD.

Our liquid biopsy-based approach aims to pave the way to new valuable, non-invasive molecular tools for ASD, TS and AC syndrome and to further studies on their role in the pathogenesis of such elusive disorders.

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