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Multi neuro-functional biomarkers for monitoring developmental trajectories in early onset psychopathology

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Vivere la vita è una cosa veramente grossa
C'è tutto il mondo tra la culla e la fossa
Sei partito da un piccolo porto
Dove la sete era tanta e il fiasco era corto
E adesso vivi.
Perché non avrai niente di meglio da fare
finché non sarai morto.
La vita è la più grande ubriacatura
Mentre stai bevendo intorno a te tutto gira
E incontri un sacco di gente
Ma quando passerà non ti ricorderai più niente
Ma non avere paura, qualcun altro si ricorderà di te
Ma la questione è: perché?
Perché ha qualcosa che gli hai regalato
Oppure avevi un debito e non l'hai pagato?
Non c'è cosa peggiore del talento sprecato
Non c'è cosa più triste di un padre che non ha amato...
[Vivere la vita, Mannarino]

A Cecilia

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Abstract

Most adult mental disorders have roots in childhood difficulties, and most childhood disorders have consequences in adult life (Pinto et al., 2015). Globally, up to 20% of children and adolescents suffer from a mental health problem (World Health Organization, 2000) and approximately half of them will persist through adulthood. When we consider the continuity of mental health problems, we must take into account that psychopathological manifestations seem to span along constructs, reflecting a continuous rather than a dichotomy between health and illness and between one diagnosis and another. "Internalizing" and "Externalizing" traits are well established to indicate class of symptoms and behaviors expressed trans-diagnostically. Developmental trajectories of internalizing and externalizing traits show a complex mix of continuities and discontinuities, and evidence both homotypic continuity—the persistence or recurrence of the same disorder (or trait) at different ages—and heterotypic continuity, where earlier and later vulnerabilities differ in presentation (Maughan and Collishaw, 2015). These possible heterotypic trajectories represent rather than a real change, a different expression of the same underlying causal factors, which interact between each other in different ways at different ages.

The aim of the present work is a better understanding of the pathogenesis and of the courses of psychopathological traits, in order to possibly identify children more in need of intervention. Understanding the different causes of psychopathology is the first step in providing a better intervention, which is an absolute priority in terms of social and economic costs. My thesis focused on the identification of psychopathological traits with *bottom-up* approaches, in order to evaluate the presence of internalizing and externalizing problems in developmental ages. Special attention has been paid to the presence of emotional/behavioral dysregulation, as a specific risk factor for worse psychopathological developmental trajectory. I was interested in evaluating the role of environmental and genetic factors in influencing these psychopathological traits. Moreover, I investigated the relevance of epigenetic variables. Lastly, I considered the development throughout the lifespan of psychopathological traits, in order to early identify individuals at higher risk of worse development.

To achieve the identification of clusters of symptoms that characterize individuals at higher risk for persistent mental health issues, two experimental studies, which are presented in

chapter 2, were conducted. In order to identify the presence of different psychopathological profiles, *bottom-up* person-centered approaches, without *a priori* conceptions, were used in very large and heterogeneous samples. In the first part of the chapter, the results of latent class analysis (LCA) performed on a sample including both general and referred children and adolescents are presented. To achieve the above-mentioned goal, we performed the LCA on the full spectrum of the psychopathological traits measured with a standardized screening questionnaire, the Child Behavior Checklist (CBCL/6–18, Achenbach and Rescorla, 2001). The results underline the presence of a profile characterized by severe emotional/behavioral dysregulation, which is associated with the presence of multiple categorical diagnoses. In the second part of chapter 2, the aim was to test the cross-societal generalizability of emotional/behavioral dysregulation profile. LCA was replicated, as a suitable method to understand this particular psychopathological trait apart from cultural differences, with data of 9238 subjects from 10 different societies. Results show that the presence of a "dysregulated class" is detectable in all the societies.

Subsequently, two experimental studies, which are reported in chapter 3, were conducted to evaluate the different exposures of individuals with higher psychopathology to previously identified genetic and environmental risk factors. The first study investigated the association between parents' internalizing/externalizing symptoms, parenting practices and children's internalizing/externalizing symptoms. Parent and child psychopathology were assessed with the Internalizing and Externalizing scales of the Adult Self Report (ASR, Achenbach and Rescorla, 2003) and CBCL/6–18, respectively. Results show that high levels of maternal pathology predict high levels of children's psychopathology and a total mediating effect of parenting measures within this relationship. The second study presented in chapter 3, using a proof-of-concept imaging genetics mediation design, investigated the genetic (specifically, GRIN2B gene variants) and neural determinants (specifically, cortical thickness, cortical surface area, and gray matter volume evaluated with structural magnetic resonance imaging) of attention and hyperactivity problems evaluated with CBCL/6–18. The mediation results show that GRIN2B variants effect on inattention/hyperactivity is partially mediated by volume in the left isthmus of the cingulate cortex, suggesting a key role of this region in translating glutamatergic GRIN2B variations to attention/hyperactivity problems.

The fourth chapter focuses on the role of gene-environment interaction in shaping psychopathological traits. With *machine learning* approaches clusters of symptoms that characterize individuals for internalizing and externalizing psychopathology during adolescence were identified. Subsequently, with a decision tree classifier, the role of

environmental and epigenetic variables in determining these different clusters were evaluated. The role of perinatal adversities and obstetric complications, stressful life events, and DNA methylation of candidate genes previously involved in psychopathology (i.e., BDNF, OXT, FKBP5, IGF2) was tested. Taking into account both internalizing and externalizing problems, two homogeneous clusters of psychopathology have been identified: "high" and "low". The variables which mostly discriminated between the two clusters is the presence of perinatal risk factors, followed by percentages of methylation in BDNF CpGs.

In chapter 5, a preliminary work on three-time points data is presented, with the aim of testing the presence of specific trajectories in internalizing and externalizing areas. Clusters of subjects presenting peculiar symptom trajectories in anxious/depressed, withdrawn, and somatic problems were identified, whilst internalizing and externalizing symptoms resulted in overall stability. Individuals belonging to clusters characterized by stable high presence of anxious, depressive and somatic problems, presented higher emotional/behavioral dysregulation during preadolescence, with higher internalizing and externalizing problems. This preliminary evidence suggest the importance for clinicians and researchers to account for both homotypic and heterotypic continuity of psychopathological traits when planning interventions of treatment and prevention.

Chapter 1
Introduction: psychopathological developmental traits

1.1 Multifactoriality and complexity

Historically, mental health has not been seen as a priority in global health politics, especially when compared with communicable diseases and non-communicable diseases (Whiteford et al., 2013). Psychiatric disorders always suffered from the fact that the impact of disorders were measured as mortality. In the last 20 years, the focus moved and new findings highlighted that mental health problems are a worldwide major burden in terms of suffering and public health costs. In fact, mental and substance use disorders, which often originate during childhood and adolescence, are the first leading cause of all non-fatal burden of disease as measured with years lived with disability (YLD) and the fifth causes of disability-adjusted life years (DALYs), which combines premature mortality as years of life lost (YLLs) and disability as YLDs (Whiteford et al., 2013).

So far, despite this clear burden of mental disorders, research has not led to complete understanding of psychopathology. This situation causes a vicious circle into which possible mis-conceptualization might cause worse prognosis, firstly due to misdiagnosis, non-optimal rehabilitation treatments, and unpersonalized interventions. The final results of this chain unfortunately are increased individual suffering and higher social and economic costs (Nees et al., 2021).

It is undeniable that the nature of the research object is partially responsible for this lack: when referring to complexity of mental disorders, we must take into account their multifactorial etiology, their different manifestation and their possible developmental trajectories.

1.1.1 Multifactorial etiopathology theory: genes, environment and their interaction

Talking about etiological complexity, the multifactorial etiology thesis aims to integrate social and psychological with biological and neural aspects when working on diagnostic conceptualizations, treatment, and prevention of mental disorders.

It is well established the need to account for genetic and environmental risk and resilience factors when trying to understand mental disorders (Hirschhorn et al., 2002, Nees et al., 2021, Pinto et al., 2015, State and Thapar, 2015).

Twin studies, the most traditional approach in the study of etiopathogenesis, enable researchers to understand the contribution of genetic and environmental factors in causing specific phenotypes. These studies suggest that the biological-genetic risk factors, measured as the overall genetic contribution or the heritability, are responsible for continuity in mental health problems, but also that the environmental contribution is essential in the explanation of changes over time (Nees et al., 2021): literature identifies that before the age of 5, the influence of the environment is even more important than genetics.

The study of the biological contributions to the development of psychopathology is a continuous "come and go" from behavioral genetics, with the methodologies of family, twin, and adoption studies, to molecular genetics.

Molecular genetics firstly approached the study of psychiatric disorders with linkage studies. These kink of studies are used to examine phenotypes that follow the rules of Mendelian inheritance: they are based on the idea that the position within the genome of an unknown disease mutation may be found by examining the transmission of known genetic variations (or markers) in an affected family and group of families (State and Thapar, 2015). Anyway, the majority of mental disorders has been found to be the result of complex inheritance, involving many genes (and environmental factors), rather than one or a small number of them, demonstrating a probabilistic relationship between having a mutation and any given phenotypic outcome (State and Thapar, 2015). It is highly probable that common genetic variants, including single nucleotide polymorphisms (SNPs), influence susceptibility to diseases (Hirschhorn et al., 2002).

This gave way to a dramatic increase in the number of studies using the candidate gene approach, which initially involved one or a small number of common variations or SNPs, in genes selected on the basis of biological plausibility. Another important methodology involves the detection of copy number variations, which often accumulate at particular regions and genes in affected individuals. Recently, the majority of the studies evaluates a large number of candidate genes (State and Thapar, 2015). In psychiatry they include multiple genes encoding components of neurotransmitter systems: BDNF, COMT, DRD4, GRIN2B, IGF2, MAOA, OXTR, SLC6A4, TPH2 (Hirschhorn et al., 2002, Nobile et al., 2007, 2009, 2010, 2016, 2021; Riva et al., 2015(a), 2015(b), State and Thapar, 2015, Zohsel et al., 2015), which are just a small part of all the genes studied in psychiatry, have been demonstrated to be involved in brain development and neurotransmission.

Even though candidate genes studies are essential in psychiatric research, it is important to note that they suffer from lack of reproducibility, mostly because often the genetic effect is real but weak, so not detectable due to small sample size. In other cases, this is because of false positives due to population stratification in case-control studies or to differentiate linkage disequilibrium of the same polymorphism in different populations (Hirschhorn et al., 2002). Genome-wide association studies, which focus on the contribution of common variation to common psychiatric disorders, but take a hypothesis-free approach, helped with these limits.

Another potential source of variable findings of these studies is gene–environment interaction. This occurs when the relation between environmental risk or resilience factors and a phenotype is conditional on individual differences in genetics, or, on the other side, when the relation between individual genotype and a phenotype is conditional on environmental experiences (Pinto et al., 2015). The interplay between genes and environment is essentially of two types: gene-environment correlation and gene-environment interaction. Environmental risks are not randomly allocated, they are mostly shaped by genetic influenced dispositions and behaviors. In this case we are facing what is known as gene-environment correlation. The gene-environment interaction is present in all the cases when a specific phenotype, of which genes are responsible for susceptibility, presents only at specific environmental conditions. So, the literature has been focusing on variations in DNA that can lead to different outcomes of environmental experience in determining the development of psychopathology (State and Thapar, 2015, Pinto et al., 2015).

In the category of the environmental risk factors, they are listed a wide range of different events and adversities, which the individual may face at very different points in life, from prebirth period to adolescence. Frequently, the association between an environmental risk (e.g., maternal perinatal anxiety and/or depression or low socioeconomic status - SES) and poor child and adolescent mental health is not linked to the presence of a specific disorder in offspring.

Environmental risk factors comprehends pre and perinatal experiences, which have been demonstrated might have a long term impact on development of psychopathological traits liability (Rutter and Azis-Clauson, 2015). It is important to note that often environmental risks are interdependent between each other.

Exposure to maternal alcohol abuse, smoking, and drugs during pregnancy have been associated with impaired fetal growth, low birth weight, neural alterations, craniofacial

malformations, lower intelligence quotient (IQ), and presence of psychopathology, but, because it is impossible to isolate the effect of this kind of risks in natural experiments, the effects of confounding factors cannot be controlled and the risk factors remain non-specific (Rutter and Azis-Clauson, 2015).

There is evidence also of an effect of prenatal maternal psychopathology or exposure to stress on psychopathology of children. The experience of stress during pregnancy is associated with increased preterm birth, reduced birth weight, and smaller head circumference (Peña et al., 2012). Depression during pregnancy, which is also a significant risk factor for postpartum depression (i.e., another environmental risk for the child), is associated with a significantly lower IQ and with a trend of higher rates of problematic behaviors (Nulman et al., 2012). Moreover, maternal antenatal trait anxiety is associated with internalizing problems in toddlers (Frigerio et al., 2021). In order to enlighten the complexity under these results, it is essential to note that the rate of maternal depression and anxiety tends to be increased in the context of poverty and disadvantage (i.e., again, another environmental risk for the child).

Pregnancy-related and birth complications, such as preeclampsia, maternal obesity, maternal diabetes, maternal hypertensive disorder, maternal infection/inflammation, maternal exposure to heavy metals or other neurotoxicants, cesarean section, perinatal hypoxia, preterm birth, have been associated with increased risk of psychopathological behaviors and disorders in children (Giannopoulou et al., 2018, Modabbernia et al., 2017, Monk et al., 2019, Tien et al., 2020).

During the first days of life the infant brain is extremely sensitive to the environment. As an example of a stressful environment we might consider the experience of neonatal intensive care units (NICU). The NICU, even in absence of medical complications, might be a source of enormous distress for the infants because of physical and sensorial stimulations, painful procedures and maternal separation. This experience has been associated with long-lasting effects on the development of stress regulation systems in preterm infants (Provenzi et al., 2018).

Later in life stressful experiences continue to have a great impact on mental health: adverse childhood experiences (ACEs) have been widely associated with long term impact on individuals physical and mental health, with a significantly increased risk for sexual risk taking behaviors, mental health problems, problematic alcohol use, drug use and interpersonal and self-directed violence (Hughes et al., 2017). ACEs include childhood physical or sexual abuses, household of substance abuses, criminality or mental illnesses, exposure to domestic violence, emotional, psychological or verbal violence, divorce or

separation of parents, neglect, familiar financial problems or conflicts, bullying, death of a parent or a close relative or friend, separation from family, serious childhood illness or injury.

Hypothalamic-pituitary-adrenal (HPA) axis, inflammation and epigenetics are different mechanisms that explain how environmental experiences, which an individual may have faced pre birth as well as during childhood, might influence the development of psychopathology later in life.

HPA is central in stress response: it secretes glucocorticoids, including cortisol, which alerts the organism to potential threats and activates the sympathetic nervous system that enables adaptation to environmental demands. HPA abnormal functioning is associated with pathogenetic mechanisms; in fact, overactivation of these mechanisms, in case of chronic stress, traumatic experiences or ineffective coping, might trigger systemic biological responses that exert deleterious effects (Misiak et al., 2020). High circulating glucocorticoids during pregnancy may alter placenta functioning, reach the fetus and alter offspring's HPA circuitry and brain development, increasing risk of psychopathology (Monk et al., 2019). Given that the gut microbiome has a significant role in psychopathology, it is important to note that HPA is a component of the "gut-brain axis" (Misiak et al., 2020). The brain developing demands high metabolic exertions, so the microbial may influence its maturation and potentially the risk for mental disorders (Monk et al., 2019). Maternal stress during pregnancy may alter maternal microbiome and, as a consequence, alter offspring microbiome, which is known to be linked to maternal one (Monk et al., 2019).

Immune activation and inflammation impact on psychopathological development through cytokines, which are involved in dendritic growth and neuronal survival and differentiation (Monk et al., 2019). Maternal infection and cytokines may impact the fetus affecting placenta and creating fetus immune dysregulations (Monk et al., 2019).

The study of epigenetic mechanisms is another way in which it is possible to evaluate the effect of the interaction between environment and heritable traits. In fact, through epigenetic mechanisms, environmental events influence modulation of DNA transcriptional activity. Epigenetics is defined as chemical modifications of DNA, or of the structural regulatory proteins bound to it, that alter DNA transcription and protein production, without changing nucleotide sequences (Provenzi et al., 2018). There are several examples of such modifications including, but not limited to, acetylation, phosphorylation, methylation and ubiquitination. Epigenetic mechanisms regulate normal brain function, and histone modifications, along with other forms of chromatin remodeling, have been linked to neural

plasticity and multiple forms of behavioral memory (Maze et al., 2013). Mutations in epigenetic regulators can alter chromatin structure and DNA transcription and induce a broad spectrum of neurological and behavioral deficits (Maze et al., 2013).

DNA methylation, which is also responsible for cell differentiation, so it is extremely important in pre-birth period, is the most studied of these mechanisms in psychopathology: it works through the binding of a methyl group to specific 5′-cytosine guanine-3′ dinucleotides (i.e., CpG sites) (Provenzi et al., 2018). Stability is an important feature of histone methylation; unlike acetylation, methylation marks can persist and have been shown to directly mediate the stable influence of environmental signals on gene transcription (Meaney and O'Donnell, 2015).

The presence of altered methylation profiles, as a consequence of environmental events, has been seen in both animal and human studies. Animal studies found that environmental manipulations are associated with long term effects in behaviors, in particular the ones related to stress responses, and with methylation alterations which are associated with alterations in neurotransmitter functioning and structural brain changes (Champagne et al. 2008, Meaney 2010, Stevens et al. 2009).

These results have been also replicate in human studies: there is a consistent amount of studies that documented altered DNA methylation of various genes in individuals exposed to stressful adverse experiences during prenatal (e.g., maternal depression) and postnatal life (e.g., childhood abuse) (Beach et al., 2011, Maud et al., 2018, Provenzi et al., 2018, Thaler 2014).

1.1.2 Transdiagnostic psychopathological traits

Given the above mentioned complex multifactorial ethology, it is not surprising that the manifestation of psychopathology is often a complex phenotype that might appear different if studied from different perspectives.

Categorical diagnoses are constructs researchers and clinicians use to navigate phenotypic complexity. In fact, diagnostic labels allow professionals of mental health care to communicate the essential characteristics of mental disorders presented by their patients. For example, the diagnostic criteria proposed by the American Psychiatric Association for mental health disorders, collected in the Diagnostic and Statistical Manual of Mental Disorders (DSM) are concise and explicit. These characteristics allow medicians and psychologists to

assess symptom presentations as objectively as possible, in a variety of clinical settings and in general community epidemiological studies of mental disorders (APA 2013 - DSM 5).

It is therefore also true that a wide range, rather unspecific, pattern of psychopathological manifestations can be associated with a specific diagnosis of mental disorder. For example, for a diagnosis of attention deficit hyperactivity disorder (ADHD), the most common neurodevelopmental disorder, there is the necessity to meet 6 out of 18 possible hyperactivity and/or inattention manifestations (APA 2013 - DSM 5). It introduces high heterogeneity between individuals within the same diagnosis, without an univocal association between manifestation and disorder (Nees et al., 2021). One of the possible way to parse this heterogeneity is to find specific markers of mental disorders, but the results are still lacking; taking into consideration ADHD again and remaining onto a behavioral level, different associations have been found between different neuropsychological responses and ADHD (Mauri et al., 2017,2020; Pievsky and McGrath, 2017), and for this reason different studies tried to find a neuropsychological biomarker of ADHD, but results are not always consistent and the association is not exclusive (Pievsky and McGrath, 2017). Therefore, the presence of peculiarities in neuropsychological responses is not a diagnostic criterion (Thapar and Cooper, 2015). If we move to bio-markers for specific psychiatric diagnosis, the results are similar. For example, abnormal functioning of dorsolateral prefrontal cortex, which have been linked to deficits in sustained attention performances, have been found in different mental disorders concerning executive functions deficit, including schizophrenia, mania, depression, anxiety and ADHD (Cortese, 2012).

1.1.2.1 Internalizing and externalizing problems

Psychopathological manifestations seem therefore to span along constructs, reflecting a continuous rather than a dichotomy between health and illness and between one diagnosis and another. Such distinctions have been proposed by the Research Domain Criteria (RDoC) Project, an initiative of the National Institute of Mental Health. Specifically, the RDoC project suggests to base the classification of mental disorders on dimensions of observable behavior and neurobiological measures rather than on symptom-based descriptive categorical diagnoses.

For the same reasons, much research focuses on psychopathological traits rather than on psychiatric categorical diagnosis. In neurodevelopmental psychopathology, the use of the terms “internalizing” and “externalizing” traits is well established to indicate class of

symptoms and behaviors which may be found associated trans-diagnostically with psychopathology.

Internalizing problems refer to inwardly focused negative behaviors such as anxiety, depression, and somatic symptoms, while externalizing problems refer to outwardly focused negative behaviors such as hyperactivity, aggression, disruptive conduct, and substance use (Achenbach and Rescorla, 2001, Tien et al., 2020). There are high rates of overlap between internalizing and externalizing problems. Very recent data from the longitudinal New Zealand Dunedin Multidisciplinary Health and Development Study, for example, show that less than 15% of participating individuals diagnosed with externalizing or internalizing disorders showed only homotypic symptomatology (Caspi et al., 2020).

Internalizing and externalizing behaviors during childhood are predictive of later negative adolescent and adult behavioral, emotional, cognitive, and physical health outcomes, including increased risk for aggression and violence, substance abuse, depression, anxiety disorders, lowered academic competence, and increased long-term mortality risk in adulthood (Tien et al., 2020).

Achenbach System of Empirically Based Assessment (ASEBA) provides multicultural norms and standardized instruments to assess psychopathological traits through lifespan (Achenbach, 2019). The "Child Behavior Checklist/6-18" (CBCL/6-18) (Achenbach and Rescorla, 2001), an empirically based checklist of social competence and behavioral problems filled out by parents, enables the assessment of children between the ages of 6 and 18. CBCL/6-18 lists 138 items to assess children's and adolescents' school and social competences as well as behavioral and emotional problems. It is possible to interpret the results of CBCL/6–18 considering a profile composed of eight syndrome scales (i.e., anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, aggressive behavior, rule-breaking behavior) or analyzing two major factors: the Internalizing scale (which takes into account the scores of anxious/depressed, withdrawn/depressed and somatic complaints subscales) and the Externalizing scale (which takes into account the scores of rule breaking and aggressive behavior subscales) (Achenbach and Rescorla, 2001).

1.1.2.2 Emotional/behavioral dysregulation

In the literature, children with comorbid internalizing and externalizing problems have been found to be often characterized by the presence of emotional/behavioral dysregulation, a

transdiagnostic psychopathological trait. This dysregulation has been described for the first time by Biederman and colleagues in 1995 (Biederman et al., 1995).

Response inhibition deficit refers to three interrelated processes: i) a difficulty to inhibit an initial prepotent response (i.e., a response associated with immediate reinforcement); ii) an inability to stop an ongoing response, a skill which usually enables a delay in decision process; iii) an incapacity to remain focused and to operate interference control.

In addition to the behavioral domain, impairment in response inhibition also affects the ability to control emotions, arousal, and self-regulation (Barkley, 1997). In daily living, emotional self-regulation includes the individual ability to identify and properly interpret environmental emotional stimuli, to recognize individual self-emotions, and to deal with them, generating appropriate behavioral social responses (Doumond et al., 2019).

Emotional/behavioral dysregulation (ED) is a transdiagnostic psychopathological trait which is frequently associated with poor outcomes of mental health problems and with the presence of different comorbidities within both externalizing (e.g., oppositional defiant disorder) and internalizing (e.g., depression, dysthymia) areas (Shaw et al., 2014; Wang et al., 2018).

ED refers to deficits in physiological arousal regulation and in inhibition of disruptive behavioral response to emotions, an inability to refocus attention after a strong emotional feeling and to have goal-directed behaviors after emotional activation (Biederman et al., 2012). Therefore, ED reflects both failure of cognitive control and high intensity of arousal (Soloff et al., 2015). These deficits cause higher sensitivity to emotional arousal, slower return to baseline activation, and deficits in coping strategies. Especially in children and adolescents, ED is responsible for low tolerance to frustration, impatience, easy anger and excessive emotional excitement (Biederman et al., 2012).

In literature, one of the most used instruments to evaluate the presence of ED is the CBCL/6-18, through the CBCL-Dysregulation Profile (CBCL-DP). This profile is characterized by co-occurring elevations in anxious/depressed, attention problems, and aggressive behaviors CBCL subscales. Several studies have demonstrated that the DP, rather than being a predictor of a specific disorder, is a marker for persistent psychopathology and significant impairment, as well as personality pathology, suicidality, and substance use (Rescorla et al., 2020). The majority of these studies uses an a priori identified profile that sums the T-score of the three scales, but in recent years, the number of the studies which are using person-centered statistical approaches, such as Latent class analysis (LCA), is increasing. Rather than pre-selecting specific items or subscales, LCA is a bottom-up method

in which each participant is assigned to a specific class depending on how they respond to items or scales of a questionnaire. Each class has a specific profile of responses (Bianchi et al., 2021). Several studies using LCA have identified a dysregulated class (DYS) among infants, children, and adolescents from different countries (Bianchi et al., 2017; Bianchi et al., 2022).

1.1.3 Developmental perspective: psychopathological longitudinal trajectories

The majority of adult disorders have roots in childhood difficulties, and most childhood disorders have consequences in adult life. The study of mental health problems from a developmental and longitudinal point of view has several advantages. In fact, developmental findings are able to highlight connections across the life course and parse the complexity regarding multifactoriality of etiopathogenesis. Moreover, a longitudinal perspective permits to better account for the role of biological, psychological, and social mechanisms underlying continuity and changes in psychopathological traits manifestations.

The results of different longitudinal studies have shown that some psychopathological symptoms which are present at an early age, could last throughout development into adulthood (Pinto et al., 2015). Results of studies evaluating internalizing and externalizing during childhood evidence that they predict the presence of psychopathological traits and categorical diagnosis later in life (Hofstra et al., 2000, Hofstra et al., 2002). Some individuals have constantly high externalizing symptoms, while intermediate groups of individuals shift up or down slowly or rapidly. Similar patterns were observed for internalizing symptoms (Nees et al., 2021). Generally, externalizing behavior tends to be more stable than internalizing manifestations (Blok et al., 2021). Globally, up to 20% of children and adolescents suffer from a mental health problem (World Health Organization, 2000), and, even if the specific rate differs across different psychopathological traits and diagnoses, approximately half of them will persist through adulthood. For example, meta-analysis of longitudinal follow-up studies of children with ADHD suggests that at least 15%, up to a 80% of them, continue to meet full diagnostic criteria for ADHD into young adulthood (Franke et al., 2018). Interestingly, it is likely that during childhood, in children diagnosed with ADHD, there are more externalizing problems, while during adolescence and adulthood, also internalizing problems are present.

ADHD trajectory show that a developmental point of view may highlight complex mixes of continuities and discontinuities, and evidence of both homotypic continuity—the persistence

or recurrence of the same disorder (or trait) at different ages—and heterotypic continuity, where earlier and later vulnerabilities differ in presentation (Maughan and Collishaw, 2015). These possible heterotypic trajectories represent rather than a real change, a different expression of the same underlying causal factors, which interact between each other in different ways at different ages.

Many longitudinal studies used ASEBA questionnaires in order to assess psychopathological traits through time (Achenbach, 2019). ASEBA forms are particularly suited to research that requires re-assessments across long periods because they include developmentally appropriate items, scales, constructs, and norms for ages 1½–90 + years, so the same individuals can be repeatedly assessed through successive developmental periods. Moreover, ASEBA questionnaires are standardized across developmental periods and this facilitates statistical analyses for identifying continuities and changes in individuals' functioning as they develop.

In the light of these premises, it is essential to enhance our understanding of the multifactoriality of the mechanisms underlying psychopathology. This may help parsing psychopathological traits heterogeneity and early identifying individuals at higher risk of worse prognosis. A better conceptualization and an early identification may lead to higher efficacy and timeliness of interventions and therapies. In the final instance, these represent a crucial issue for the reduction of social and economic burden which psychopathology represents.

1.2 ReMIND project

My thesis "*Multi neuro-functional biomarkers for monitoring developmental trajectories in early onset psychopathology*" work is part of a larger longitudinal project called "*ReMIND - REal Matters IN Developmental psychopathology. A 15 years follow-up study of risk and resilience factors and outcomes from childhood to adulthood*", founded by the Italian Ministry of Health, which takes place at IRCCS Eugenio Medea - Associazione La Nostra Famiglia in Bosisio Parini (Lecco).

The ReMIND project's primary aims are to achieve a better understanding of the heterogeneity of neuropsychiatric conditions and to identify and characterize children which are most in need of intervention.

Specific aims of the project are:

1) Identify developmental trajectories of internalizing and externalizing problems from childhood towards early adulthood. Specific attention is given to the roles of multifactorial risk as resilience factors (e.g., genetic makeup, environment, life events, epigenetic contribution) in shaping these developmental trajectories.
2) Integrate developmental emotional and behavioral data with neuroendocrine and multimodal neurocognitive data. Specific attention is given to changes in brain organization during puberty and to brain imaging measures more sensitive to individual differences due to stress.
3) Develop algorithms for early identification and appropriate preventive and therapeutic interventions towards adult life. The project aims also to implement diagnostic guidelines and treatment protocols for youth emotional-behavioral problems.

To reach the above-mentioned goals, ReMIND project recruits young adults which have already been evaluated during childhood and adolescence throughout a 20 years of age range (2003-2023).

1.2.1 ReMIND: study design and sample

The sample of ReMIND project is composed of two different subsamples: individuals belonging to general population recruited during the "*Italian preadolescent mental health project - PrISMA*" (*Progetto Italiano Salute Mentale Adolescenti*) study (Frigerio et al., 2006, 2009) and help seeking for behavioral and emotional problems individuals recruited during the CABALA and successively the GENESIS projects (Bellina et al., 2013, Bianchi et al., 2017).

In the following sections, specific characteristics regarding recruitment and evaluations of these two unique subsamples are reported. A specific paragraph is reserved to ASEBA instruments, through which the majority of the emotional and behavioral data subsequently presented are collected (see paragraphs 1.1.2.1 and 1.2.2.2)

1.2.2 Wave 1 and wave 2

1.2.2.1 General Population Subsample

At the first evaluation, the PrISMA sample was composed of Italian preadolescents, aged 10–14 years, attending secondary school (6th–8th grade) and living in seven urban areas,

including two metropolitan areas (the cities of Rome and Milan) and five small- to average-sized urban areas. This evaluation started in 2003. This first evaluation consisted of two different phases. In the first phase, 3418 subjects completed the screening phase (Phase 1) which consisted in the filling of CBCL/6-18 questionnaires. Phase 1 participants were stratified according to whether they exceeded the 90th percentile of the internalizing and/or the externalizing scales of the CBCL/6-18. Phase 2 was proposed to all screen-positive participants (N=661) and to a 10% random sample of screen–negative participants (N=311). The phase 2 sample was composed of 631 subjects who completed the diagnostic assessment (451 from the positive screened subgroup, 180 from the negative screened subgroup) with the administration of semi structured interview "Development and well being assessment - DAWBA" (Goodman et al., 2000).

The second wave evaluation started in 2008 (Bianchi et al., 2017, Nobile et al., 2013). Of the 1205 possible candidates identified within children that were assessed during wave 1, 420 subjects (aged 15–19 years) accepted to participate. In this project phase, socio-demographic data (including socio-economic status - SES, parent years of education, family structure), clinical evaluation were recollected as described above. Moreover, neuropsychological evaluation, environmental risk factors evaluation (SLE, perinatal adversities), genetic evaluation were performed.

Birth assigned sex (BAS) and age distributions are reported in Table 1.1

Table 1.1: General population subsample characteristics

	wave 1 - phase 1 (N = 3418)	wave 1 - phase 2 (N = 631)	wave 2 (N = 420)
BAS (males:females) (N)	1695:1723	290:341	207:213
Age (mean ± SD) (years)	12.08 ± 0.90	12.16 ± 0.91	17.71 ± 0.93

Note: BAS = birth assigned sex; SD = standard deviation

1.2.2.2 Clinical Subsample

First evaluation of clinical sample started recruiting, between January 2003 and December 2008, children that were referred, for the first time, for mental health problems to the Child

Psychiatry Unit of Eugenio Medea Scientific Institute - Associazione La Nostra Famiglia in Bosisio Parini (LC), Conegliano Veneto (TV), Pasian di Prato (UD), and San Vito al Tagliamento (PN). At wave 1, the sample included 1225 subjects (aged 6–17 years). Presence of psychiatric and neurodevelopmental diagnosis were evaluated with "Kiddie schedule for affective disorders and schizophrenia for school age children—present and lifetime version" semi-structured interview (Kaufman et al., 1997). Furthermore, parents were asked to fill in the CBCL/6–18, and total IQ was assessed with the Wechsler intelligence scale for children-revised (WISC-R, Wechsler, 1986).

During the second wave evaluation of the clinical sample, which has been conducted 5.35 ± 1.6 years after the first one, 331 subjects were recruited. The adolescents and their parents were characterized for emotional behavioral characteristics with ASEBA questionnaires. Moreover, socio-demographic data (including socio-economic status - SES, parent years of education, family structure), neuroimaging (with functional and structural magnetic resonance imaging), neuropsychological, and genetic and epigenetic evaluations were conducted. Also in this case, environmental risk factors (i.e., SLEs and obstetric complications) were collected.

Birth assigned sex assigned at birth and age distributions are reported in Table 1.2.

Table 1.2: Clinical subsample characteristics

	wave 1 (N = 1225)	wave 2 (N = 331)
BAS (males:females) (N)	942:283	252:79
Age (mean ± SD) (years)	9.11 ± 2.34	14.52 ± 2.41

Note: BAS = birth assigned sex; SD = standard deviation

1.2.3 Third wave evaluation

The third evaluation of these subsamples was therefore foreseen within the ReMIND project, which started in December 2018. The project has been approved by Eugenio Medea IRCCS -

Associazione La Nostra Famiglia (Bosisio Parini, LC) ethics committee and written informed consent and assent were obtained from all participants.

In primis, the selection of assessment instruments has been done. The third evaluation is a multimodal characterization of participants, in which psychological-behavioral profiles, environmental informations, neuropsychological measures, epigenetic data, neuroimaging evaluation have been reassessed and collected again in the above described subsamples.

1.2.3.1 Participants

296 subjects belonging to the general population sample and 103 belonging to the Bosisio Parini clinical sample have been invited by mail and/or by telephone to participate in the study. 113 subjects accepted to take part in the research project. Figure 1.1 and 1.2 illustrate the subjects' flow, within the planned multimodal assessments, at September 2022. The recruitment is ongoing and the ReMIND project ends in December 2023.

Figure 1.1: General population sample third wave recruitment flowchart

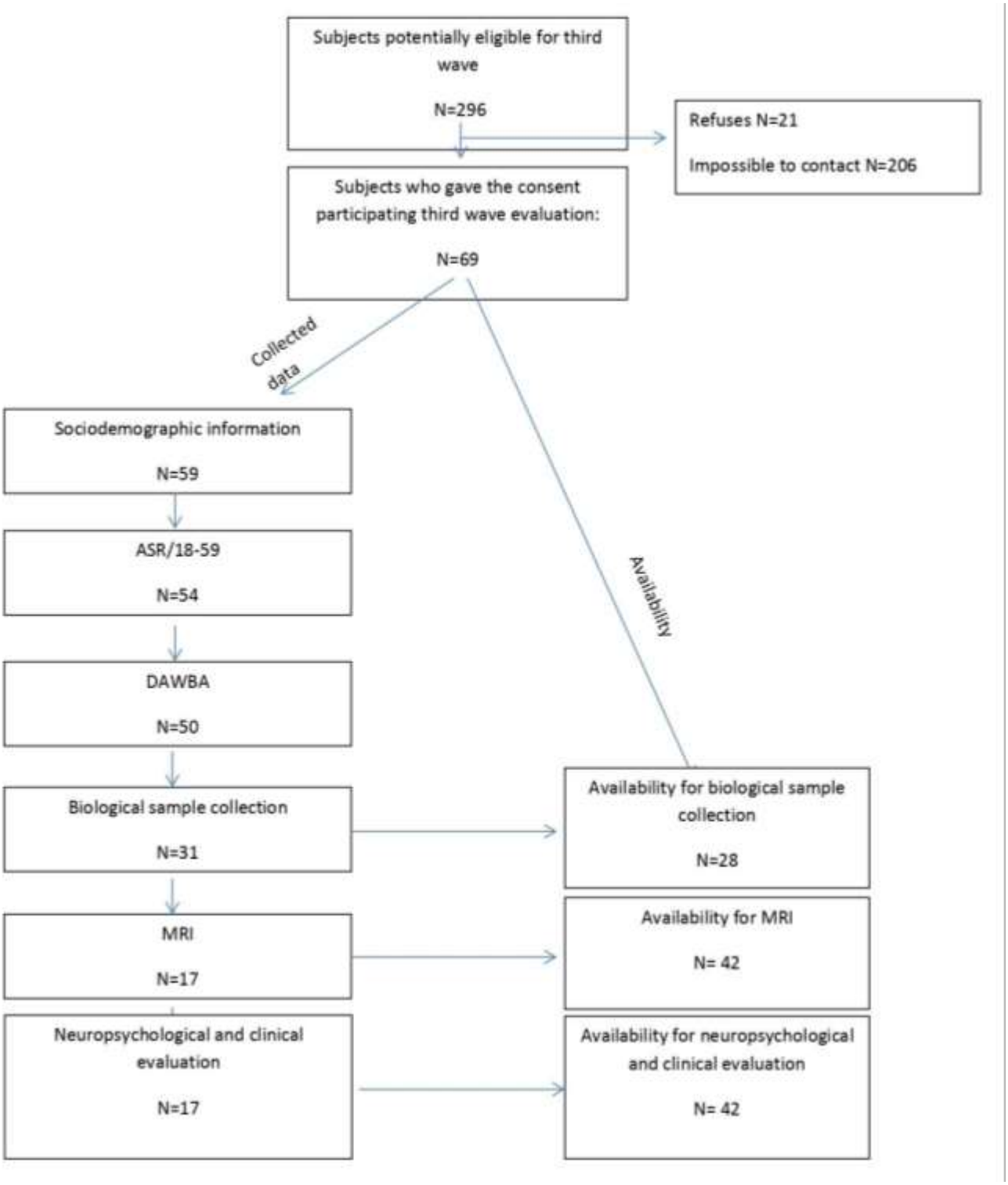
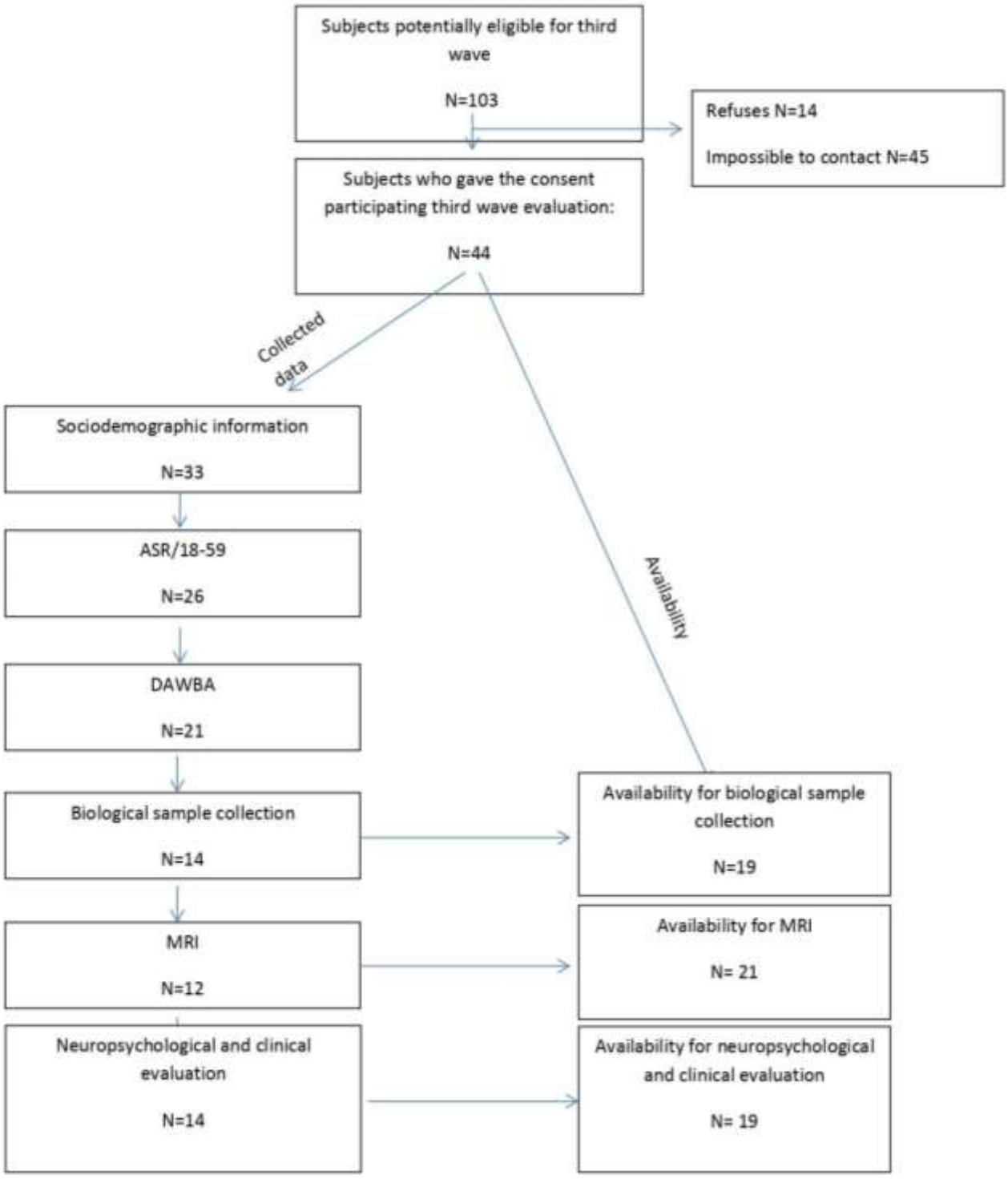


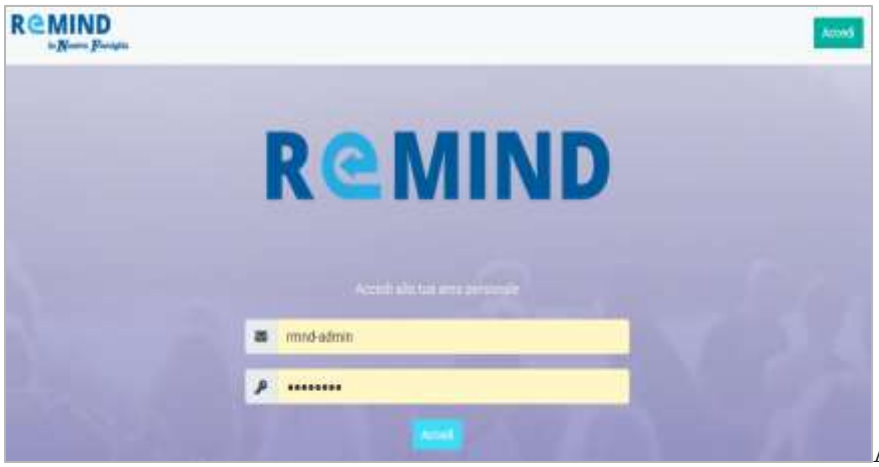
Figure 1.2: Clinical sample third wave recruitment flowchart



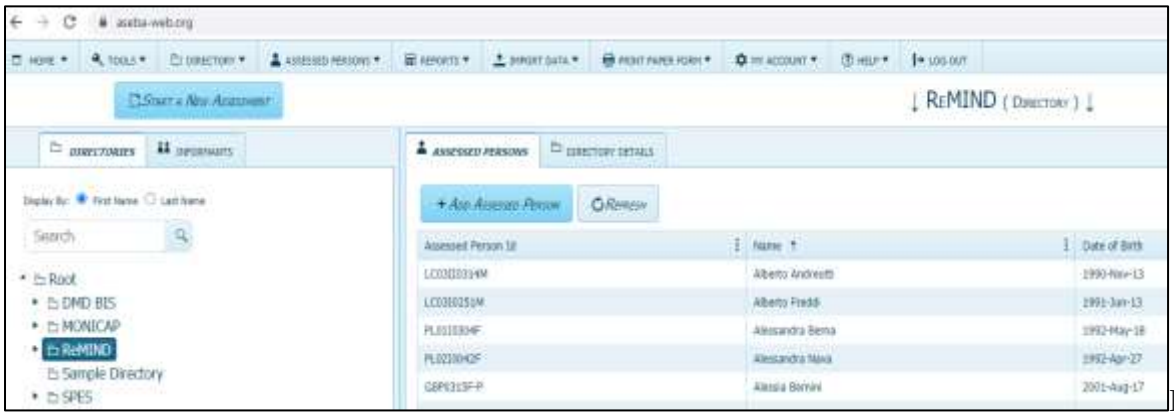
1.2.3.2 Online data collection

Within the ReMIND project, we created an online platform to aggregate information collected through standardized and *ad hoc* questionnaires. Through the platform, we collect psychological, psychopathological and behavioral profiles, socio-demographic data, and the presence of environmental stress factors. Figure 1.3 shows some examples of ReMIND platform web pages screens.

Figure 1.3



A



B

Note: Figure 1.3.A: ReMIND project platform login web page. Our platform unables to collect data directly or, thanks to its possibility to communicate with other online platforms, from the ASEBA questionnaires (see figure 1.3.B) or the DAWBA interview. Moreover, the ReMIND platform can aggregate information, collected with different instruments, and create datasets which facilitate data analysis.
Figure 1.3.B: "aseba.web" web page example. This platform unables to online collect and score ASEBA questionnaires, reaching participants via email.

1.2.3.3 Psychological and behavioral evaluation

ASEBA instruments

Psychological and behavioral profiles were assessed through the Achenbach System of Empirically Based Assessment - ASEBA instruments. The questionnaire comprehended in the evaluation are the Adult Behavior Checklist (ABCL/18) and the Adult Self Report (ASR/18-59 - Achenbach & Rescorla, 2003).

The ABCL and the ASR (Achenbach & Rescorla, 2003) are respectively 126-items other-rater and self-report questionnaires for adults comprehended between 18 and 59 years of age. Items are rated on a 3-point scale: 0 = Not True, 1 = Somewhat or Sometimes True, 2 = Very True or Often True.

ABCL and ASR assess emotional and behavioral functioning aspects. The questionnaires provide scores for the following syndrome scales: Anxious/depressed, Withdrawn, Somatic complaints, Thought problems, Attention problems, Aggressive behavior, Rule-breaking behavior and Intrusive behavior. In addition to the syndrome scale, the questionnaires' problem items can be scored in two broad groupings of syndromes. One grouping, designated as "Internalizing", takes together three syndrome scales: Anxious/depressed, Withdrawn and Somatic complaints. This grouping is defined as Internalizing because it comprises problems that exist mainly within the self. The second grouping, designated as "Externalizing", takes together three other syndromes scales: Aggressive behavior, Rule-breaking behavior and Intrusive behavior. The problems comprising the Externalizing grouping mainly involve conflicts with other people and with social mores. Lastly, the questionnaire provides scores for DSM-oriented scales: Depressive Problems, Anxiety Problems, Somatic Problems, Avoidant Personality Problems, Attention Deficit/ Hyperactivity Problems (Inattention and Hyperactivity/Impulsivity subscales), and Antisocial Personality Problems.

DAWBA

The "Development and well being assessment - DAWBA" (Goodman et al., 2000) is a diagnostic interview that combines a structured with a semi-structured part, and it is designed to generate the present-state psychiatric diagnoses for children, adolescents and adults following DSM-V criteria.

1.2.3.4 Environmental risk factors data

The following socio-economic and socio-demographic data are collected with the online platform:

- Socio-economic status: employment is used as a measure of socio-economic status (SES) coded according to the Hollingshead 9-point scale for occupation (Hollingshead, 1975).
- Information regarding family structure (number of family members, occupation and levels of education, marital status)
- Information regarding contacts with mental health services.

19 SLEs are collected with an *ad hoc* questionnaire filled out by the subjects:

1. parent had separated or broken up in a steady relationship;
2. parent had a serious illness which required a stay in hospital;
3. subject had separated or broken up in a steady relationship;
4. subject had a serious illness which required a stay in hospital;
5. subject had been in a serious accident or badly hurt in an accident;
6. parent death;
7. brother or sister death;
8. grandparent death
9. subject close friend death;
10. pet death;
11. subject had broken off close friendship;
12. subject had broken off affective relationship;
13. family had a major financial crisis;
14. parent had a problem with the police involving a court appearance;
15. subject had a problem with the police involving a court appearance;
16. subject had a pregnancy or abortion;
17. subject became parent;
18. subject had been repetitively humiliated, bullied, or physically assaulted
19. subject had been a victim of sexual harassment

The presence of pre- and perinatal risk factors is evaluated with an *ad hoc* questionnaire filled out by the subjects. The questionnaire asks for the presence of threat of abortion, twin birth - simple or complicated, incubator / resuscitation / "blue baby", maternal use of drugs, alcohol or tobacco during pregnancy, duration of gestation and birth weight.

1.2.3.5 Genetic and Epigenetic

Saliva samples are re-collected and all samples will be genotyped for candidate genes involved in psychopathology. The methylation status of a panel of candidate regions containing CpG residues thought to be differentially methylated in blood tissue of individuals affected by mental disorders will be analyzed.

1.2.3.6 Neuroimaging

A subgroup of patients undergoes a magnetic resonance imaging (MRI) session. A 3T Philips Achieva scanner is used. 3D MPR T1, T2, 64-directions DTI, MRS and fMRI are collected. Spectroscopy is acquired for the anterior cingulate gyrus. Volumes and thickness of gray matter of brain regions will be estimated using FreeSurfer. DTI data will be used to derive diffusion indexes, which reflect the integrity of axons and brain networks. MRS will allow the estimation of the concentration of metabolites as N-acetyl Aspartate (NAA), related to axonal integrity, Choline, related to demyelination, creatine and phosphocreatine, connected to brain energy. fMRI data will be useful to determine the functionality of brain networks.

Preliminary results regarding Third Wave evaluation are presented in Chapter 5.

1.3 The present work: Multi neuro-functional biomarkers for monitoring developmental trajectories in early onset psychopathology

My research work is part of the ReMIND project. I was interested in better understanding the pathogenesis and the courses of psychopathological traits, in order to possibly identify children more in need of intervention. I think that understanding the different causes of psychopathology is the first step in providing a better intervention, which is an absolute priority in terms of social and economic costs.

Specifically, I focused on the identification of psychopathological traits with *bottom up* approaches, in order to evaluate the presence of internalizing and externalizing problems in developmental ages. Special attention has been paid to the presence of emotional/behavioral dysregulation, as a specific risk factor for worse psychopathological developmental trajectories.

I was interested in evaluating the role of environmental (i.e., SLEs, perinatal adversities, SES, parenting style) and genetic factors in influencing these psychopathological traits. Moreover, I investigated the relevance of epigenetics variables.

Lastly, I took into account the development throughout the lifespan of psychopathological traits, in order to identify early individuals at higher risk of worse development.

1.3.1 Study aim

Specific study aims are:

- Identify clusters of symptoms that characterize individuals at higher risk for persistent mental health issues;
- Evaluate whether these patients' subgroups have been differently exposed to environmental and biological risk factors;
- Identify different psychopathological developmental trajectories.

1.3.2 Thesis structure presentation

To achieve the identification of clusters of symptoms that characterize individuals at higher risk for persistent mental health issues, we conducted two experimental studies which are presented in Chapter 2. In order to identify the presence of different psychopathological profiles, we used *bottom-up* person centered approaches, without *a priori* conceptions, in very large and heterogeneous samples. In the first part of the chapter, the results of latent class analysis (LCA) performed on the wave 1- ReMIND sample (including both general population and referred children and adolescents) are presented. To achieve the above mentioned goal, we performed the LCA on the full spectrum of the CBCL/6–18 syndromic scales. The results underline the presence of a profile characterized by severe emotional/behavioral dysregulation, which is associated with the presence of multiple categorical diagnoses. In the second part of chapter 2, the aim was to test the cross-societal generalizability of emotional/behavioral dysregulation profile. We replicated the LCA, as a suitable method to understand this particular psychopathological trait apart from cultural differences, with data of 9238 sobjects from 10 different societies. Results show that the presence of a "dysregulated class" is detectable in all the societies.

Subsequently, we conducted two experimental studies, which are reported in Chapter 3, to evaluate the different exposures of individuals with higher psychopathology to previously identified genetic and environmental risk factors. The first study investigated the association between parents' internalizing/externalizing symptoms, parenting practices and children's internalizing/externalizing symptoms. At wave 2, parent and child psychopathology were assessed with the Internalizing and Externalizing scales of the ASR and CBCL/6–18, respectively. Results show that high levels of maternal pathology predict high levels of children's psychopathology and a total mediating effect of parenting measures within this

relationship. The second study presented in Chapter 3, using a proof-of-concept imaging genetics mediation design, investigated the genetic (specifically, GRIN2B gene variants) and neural determinants (specifically, cortical thickness, cortical surface area, and gray matter volume evaluated with structural magnetic resonance imaging) of attention and hyperactivity problems evaluated with CBCL/6–18 at wave 1 and 2. The mediation results show that GRIN2B variants effect on inattention/hyperactivity is partially mediated by volume in the left isthmus of the cingulate cortex, suggesting a key role of this region in translating glutamatergic GRIN2B variations to attention/hyperactivity problems.

The fourth Chapter focuses on the role of gene-environment interaction in shaping psychopathological traits. With *machine learning* approaches we identified clusters of symptoms that characterize individuals for internalizing and externalizing psychopathology during adolescence (wave 2). Subsequently, with a decision tree classifier, we evaluated the role of environmental and epigenetic variables in determining these different clusters. We tested the role of perinatal adversities and obstetric complications, stressful life events, DNA methylation of candidate genes previously involved in psychopathology (i.e., BDNF, OXT, FKBP5, IGF2). Taking into account both internalizing and externalizing problems, two homogeneous clusters of psychopathology have been identified: "high" and "low". The variables which mostly discriminated between the two clusters is the presence of perinatal risk factors, followed by percentages of methylation in BDNF CpGs.

In Chapter 5, a preliminary work on the three time points data is presented, with the aim of testing the presence of specific trajectories in internalizing and externalizing areas, from childhood to adulthood. Clusters of subjects presenting peculiar symptom trajectories in anxious/depressed, withdrawn, and somatic problems were identified, whilst internalizing and externalizing symptoms resulted in overall stability. Individuals belonging to clusters characterized by stable high presence of anxious, depressive and somatic problems, presented higher emotional/behavioral dysregulation during preadolescence, with higher internalizing and externalizing problems. This preliminary evidence suggested the importance for clinicians and researchers to account for both homotypic and heterotypic continuity in psychopathological traits when planning interventions of treatment and prevention.

Chapter 2

The identification of psychopathological traits with bottom-up approach: Latent Class Analysis results from Remind Project and 10 society replication

2.1 Introduction

Clinicians and researchers agree that psychopathological manifestations span along a continuum, rather than being conceptualized in a dichotomy, between health and illness and between one diagnosis and another. For this reason, much research focuses on psychopathological traits rather than on psychiatric categorical diagnosis.

In neurodevelopmental psychopathology, the use of the terms "internalizing" and "externalizing" traits is well established to indicate class of symptoms and behaviors which may be found associated trans-diagnostically with psychopathology. Internalizing problems refer to inwardly focused negative behaviors such as anxiety, depression, and somatic symptoms, while externalizing problems refer to outwardly focused negative behaviors such as hyperactivity, aggression, disruptive conduct, and substance use (Achenbach and Rescorla, 2001, Tien et al., 2020). There are high rates of overlap between internalizing and externalizing problems. In the literature children with comorbid internalizing and externalizing problems have been found to be often characterized by the presence of emotional/behavioral dysregulation (ED), a transdiagnostic psychopathological trait, associated with worst outcomes in both internalizing and externalizing disorders (Shaw et al., 2014, Wang et al., 2018). ED refers to deficits in physiological arousal regulation and in inhibition of disruptive behavioral response to emotions, an inability to refocus attention after a strong emotional feeling and to have goal-directed behaviors after emotional activation (Biederman et al., 2012). Therefore, ED reflects both failure of cognitive control and high intensity of arousal (Soloff et al., 2015). These deficits cause higher sensitivity to emotional arousal, slower return to baseline activation, and deficits in coping strategies. Especially in children and adolescents, ED is responsible for low tolerance to frustration, impatience, easy anger and excessive emotional excitement (Biederman et al., 2012).

The study of psychopathological traits was also conducted in multicultural perspectives: the replicability of psychological categorical and dimensional constructs in different societies has become a relevant research field with significant influences on clinical practice (Ivanova et al., 2015a). Hence, several cross-cultural studies, using different instruments and approaches, have been conducted to evaluate the generalizability of psychological profiles in societies with very different social, political, and economic systems as well as languages, ethnicities,

religions, and geographical regions (Gardiner et al., 2019, Ivanova et al., 2015b, Vindbjerg et al., 2019).

Child Behavior Checklist (CBCL/6-18) questionnaire is a worldwide used instrument to screen for emotional, behavioral, and social problems in children and adolescents aged 6–18. The checklist returns a profile of scores on eight syndrome scales and six DSM-oriented scales. CBCL also evaluates the presence of internalizing and externalizing symptoms in children and adolescents thanks to its "Internalizing" scale (which takes into account the scores of anxious/depressed, withdrawn/depressed and somatic complaints subscales) and "Externalizing" scale (which takes into account the scores of rule breaking and aggressive behavior subscales) (Achenbach and Rescorla, 2001). Moreover, CBCL/6-18 allows the identification of recurrent profiles characterized by concurrent elevations on more scales that cut across internalizing and externalizing spectrum. CBCL Dysregulation Profile was identified by Biederman and colleagues in 1995 (Biederman et al., 1995) as the simultaneous elevation of "anxious/depressed", "attention problems" and "aggressive behaviors" CBCL subscales. In the beginning, this profile was associated with juvenile bipolar disorder, anyway, more recent findings suggest that the elevation of these three scales is a risk factor for greater number of comorbidities and longer persistence of mental health problems (Biederman et al., 2012).

In the last decade, the research on psychopathological traits started moving from top-down to bottom-up approaches. Rather than pre-selecting specific items or subscales, bottom-up methods involve identifying profiles via person-centered statistical approaches. Among these, latent class analysis (LCA) is one of the most common. LCA is a finite mixture model in which each participant is assigned to a specific class depending on how they respond to items or scales of a questionnaire (McCutcheon, 1987). Each class has a specific profile of responses (Goodman, 1974). LCA provides models based on the sample data, to find the solution that maximizes the difference between the identified classes and minimizes the heterogeneity within each class (Jung & Wickrama 2008). LCA is well suited for ASEBA syndromic data from various societies because it enables researchers to test whether similar concurrent elevations in syndrome scales can be detected among members of different populations, without a priori conception and apart from cultural differences.

2.2 Aim of the chapter

The chapter aim is to identify clusters of symptoms that characterize individuals at higher risk for persistent mental health issues. For this, we conducted two experimental studies which

enabled us to identify the presence of different psychopathological profiles.

We used *bottom-up* person centered approaches, without *a priori* conceptions, in very large and heterogeneous samples.

The first experimental study, published by Bianchi and colleagues (2017), was conducted on the wave 1- ReMIND sample (including both general population sample and referred children and adolescents). Our research group performed a LCA on the full spectrum of the CBCL syndromic scales. Moreover, the relationship between the identified classes and DSM-IV diagnoses was evaluated, also taking the presence/absence of comorbidity into consideration.

The second experimental study focused on ED trait: we replicated the LCA on 9238 subjects from 10 different societies to test the cross-societal generalizability of ED profiles in adult populations assessed with the Adult Self Report (ASR, Achenbach & Rescorla 2003). The second study results are published in Bianchi and colleagues (2022).

2.3 Experimental study 1

The study protocols were approved by the Research Ethical Committee of IRCCS Eugenio Medea Scientific Institute and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Parents' written informed consent was obtained for all participants.

2.3.1 Methods

2.3.1.1 Sample

First wave ReMIND sample was included in the study, which therefore comprehends both general-population subjects and referred subjects. The general population subsample consisted of 3418 subjects at the screening phase (49.6% males; 10–14 years old, M = 12.08, SD = 0.90) and of 631 subjects in the second diagnostic phase (46.0% males; 10–14 years old, M = 12.16, SD = 0.91). The clinically referred sample was composed of 1226 subjects (76.9% males; 6–17 years old, M = 9.11, SD = 2.34).

2.3.1.2 Measures

For this study, fathers' and mothers' educational levels were selected as indicators of socio-economic status. The parents' education level was divided for analysis into two classes: "at risk" (less than 10 years of school) and "not at risk" (10 years or above).

Behavioral and emotional screening was conducted with CBCL/6-18 (Achenbach & Rescorla 2001), filled out by parents. CBCL/6-18 is divided into eight syndrome scales: Anxious/depressed, Withdrawn/depressed, Somatic complaints, Social problems, Thought problems, Attention problems, Rule-breaking behavior, and Aggressive behavior. In this study, we used the T-score based on the set of multicultural norms 'group 2', which applies to the normative sample of the Italian population (Achenbach & Rescorla 2007; Ivanova et al., 2007). Scores on the eight clinical scales were dichotomized as "not at risk" ($T < 65$) or "at risk" ($T \geq 65$).

Diagnoses were evaluated with two instruments: Development and Well-Being Assessment (DAWBA, Goodman et al., 2000) and Kiddie schedule for affective disorders and Schizophrenia for school-age children-present and lifetime version (K-SADS-PL, Kaufman et al., 1997). DAWBA is a diagnostic interview that combines a structured and a semi-structured part and is designed to generate the present-state psychiatric diagnoses for children and adolescents following DSM criteria, with satisfactory validity and inter-rater reliability. This interview was administered to the 631 subjects that took part in the second phase of the PrISMA project (general population sample). The diagnostic evaluation was conducted on probable cases of mental disorders and on a sample of non-probable cases: all subjects exceeding the cutoff scores (90th percentile of the frequency distribution) of CBCL internalizing and/or externalizing scales and a 10% random sample of those who did not exceed the cutoff scores were selected for this second phase. Full details concerning research design and methods are available elsewhere and reported in chapter 1 (see paragraph 1.2.2.1) (Frigerio et al., 2009).

K-SADS-PL is a semi-structured diagnostic interview created to assess current and past episodes of psychopathology in children and adolescents according to DSM criteria. All subjects in the Cabala-Genesis project (clinically referred sample) were assessed through K-SADS-PL interview.

Characteristics of the sample are depicted in table 2.1.

Table 2.1: Sociodemigraphic, emotional behavioral and diagnostic characteristics of the sample

	General population sample	Clinical sample	Total sample
N	3418	1225	4643
Birth assigned sex (males:females) (N)	1695:1723	942:283	2637:2006
Age (mean ± SD) (years)	12.08 ± 0.90	9.11 ± 2.34	11.30 ± 1.94
Mother education at risk (n %)	872 (25.5%)	456 (37.2%)	1328 (28.6%)
Father education at risk (n %)	1011 (29.6%)	515 (42.0%)	1526 (32.9%)
Frequencies of syndrome scales score in the clinical range (n %)			
Anxious/depressed	494 (14.5%)	489 (39.9%)	983 (21.2%)
Withdrawn/depressed	409 (12.0%)	428 (34.9%)	837 (18.0%)
Somatic complaints	373 (10.9%)	187 (15.3%)	560 (12.1%)
Social problems	308 (9%)	431 (35.2%)	739 (15.9%)
Thought problems	263 (7.7%)	298 (24.3%)	561 (12.1%)
Attention problems	382 (11.2%)	598 (48.8%)	980 (21.1%)
Rule-breaking behavior	69 (2.0%)	207 (16.9%)	276 (5.9%)
Aggressive behavior	240 (7.0%)	412 (33.6%)	652 (14.0%)
Subsample which conducted the diagnostic phased			
N	631	1225	1856
Birth assigned sex (males:females) (N)	290:341	942:283	1232:624
Age (mean ± SD) (years)	12.16 ± 0.91	9.11 ± 2.34	11.29 ± 1.94
Frequencies of DSM-IV diagnoses (n %)			
Any diagnosis	104 (16.5%)	1058 (86.4%)	1162 (62.6%)
ADHD	21 (3.3%)	386 (31.5%)	407 (21.9%)
Any behavior disorder	13 (2.1%)	162 (13.2%)	175 (9.4%)

Any mood disorder	13 (2.1%)	372 (30.4%)	385 (20.7%)
Any anxiety disorder	77 (12.2%)	537 (43.8%)	614 (33.1%)
Other diagnoses	3 (0.5%)	110 (9.0%)	113 (6.1%)
Presence of comorbidity	34 (5.4%)	451 (36.8%)	485 (26.1%)

Note: SD = Standard deviation; ADHD = attention deficit hyperactivity disorder; Behavior disorders = oppositional defiant disorder, conduct disorder, and disruptive disorder not otherwise specified (NOS); Mood disorder = depressive disorder, dysthymic disorder, and depressive disorder NOS; Anxiety disorder = generalized anxiety disorder, specific phobia, panic disorder, social phobia, separation anxiety, obsessive–compulsive disorder, post-traumatic stress disorder, mixed anxiety depressive disorder, and other anxiety disorders NOS; Other diagnoses = tic disorder, stuttering, enuresis, and selective mutism.

2.3.1.3 Data analyses

Preliminary analysis

$\chi 2$ and t tests were used to identify differences between the two subsamples in BAS, age, parents' educational levels, and CBCL/6–18 scores according to variables distributions.

Latent class analysis

After checking for model assumption, a Latent Class Analysis (LCA) was performed in order to identify distinct clusters of symptoms. LCA is a person-centered statistical approach able to assign subjects to a statistically independent class when they respond in the same way to items (or scales) of a questionnaire. Thus, each class has a specific symptom (item or scale) endorsement profile (Goodman 1974, McCutcheon, 1987). LCA on the CBCL/6–18 syndrome scales in the two samples separately (with age and BAS as covariates) and in the total sample (introducing the clinical status as covariate) was performed using Mplus 6.11 software (Muthén & Muthén, 1998). Models estimating 1-class through 5-class solutions were compared. The best solution was determined according with the Bayesian Information Criterion (BIC, Kass & Wasserman, 1995), the Lo–Mendell–Rubin test (Lo et al., 2001) and the Bootstrapped Likelihood Ratio test (BLRT, McLachlan & Peel, 2004). To determine the number of classes, the rule of parsimony and the substantive relevance of a class was also considered (Nylund et al., 2007). Descriptive labels were given to the identified classes. During the validation phase of latent classes, each subject was assigned to their highest probability class using the "knownclasses" algorithm. After conducting separate LCAs for the

two samples and for the total sample, the classification agreement using the Cohen's Kappa coefficient was evaluated. The degree of agreement was interpreted according to the magnitude guidelines defined by Landis and Koch (1977).

Relationship between classes and diagnoses

Subjects who completed the diagnostic phase entered in this second step analysis.

The $\chi 2$ test was used to check differences in diagnosis distribution between classes. Diagnoses were coded in the following categories: ADHD, Behavior Disorders, Mood Disorder, Anxiety Disorder, and Other Diagnoses. Our research group was interested in evaluating the association between classes and the presence of diagnosis and comorbidities, for this reason the categories were re-coded as: 0 = absence of diagnosis, 1 = one diagnosis, and 2 = two or more diagnoses (i.e., comorbidity).

To evaluate whether the membership to a class might predict the presence/absence of diagnosis, a backward multinomial logistic regression with diagnostic status as dependent variable (with absence of diagnosis as reference category) and classes as predictors was performed. Age, BAS, and parents' educational levels were entered as covariates.

The likelihood ratio $\chi 2$ was used to test the significance ($p < 0.05$) of all models, whereas the Wald statistic was used to test the significance ($p < 0.05$) of the independent variables. For each model, we also reported Nagelkerke pseudo-R2 to show each model's ft. We used the odd ratio (OR) as a measure of effect size and Monson's classification of OR (Monson, 1990) to describe the strength of the association between the dependent variables and the predictors.

2.3.2 Results

Preliminary analysis

The two subsamples significantly differed for age ($t = 43.317$, $p = 0.000$), BAS distribution ($\chi 2 = 272.940$, $p = 0.000$), mothers' and fathers' educational levels ($\chi 2 = 113.221$, $p = 0.000$; $\chi 2 = 125.368$, $p = 0.000$, respectively), and the percentage of subjects in the clinical range ($\chi 2$ from 15.778 to 765.010, $p = 0.000$), with the referred subsample characterized by younger subjects, a higher rate of male subjects, a higher percentage of subjects with a lower socio-economic status, and a higher percentage of subjects in the clinical range in all CBCL/6-18 scales.

Latent class analysis

According to model fit indices, the 4-class solution was the chosen one, results are reported in table 2.2. The 4-class solution resulted in clearly distinct classes. The four classes identified in the three LCAs (general population, clinical and total sample) had a very similar structure, with limited differences between the two subsamples.

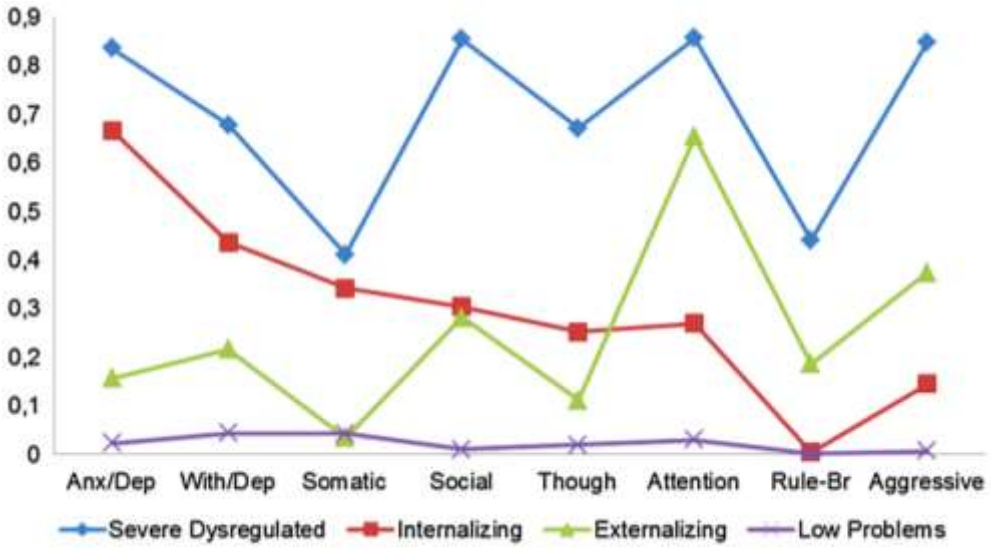
Table 2.2: Fit statistics for latent class models

	Log-likelihood	BIC	LMRT (p)	BLRT (p)
General population subsample				
2 Classes	−6986.79	14128.18	2414.22 (0.00)	2441.19 (0.00)
3 Classes	−6896.90	14037.91	177.19 (0.00)	179.78 (0.00)
4 Classes	−6841.55	14016.71	109.48 (0.00)	110.70 (0.00)
5 Classes	−6811.38	14045.87	29.68 (0.28)	60.35 (0.00)
Clinical sample				
2 Classes	−5192.34	10519.78	1201.48 (0.00)	1216.85 (0.00)
3 Classes	−5119.34	10451.99	144.16 (0.01)	146.01 (0.00)
4 Classes	−5054.25	10400.03	128.54 (0.00)	130.18 (0.00)
5 Classes	−5032.06	10419.34	43.81 (0.09)	44.369 (0.00)
Total sample				
2 Classes	−12442.97	25054.80	5788.68 (0.00)	5845.82 (0.00)
3 Classes	−12149.40	24568.97	581.41 (0.00)	587.14 (0.00)
4 Classes	−11982.00	24335.50	331.52 (0.00)	334.79 (0.00)
5 Classes	−11940.07	24352.95	83.05 (0.01)	83.87 (0.00)

The Cohen's Kappa coefficient indicated a substantial agreement ($\kappa = 0.766$; 95% CI, 0.590 to 0.942, $p < 0.0005$) between subject classifications obtained by running separate LCAs on the subsamples and on the total sample. In subsequent analyses, the classification obtained in the total sample was used.

The analysis of the features revealed important differences between classes (Figure 2.1). The first class, labeled "Severe Dysregulated–DYS" (7.82% of the sample), included subjects with an elevated probability (>60%) of being in the clinical range for all CBCL scales, with the exception for somatic complaints and rule-breaking behavior. The second class, called "Internalizing Problems–INT" (15.68%), is characterized by a high probability of being in the clinical range only for the anxious/depressed scale. The third class, "Attention/Hyperactivity–ADHD" (10.19%), has an elevated probability of being in the clinical range for the attention problems scale. Finally, the fourth class, labeled "Low Problems–LOW" (66.32%), includes those subjects with a low probability of clinical range results for each CBCL syndrome scale.

Figure 2.1: CBCL/6–18 profiles according to the 4-class solution in the total sample



Relationship between classes and diagnostic profiles

Table 2.3 shows the distribution of diagnostic categories in the four identified classes and relative $\chi 2$ indices. The results of the logistic regression analysis are reported in Table 2.4. All classes with the exception of Attention/Hyperactivity Class significantly predicted the

absence of diagnosis. More specifically, there is a strong association between the absence of diagnosis and the Low Problems class (OR = 8.17; 95% CI 5.61–11.91). There is also a moderate negative association between the absence of diagnosis and the Severe Dysregulated and Internalizing Classes (OR = 0.58; 95% CI 0.36–0.94; OR = 2.02; 95% CI 1.40–2.98, respectively).

Table 2.3: Classes clinical description

	Class 1: DYS (339)	Class 2: INT (500)	Class 3: ADHD (416)	Class 4: LOW (601)	
Diagnoses (n %)					
Any diagnosis	302 (89.1%)	296 (59.2%)	351 (84.4%)	213 (35.4%)	$\chi2 = 377.667$ $p = 0.000$ a, c, d, e, f
ADHD	104 (30.7%)	32 (6.4%)	204 (49.0%)	67 (11.1%)	$\chi2 = 304.967$ $p = 0.000$ a, b, c, d, e, f
Any behavior disorder	64 (18.9%)	16 (3.2%)	66 (15.9%)	29 (4.8%)	$\chi2 = 93.263$ $p = 0.000$ a, c, d, f
Any mood disorder	151 (44.5%)	132 (26.4%)	66 (15.9%)	36 (6.0%)	$\chi2 = 212.112$ $p = 0.000$ a, b, c, d, e, f
Any anxiety disorder	184 (54.3%)	214 (42.8%)	99 (23.8%)	117 (19.5%)	$\chi2 = 156.639$ $p = 0.000$ a, b, c, d, e
Presence of comorbidity	177 (52.2%)	144 (28.8%)	100 (24.0%)	64 (10.6%)	$\chi2 = 196.882$ $p = 0.000$ a, b, c, e, f

Note: a = Class 1 vs. class 2 ($p < 0.05$); b = Class 1 vs. class 3 ($p < 0.05$); c = Class 1 vs. class 4 ($p < 0.05$); d = Class 2 vs. class 3 ($p < 0.05$); e = Class 2 vs. class 4 ($p < 0.05$); f = Class 3 vs. class 4 ($p < 0.05$).

Table 2.4: Significant OR ($p < 0.05$) in the logistic regression and multinomial logistic regression analysis with classes as predictors and age, BAS, and parental education as covariates

	Logistic regression	Multinomial regression (absence of diagnosis is the reference category)	
	Absence of diagnosis (N = 694)	One diagnosis (N = 677)	Comorbidity (N = 486)
classes	OR (95% confidence interval)		
Severe Dysregulated	0.58** (0.36–0.94)	8.57*** (5.38–13.66)	27.69**** (16.78–45.70)
Internalizing	2.02** (1.40–2.98)	2.93** (2.08–4.12)	6.63*** (4.43–9.94)
Attention/Hyperactivity	–	8.51*** (5.74–12.60)	7.27*** (4.54–11.65)
Low problems	8.17*** (5.61–11.91)	–	–
Age	1.55** (1.46–1.65)	0.66** (0.62–0.71)	0.62** (0.58–0.66)
Gender	1.77** (1.37–2.30)	1.79** (1.34–2.37)	1.76** (1.28–2.41)
Mother's education	0.63** (0.47–0.83)	1.56** (1.16–2.11)	1.65** (1.18–2.29)
Father's education	–	–	–
Nagelkerke pseudo-R2	0.41	0.41	

Note: * weak association, ** moderate association, *** strong association, **** very strong association

The multinomial logistic regression revealed that the "One Diagnosis" condition was significantly associated with the Severe Dysregulated, Internalizing, and Attention/Hyperactivity Classes (respectively, OR = 8.57; 95% CI 5.38–13.66; OR = 2.93; 95% CI 2.08–4.12; OR = 8.51; 95% CI 5.74–12.60 for one diagnosis vs. absence of diagnosis) with a moderate association in the second case and strong associations for the other classes. The "Comorbidity" condition, defined as two or more diagnoses, was significantly associated with the Severe Dysregulated, Internalizing, and Attention/Hyperactivity classes (respectively, OR = 27.69; 95% CI 16.78–45.70; OR = 6.63; 95% CI 4.43–9.94; OR = 7.27; 95% CI 4.54–11.65 for Comorbidity vs. Absence of Diagnosis) with a very strong association in the first case and strong associations for the other classes. The Nagelkerke pseudo-R2 values concerning the two models are reported in Table 2.4.

The use of "most likely class membership" as a variable for further analysis may be problematic when the entropy goes much lower than 0.8 because the precision in assigning class membership is less than optimal (Asparouhov & Muthén, 2014). As in our model entropy was 0.77, the analysis was repeated by directly introducing the categorical outcome (i.e., diagnostic status) in the LCA 4-classes model (Lanza et al., 2013), and the risk of having two or more diagnoses was identified for each latent class. Results confirmed that the Comorbidity condition is more likely for subjects in the Severe Dysregulated class (64.3%), followed by Internalizing class (51.1%), Attention/Hyperactivity class (24.9%) and, finally, by Low Problems class (7.9%). Moreover, the probability of having two or more diagnoses with respect to one diagnosis is significantly higher for individuals in Severe Dysregulated class with respect to individuals in Attention/Hyperactivity and Low Problems classes (respectively, OR = 5.42; 95% CI 2.55–11.53; OR = 21.12; 95% CI 7.62–58.59). Instead, subjects in Internalizing class have a probability only moderately lower (but significant) than those in Severe Dysregulated class to have two diagnoses (OR = 0.58; 95% CI 0.21–1.635).

2.4 Experimental study 2

Investigators from the 10 different countries/societies followed local ethical protocols and obtained informed consent from participants. All data were de-identified. The University of Vermont Committee on Human Research in the Medical Sciences (CHRMS) approved this study. The University of Vermont (UVM) IRB protocol number is 14-237.

2.4.1 Methods

2.4.1.1 Sample

Adult Self Report (ASR, Achenbach & Rescorla, 2003) data were obtained from population samples of ≥400 adults in each of 10 societies, with most being countries. The societies differed with respect to ethnicity, religion, geographical location, socioeconomic and mental health systems, and population size. Specific data on cultural differences and sociodemographic level were not taken into account.

Data were analyzed from 9238 18- to 59-year-olds living in Albania, Belgium (Flanders), Brazil, Czech Republic, Italy, Japan, Kenya, Korea, Lithuania, and USA, as summarized in

Table 2.5. The sample sizes ranged from 427 in Kenya to 2020 in the USA; the percentage of male subjects ranged from 38.9% in Kenya to 50.9% in the Czech Republic; and the mean age ranged from 34.54 years (SD = 11.75) in Brazil to 39.07 (SD = 11.97) in the USA.

2.4.1.2 Measures

In this second experimental study, the behavioral and emotional information regarding participants have been collected with the Adult Self Report (ASR, Achenbach & Rescorla, 2003). The ASR (Achenbach & Rescorla, 2003) has been shown to be a valid transcultural self-report measure of psychopathological problems for ages 18-59 in all the societies included in our study, presenting a homogeneous structure across cultures (Achenbach, 2019; Ivanova et al., 2015a). The ASR obtains self-ratings of 120 items assessing behavioral, emotional, social, and thought problems, based on the preceding 6 months, and scored on eight syndromes derived from factor analysis (Achenbach & Rescorla, 2003). The problem items are rated 0 = not true, 1 = somewhat or sometimes true, 2 = very true or often true. The 0-1-2 ratings are summed to provide scale scores for syndromes designated as Anxious/Depressed, Withdrawn, Somatic Complaints, Thought Problems, Attention Problems, Aggressive Behavior, Rule-Breaking Behavior, and Intrusive.

Achenbach and Rescorla (2003) reported that the test-retest reliability of the ASR syndrome scales in the U.S. national sample ranged from .83 to .91, while Cronbach's internal consistency alphas ranged from .51 to .88 for the eight syndrome scales. The Multicultural Supplement to the Manual for the ASEBA Adult Forms & Profiles (Achenbach & Rescorla, 2015) and the Manual for the ASEBA Adult Forms & Profiles (Achenbach & Rescorla, 2003) report additional psychometric properties.

Researchers from all societies except the USA used translations of the ASR that were validated through independent back-translations and were approved by the authors.

For each of the 10 samples, T-scores (Table 5) were calculated for the eight syndrome scales, separately for each sex at ages 18-35 and 36-59, using the following formula:

$$50 + [10 * (\text{raw score} - \text{mean score}) / \text{standard deviation}]$$

The T-scores then were dichotomized to create an additional variable as "not at risk" (< 85th percentile) versus "at risk" (85th percentile).

Table 2.5: Descriptive statistics and frequencies of syndrome scales T−scores in the clinical range (≥85th percentile) (*n*, %), for each included society

Society	*n (% males)*	Mean age (*SD*)	ANX/D	WIT	SOM	THO	ATT	AGG	RBB	INTR
Albania	750 (50.3%)	37.32 (12.75)	132 (17.6%)	147 (19.6%)	139 (18.5%)	171 (22.8%)	136 (18.1%)	137 (18.3%)	162 (21.6%)	172 (22.6%)
Belgium (Flanders)	1548 (50%)	38.57 (12.18)	269 (17.4%)	297 (19.2%)	312 (20.2%)	360 (23.3%)	291 (18.8%)	313 (20.2%)	319 (20.6%)	383 (24.7%)
Brazil	813 (40.8%)	34.54 (11.75)	151 (18.6%)	166 (20.4%)	162 (19.9%)	154 (18.9%)	150 (18.5%)	143 (17.6%)	157 (19.3%)	170 (20.9%)
Czech Republic	588 (50.9%)	37.84 (12.37)	105 (17.9%)	109 (18.5%)	113 (19.2%)	136 (23.1%)	104 (17.7%)	110 (18.7%)	107 (18.2%)	128 (21.8%)
Italy	519 (46.2%)	38.03 (12.37)	96 (18.5%)	106 (20.4%)	105 (20.2%)	102 (19.7%)	104 (20.0%)	101 (19.5%)	138 (26.6%)	106 (20.4%)
Japan	1000 (46.5%)	38.23 (10.70)	184 (18.4%)	191 (19.1%)	180 (18.0%)	244 (24.4%)	189 (18.9%)	186 (18.6%)	195 (19.5%)	193 (19.3%)
Kenya	427 (39.8%)	38.91 (8.53)	73 (17.1%)	77 (18.0%)	87 (20.4%)	85 (19.9%)	82 (19.2%)	81 (19.0%)	75 (17.6%)	91 (21.3%)
Korea	1000 (50.5%)	37.91 (9.84)	177 (17.7%)	189 (18.9%)	198 (19.8%)	231 (23.1%)	183 (18.3%)	188 (18.8%)	214 (21.4%)	219 (21.9%)
Lithuania	573 (47.6%)	35.27 (11.13)	123 (21.5%)	111 (19.4%)	118 (20.6%)	135 (23.6%)	109 (19.0%)	98 (17.1%)	112 (19.5%)	127 (22.2%)
USA	2020 (41.1%)	39.07 (11.97)	361 (17.9%)	480 (23.8%)	372 (18.4%)	419 (20.7%)	377 (18.7%)	344 (17.0%)	413 (20.4%)	402 (19.9%)

Note*:* ANX/D = Anxious/Depressed; AGG = Aggressive Behavior; ATT = Attention Problems; INTR = Intrusive; *SD* = standard deviation; SOM = Somatic Complaint; THO = Thought Problems; WIT = Withdrawn; RBB = Rule-Breaking Behavior

2.4.1.3 Data analyses

After checking for the model assumptions, we performed a LCA to examine whether specific homogeneous groups of subjects could be identified in each sample. Specifically, LCA was performed on the dichotomized ASR scales (as explained in the Instruments section, see paragraph 2.4.1.2) in each sample, separately. Models estimating solutions from two classes to N classes were compared. The number of classes was increased until the log-likelihood (LLH) value stopped replicating or until N-class solution fit-indexes were worse than N-1 class solution fit-indexes. The best solution was determined following the LCA procedures used by Althoff and colleagues (2010): we assessed model fit with the sample size-adjusted Bayesian information criterion (adj-BIC), the VoungLoMendelRubin likelihood ratio test (VLMRT), and the bootstrapped likelihood-ratio test (BLRT). Based on procedures described by Althoff and colleagues (2010), we took the BLRT as being more definitive than the VLMRT when the two did not agree. Therefore, the model considered as having the best fit should have the lowest adj-BIC and a significant BLRT when compared with the k 1 class model. In addition, we considered the rule of parsimony, the substantive relevance of a class, and the value of entropy—ranging from 0 to 1, with a value of 1 when all respondents have a probability of 1 of being in one class, and a value of 0 when the probabilities of being assigned to a class are constant for all subjects. We aimed to choose the highest number of classes, such that none of the classes would be too small (e.g., less than 5% of the sample). This rule has long been used in practice as a part of the idea of domain-usefulness but also has been discovered to have theoretical justification (Nasserinejad et al., 2017).

The identified classes were given descriptive labels based on the authors' consensus after reviewing each class's unique profile. Specifically, the dysregulated class (DYS) was identified as the one presenting the highest scores on the Anxious/Depressed, Attention Problems, and Aggressive Behavior scales.

During the validation phase of the latent classes, each subject was assigned to their highest probability class using the "known classes" algorithm. LCA were performed using Mplus 6.11 (Muthen & Muthen, 1998).

After identifying the best class solution and checking for model assumptions, to detect between-classes statistical differences between each society, we performed ANOVAs using the eight syndrome scales' T-values as the dependent variables and class assignment as the independent variable. ANOVAs were performed using SPSS (Version 21).

2.4.2 Results

For five of the 10 societies (i.e., Brazil, the Czech Republic, Italy, South Korea, and the USA), LCA was tested for models with two to five classes. Regarding the other five, the data model testing for Kenya ended at three classes; in addition, a six-class solution was tested for Albania, Belgium, Japan, and Lithuania. Because multiple indices were considered in selecting the optimal LCA model and those indices did not always agree, some degree of judgment was involved in the selection process. In eight of the 10 societies (i.e., Brazil, the Czech Republic, Italy, Japan, Kenya, Lithuania, South Korea, and the USA), the BLRT and the adj-BIC agreed in identifying the best class solution, and in four of these societies (Czech, Kenya, South Korea, the USA), the indexes also were in line with the VMLR. For the remaining four (Brazil, Italy, Japan, and Lithuania), the BLRT was considered the more definitive test of model fit. For one society (Belgium), the adj-BIC was lower in the model, with one less class than the chosen model, but the entropy and BLRT were better in the latter model. When selecting the final model, in this case, we also considered the rule of parsimony, the frequencies of subjects in each class, and the clinical features of the classes. Regarding the Albanian data, the five-class solution was excluded because it included one class with a percentage of subjects lower than 5%. Concerning entropy, most of our models showed good but not optimal values. Only the entropies for Kenya, Japan, and Italy were adequate (i.e., .80) and indicated a good separation between classes. For the other societies, the values ranged from .70 for Brazil to .79 for Albania, suggesting that researchers should pay attention when using "most likely class membership" as a variable for further analysis because some of the classes did not seem to be distinguished.

The model selected as best involved five classes in three societies (i.e., Belgium, Japan, and Lithuania), four classes in six societies (i.e., Albania, Brazil, Czech, Italy, South Korea, and the USA), and three classes in one society (i.e., Kenya).

A DYS class was identified in each society. The omnicultural mean prevalence of DYS was 9.2%, ranging from 6.1% (Lithuania) to 12.7% (Japan) (see Table 2.6).

The ANOVAs conducted for each society on the eight syndrome scales' T-values (as dependent variables) and class assignment (as the independent variable) showed that the participants in the DYS class had different scores in all ASR scales, as compared to adults in the other classes ($p < .01$). Specifically, subjects falling into the DYS class had higher scale scores on Anxious/Depressed, Attention Problems, Aggressive Behavior and, more generally, in both internalizing and externalizing areas. This effect was significant for all scales in each

society, with few exceptions. Participants in the DYS class and in the INT class had similar scores on the Withdrawn and Somatic Problems scales in Italy and Albania, and on the Withdrawn and Anxious/Depressed scales in Lithuania. In the Belgian sample, Rule-Breaking Behavior scores were similar between the participants in the DYS and EXT classes.

Table 2.6: T-scores means and prevalence for each class in each sample

Class	ANX/D	WIT	SOM	THO	ATT	AGG	RBB	INTR	Prevalence
Kenya									
C1	45.81	46.32	46.65	46.24	45.69	45.56	45.79	46.86	71.2%
C2	**68.99**	**66.45**	**64.36**	**69.32**	**67.08**	**68.46**	**73.31**	**64.68**	**7.5%**
C3	57.33	56.52	56.14	55.78	58.41	58.34	55.86	55.32	21.3%
Brazil									
C1	**66.89**	**62.65**	**64.96**	**64.30**	**65.33**	**64.34**	**63.94**	**56.03**	**10.1%**
C2	59.56	59.61	58.42	55.19	54.12	55.20	48.20	47.57	11.4%
C3	50.50	50.30	49.21	55.46	55.05	56.35	59.27	62.16	13.5%
C4	45.59	46.28	46.36	45.73	45.84	45.53	46.22	46.96	64.9%
USA									
C1	59.16	60.42	54.96	56.00	56.39	52.25	52.22	47.04	11.9%
C2	45.32	45.77	46.41	45.91	45.49	45.49	45.71	46.65	63.8%
C3	51.53	50.42	52.28	53.32	55.16	56.25	57.67	60.95	15.6%
C4	**68.98**	**65.95**	**65.40**	**65.80**	**65.05**	**68.74**	**64.67**	**58.99**	**8.7%**
Korea									
C1	**65.30**	**63.40**	**62.96**	**65.33**	**65.88**	**67.38**	**69.59**	**63.43**	**9.5%**
C2	62.94	66.50	57.39	55.58	61.07	58.26	53.39	46.84	7.3%
C3	53.83	50.66	53.36	54.19	54.54	55.38	55.37	56.74	21.4%
C4	44.79	45.76	45.97	45.53	44.68	44.49	44.73	45.97	61.8%
Czech Republic									
C1	51.62	49.10	51.07	55.75	55.61	58.98	59.11	63.79	14.6%
C2	**67.08**	**66.86**	**62.63**	**61.66**	**66.22**	**63.85**	**63.65**	**55.64**	**10.7%**
C3	44.94	45.24	45.83	45.57	44.49	45.32	45.54	46.72	58.7%

C4	55.66	56.99	55.87	53.18	54.21	49.69	48.88	45.63	16.0%
Italy									
C1	**64.91**	**57.71**	**59.13**	**65.20**	**65.73**	**67.79**	**66.17**	**58.88**	**7.5%**
C2	48.64	47.84	50.74	51.32	52.37	57.78	58.46	64.42	11.9%
C3	45.08	46.35	46.39	45.99	45.48	44.87	46.82	46.84	62.0%
C4	61.31	60.50	57.94	56.42	57.25	54.96	48.65	47.66	18.5%
Albania									
C1	**66.30**	**63.68**	**59.36**	**59.77**	**65.48**	**68.20**	**68.70**	**62.32**	**9.9%**
C2	51.32	49.96	51.54	53.55	53.45	57.44	57.54	56.95	16.7%
C3	45.12	45.94	46.33	46.95	45.12	44.97	45.04	46.90	63.3%
C4	62.45	62.12	61.28	53.73	59.74	51.48	50.41	45.94	10.1%
Lithuania									
C1	45.10	46.05	45.96	45.85	44.95	44.88	45.55	45.75	59.2%
C2	**64.59**	**63.61**	**61.20**	**65.95**	**65.74**	**67.47**	**71.18**	**66.46**	**6.1%**
C3	52.85	51.85	54.35	52.40	54.97	56.91	54.68	56.51	15.5%
C4	64.30	64.04	56.55	54.68	60.78	54.04	50.66	46.94	9.1%
C5	52.68	49.38	54.38	56.73	52.72	55.18	55.40	57.62	10.1%
Belgium									
C1	**68.16**	**62.88**	**65.01**	**66.16**	**65.72**	**65.11**	**64.52**	**56.24**	**9.4%**
C2	61.34	59.38	56.99	54.43	56.72	57.96	49.11	46.17	9.7%
C3	48.59	50.34	46.15	55.00	56.16	54.14	63.68	58.85	7.5%
C4	45.66	46.66	46.86	46.09	45.87	45.37	46.46	47.40	67.8%
C5	54.39	52.11	55.90	55.88	53.81	61.21	51.73	65.54	5.7%
Japan									

C1	44.46	44.56	46.04	45.35	44.66	44.94	45.61	46.31	58.2%
C2	47.49	49.03	50.10	54.78	47.43	47.54	48.93	47.06	4.5%
C3	**67.14**	**66.13**	**63.72**	**64.85**	**66.98**	**66.36**	**64.72**	**59.09**	**12.7%**
C4	53.33	50.15	53.70	52.97	54.83	58.35	57.78	65.38	10.5%
C5	55.77	58.14	51.19	52.07	53.99	50.72	49.41	46.54	14.4%

Note: ANX/D = Anxious/Depressed; AGG = Aggressive Behavior; ATT = Attention Problems; DYS = Severe Dysregulated; INTR = Intrusive; LOW = Low Problems; MILD = Mild Problems; RBB = Rule-Breaking Behavior; SOM = Somatic Complaints; THO = Thought Problems; WIT = Withdrawn.

Class, highlighted in bold, is "DYS-Severe Dysregulated" (including subjects with elevations in ANXD, ATT, and AGG and, more generally, in both externalizing and internalizing ASR areas scales).

2.5 Discussion

In the first experimental study, our research group examined whether different profiles of psychopathology could be identified using a person-centered statistical approach in wave 1 ReMIND project sample: a large and inclusive sample of Italian children and adolescents combining general-population and referred subjects. Moreover, the relationship between these profiles and the presence of one or more categorical diagnoses was examined.

The results highlighted the presence of 4 classes of psychopathological profiles, measured with CBCL/6-18 in 4643 children and preadolescents, aged 10–14 years old. The results appear comparable when the LCA was conducted on single subsamples (i.e., general population sample from PRISMA study and referred sample from CABALA-GENESIS studies) or on total sample. One class, which was named "Low Problems" grouped subjects without a significant risk of elevation on any scales; the second class was characterized by children and adolescents with a high risk of being in the clinical range for Attention problems and the authors named it "Attention/Hyperactivity–ADHD" class; the third class, named "Internalizing Problems–INT" grouped subjects with a high risk of elevation on the Anxious/depressed scale; the fourth class included subjects with an elevated probability of being in the clinical range for all CBCL scales, with the exception of Somatic complaints and Rule-breaking behavior and for this reason named "Severe Dysregulated–DYS" class.

Previous studies which conducted LCA using the ASEBA questionnaire version for infants, the CBCL/1.5-5 (Achenbach & Rescorla, 2000), found 4 four classes, very similar to ours (Connell et al., 2008), suggesting that these psychopathological traits are present from early stages of development.

The results of the second experimental study here exposed, allow us to evaluate if these traits might be stable through lifespan and in different cultures and societies, evaluating the presence of peculiar patterns of symptoms using ASR in adult samples. When we compare the four classes solution, which was the most fittable for six societies (i.e., Albania, Brazil, Czech, Italy, South Korea, and the USA), with the four classes obtained by studies conducted on infants (Connell et al., 2008) and and children (e.g., first experimental study, Bianchi et al., 2017), it is easy to recognize the same structure: one class predominantly internalizing, with more elevated scores in "anxious/depressed" and "withdrawn", one class predominantly externalizing, with more elevated scores in "rule breaking behaviors" and "intrusive behaviors", the third characterized by normal range scores in all the subscales and the fourth characterized by severe emotional and behavioral dysregulation (DYS).

This evidence leads to the estimation that the presence of a trait characterized by simultaneous internalizing and externalizing problems, compromising the emotional and behavioral functioning of individuals, is remarkable through development with bottom-up person centered approaches.

To our knowledge, our second experimental study (Bianchi et al., 2022) is the first study using LCA and on ED in adult general populations, whereas previous studies have focused on children (e.g., Basten et al., 2013; De Caluwe et al., 2013) or adolescents (e.g., Bianchi et al., 2017; Jordan et al., 2016; Rescorla et al., 2020) and on clinical or mixed populations (e.g., Bianchi et al., 2017; Rescorla et al., 2020).

The evidence of trans-cultural nature this trait is also remarkable: two previous studies, conducted on children and adolescents revealed the trans-cultural presence of DYS class: Jordan and colleagues (2016) used YSR data from 34 societies and Rescorla and colleagues (2020) used CBCL/6-18 data from 29 societies to identify a class associated with Dysregulation Profile, in each society. They found the presence of latent classes associated with Dysregulation Profile with prevalence ranging from 1% to 26% in the YSR data and from 2% to 18% in the CBCL/6-18 data. In both studies, the Dysregulation Profile class comprised participants with higher scores in the "Anxious/Depressed", "Attention problems", and "Aggressive behaviors" syndrome scales, but, not surprisingly, in many societies, the Dysregulation Profile class showed elevated scores on all eight syndrome scales.

In our studies the criterion to select the class valuated as the "DYS" was the higher scores in "Anxious/Depressed", "Attention problems", and "Aggressive behaviors", but despite this, consistently with these previous finding on children and adolescents (Jordan et al., 2016; Rescorla et al., 2020) also our results indicated that, when using a bottom-up rather than an a-priori approach, a latent class characterized by elevated scores on only the three Dysregulation Profile syndromes is less common than classes characterized by elevations on all eight syndromes.

This raises the issue of whether this profile is simply a marker of severe psychopathology, rather than a specific phenotype. Several studies found "top-down" Dysregulation Profile to be, rather than a predictor of a specific disorder, a marker for persistent psychopathology and significant impairment, as well as personality pathology, suicidality, and substance use (Rescorla et al., 2020). This fact is confirmed by our second step analysis results from the first experimental study, which evaluated the diagnostic sensitivity of each class. For all diagnostic categories, there was a very low percentage of subjects in the LOW class (between 4 and 20%, approximately). In the INT class, the higher percentages of diagnosis were for

anxiety disorders (42.8%) and Mood disorders (26.4%). In the ADHD class, about half of the subjects (49.0%) had a diagnosis of attention deficit hyperactivity disorder, but we also found that 23.8% of subjects had an anxiety disorder. This result is not surprising considering that anxiety disorders in both children and adults are among the disorders that most commonly co-occur with Attention Deficit Hyperactivity Disorder (Biederman et al., 1991). Finally, in the DYS class the highest percentages we found were for Anxiety Disorders (54.3%), Mood Disorders (44.5%), and Attention Deficit Hyperactivity Disorder (30.7%), underlining and confirming previous results suggesting a high heterogeneity of diagnoses assigned to individuals with emotional and behavioral dysregulation (Holtmann et al., 2011; Althoff et al., 2010; Caporino et al., 2016). Finally, when we analyzed how the four classes were able to predict three different diagnostic profiles (absence of diagnosis vs. one diagnosis vs. two or more diagnosis), we found that the LOW class better identified an absence of diagnosis, while all three other classes were able to predict the presence of comorbidity, with the DYS class being the significantly stronger predictor as reported by other studies (Biederman et al., 2013; Carballo et al., 2014). The same results were confirmed when analyses were repeated with a more conservative approach (i.e. by incorporating outcomes into the latent class model) to correctly account for uncertainty in class membership).

The two studies presented suffered from some limitations. First, the data, in both cases, were collected by a single informant. In the case of the previous study conducted with the ReMIND sample in 2016 the information was collected from parents, whilst in the second study conducted in 2022 on data from 10 different societies, only self-report forms were available. Especially for children, the combination of different points of view is essential, so future study may take into consideration the collection of data also with Youth Self Report (YSR/11–18) and Teacher's Report Form (TRF/6–18) (Achenbach & Rescorla 2001). The addition of other information and multiple sources could improve the validity and generalizability of the direction of our results.

It is important to underline that, differently from the first study, the most severe limitation of the second experimental study is the lack of sociodemographic data. Future studies could deepen the role of sociodemographic information to disentangle differences in prevalence and characteristics of DYS profile in adult samples.

In both cases, we did not consider longitudinal perspectives, following the same individuals during lifespan, which is fundamental to have information regarding the onset, progress, or

changes in the classes over time. Preliminary data regarding third wave evaluation of the ReMIND project are useful to achieve this aim and will be presented in chapter 5.

In some cases, arbitrary decision rules were needed to determine the optimal number of latent classes because the statistical indices were not always consistent, as was also found in previous studies (Jordan et al., 2016; Nasserinejad et al., 2017; Rescorla et al., 2020).

Finally, in both studies, the results of regressions and between-class ANOVAs should be viewed with caution, because not all of our models achieved optimal entropy values.

2.6 Conclusions

Using a person-centered statistical approach on data from the first wave of ReMIND project, we were able to identify four different psychopathological profiles in a large sample including both referred and general-population children and adolescents. Membership in these groups appears to be related to different positive and negative outcomes in terms of psychopathological diagnosis. In particular, our results underline the presence of a profile characterized by severe emotional and behavioral dysregulation, which is mostly associated with the presence of multiple diagnoses. Thanks to our second study, in which we replicated LCA on adult samples, counterparts of the DYS profile were found in adult population samples from 10 very diverse societies. The prevalence ranged from 6.1% to 12.7%, with an omnicultural mean of 9.2%. Despite major cultural differences across the samples, our findings indicate that clinicians and researchers should be alert to patterns of dysregulation among lifespan, from childhood to adulthood.

Chapter 3

The roles of genetics and environment: mediation models designs

3.1 Introduction

The study of possible causal factors of psychopathology is a very eclectic research field. Etiopathology study of mental health disorders and psychopathological traits take very different points of view and take use of very different technologies.

Researchers need to address not only neurobiological factors, but also environmental and psychosocial domains and their dynamic interplay to identify the complex etiology of mental disorders. Twin studies suggest that genetic factors contribute prominently to continuity in mental health problems, but environmental influences are a major contributor to dynamic change (Nees et al., 2021).

The risk factors categorized as "environmentals" comprehend a very wide range of experience: they range over from exposure to pollution to family structure, from obstetric complications to relationships with peers during adolescence.

The influence of perinatal risk factors, obstetric complications, exposure to substances during pregnancy, together with adverse life events are going to be discussed in Chapter 4.

There are several influences through the social environment, which is composed of three principal systems: family, peers and community. Each individual differs in their degree to which they are exposed to environmental variation and in ways they expressed this exposure, due to personal sensitivity. Loneliness and social exclusion might bias the perception of the social world to be more threatening or vice versa (Nees et al., 2021). This bias effect is related to stronger activity in the visual cortex in response to social stimuli, and thus greater attention to negative social information (Cacioppo et al., 2009).

Focusing on family contests, the quality of the relationship with the caregiver, which is one of the most complex and, at the same time, of most prominent environmental factor, influences the possibility of emotional regulation (Bernier et al., 2016, Holz et al., 2018): for example, distress of the infant can be effectively reduced by responsive caregivers, leading to decreasing the development of fear over time (Leerkes et al., 2009).

Important factors involved in the development of children's emotional and behavioral regulation are parenting practices and family functioning (Barnett et al., 2010, Gustafsson et al., 2012, Hardaway et al., 2012). The construct of parenting practices involves cognition, emotions and affects daily directed towards children as well as goal-directed behaviors such as showing children support, monitoring, nurturing, encouragement and companionship or

putting in place intrusive and punitive behaviors (Lee et al., 2006). Positive practices consist of parental warmth, support, positive affect and sensitivity and it is associated with greater levels of self-regulation in children (Moilanen et al., 2009), whereas negative parenting comprises punitions, hostility, neglect, excessive intrusiveness and over-control (Eisenberg et al., 2015) and it is inversely correlated with self-regulation in children (Lengua et al., 2006, Moilanen et al., 2009).

The construct of family functioning can be defined as the capacity of the family system to meet the needs of its members through various behaviors (Johnson et al., 2000). This dimension has a key role in the psychosocial well-being of all family members and it could represent a therapeutic target in clinical practice (Last et al., 2102).

Recent research on parents' psychological functioning, and parenting practices and family functioning highlights the existence of strong relationships between these two areas and offspring psychopathology; in fact, there is a probable mediating effect of parent-child relationship between parents and children's psychological problems. Aunola and colleagues (2015) found a full mediation effect of psychological control (conceived as a parenting dimension) between parents' depressive symptoms and offspring distress, conceptualized as negative daily emotions. Furthermore, Grasso and colleagues (2016), starting from an examination of how harsh parenting might mediate the relationship between exposure to intimate partner violence and disruptive behavior in children, found a partial mediation by psychological aggression towards the child. Similarly, Lieb and colleagues (2000), through a longitudinal study, found an association between internalizing pathology such as social phobia in children and parental overprotection and rejection; these peculiar parenting domains were also associated with parental internalizing symptoms as anxiety disorder. As opposed, Vostanis and colleagues (2006) found associations between absence of child psychopathology and rewarding and non-punitive parenting behaviors. These studies highlighted the existence of strong links between parenting, family functioning and parent and children's psychopathology, which is the focus of the first experimental study presented in this Chapter.

At these very early times of life, biological influences during pregnancy have been demonstrated to influence developing fetal brain systems, particularly those that are altered in mental disorders. For example, maternal cortisol levels during pregnancy, indicating elevated levels of psychosocial stress, were significantly related to stronger connectivity between the amygdala and brain regions relevant for the processing and integration of sensory information as well as the default mode network in females and reduced amygdala connectivity to these

regions in males (Graham et al., 2019). Further, in females, this connectivity mediated the association between maternal cortisol and higher internalizing symptoms (Graham et al., 2019).

As previously discussed, the influence of environment and of genetic factors are not independent from each other, and this became obvious especially when we are studying familial influences on individual differences. An individual shares both genetic makeup and environment with their parents.

In psychiatry the study of genetic risk factors focus on genes that have been demonstrated to be involved in brain development and neurotransmission (Hirschhorn et al., 2002, Nobile et al., 2007, 2009, 2010, 2016, 2021, Riva et al., 2015(a), 2015(b), State and Thapar, 2015, Zohsel et al., 2015).

In this chapter we discuss the influence of the GRIN2B gene. GRIN2B codes for the Glun2B subunit of N-Methyl-D-Aspartate (NMDA) receptor, which mediates the slow Ca+ component of excitatory synaptic transmission in the central nervous system. These molecular mechanisms are crucial in the development of different cognitive functions, such as memory, learning, and attention (Kim et al., 2016, Mascheretti et al., 2015). In fact, GRIN2B variants have been associated not only with ADHD (Dorval et al., 2006), but also with cognitive deficits in heterogeneous neurodevelopmental and psychiatric disorders, including autism spectrum disorders, Alzheimer's and Parkinson's diseases, bipolar disorder, and schizophrenia (Endele et al., 2010, Hassan et al., 2016, Hu et al., 2016, Lorenzi et al., 2010, Seripa et al., 2008). Among an a priori selected panel of 47 genes with well-established molecular and biological functions in animal memory, GRIN2B variants associated with episodic memory in the general population (de Quervain & Papassotiropoulos, 2006).

Therefore, it is clear that both genetics and environment might influence brain systems and functions. In particular, in recent years, the joint analysis of genomic and neuroimaging data, known as imaging genetics, has provided the opportunity to get a more complete knowledge of how genetic and neurobiological factors interplay to determine behaviors (Meyer-Lindenberg & Weinberger, 2006). Imaging genetics research sets its roots on the evidence of a close association of genetics with brain structure and function, in accord with the notion that brain physiology is etiologically closer to molecular biology than behavior (Zayed & Robinson, 2012). In this line, studies of genetic effects on behavior should account for

neuroimaging parameters as intermediate phenotypes, which may influence the link between genetic variants and complex behaviors.

In imaging genetics literature, there is evidence of an association between variants in glutamate system genes, including GRIN2B polymorphisms, and neuroimaging phenotypes in healthy and clinical populations (Kuswanto et al., 2013, Stein et al., 2010). Recent magnetic resonance studies have suggested a link between GRIN2B variants and abnormal glutamatergic neurotransmission and brain volume in children and adolescents with obsessive compulsive (Arnold et al., 2009) and alcohol use (Dalvie et al., 2016) disorders.

In the second experimental study, for the first time, we explore the possible link between GRIN2B marker variants, changes in brain morphology, and attention/hyperactivity problems in an adolescent sample.

3.2 Aim of the chapter

The chapter aim is to clarify, in our sample, the role of different risk factors in shaping psychopathological traits. For this reason, we conducted two experimental studies to evaluate the different exposures of individuals with higher psychopathology to previously identified genetic and environmental risk factors. In both studies, also the interdependence between different factors has been taken into account, and for this reason in both studies we used mediation analysis design. Both studies were conducted on different subsample of clinically referred wave 2- ReMIND sample.

In the first experiment, published in Bellina and colleagues (2020), was conducted including children and adolescents and their parents. We investigated the association between parents' internalizing/externalizing symptoms, parenting practices and children's internalizing/externalizing symptoms. Parent and child psychopathology were assessed with the Internalizing and Externalizing scales of the ASR and CBCL/6–18, respectively.

The second study presented is a genetic informed neuroimaging study, in which we implemented an exploratory mediation design with the intent to verify whether GRIN2B polymorphisms influence attention and hyperactivity phenotypes and, if yes, if such an effect is mediated (and in what proportions) by brain morphology. Second work results are published in Maggioni and Mauri and colleagues (2021).

3.3 Experimental study 1

The study protocols were approved by the Research Ethical Committee of IRCCS Eugenio Medea Scientific Institute and have been performed in accordance with the ethical standards

laid down in the 1964 Declaration of Helsinki and its later amendments. Parents' written informed consent was obtained for all participants.

3.3.1 Methods

3.3.1.1 Sample

The subsample involved in the present study consisted of 272 children and adolescents aged 9–18 and their biological parents (aged 30–65) who took part in the Cabala-Genesis Project (ReMIND project second wave data collection).

Specifically, 272 mothers and 242 fathers took part in the study; parents from 242 families were married or partners, whereas 30 participating families were characterized by divorced or separated parents. In the second case, only mothers accepted to participate in the present research project. Parents were asked to complete questionnaires about their parenting practices or family functioning and children's psychopathological symptoms. Participants were excluded if they had an associated neurologic, genetic, infectious or metabolic disorder, or a seizure disorder, cognitive disability (IQ < 70), pervasive developmental disorders, severe hypoacusia, hypovision or severe linguistic comprehension deficit.

Table 3.1 shows the children's and parents' demographic characteristics.

Table 3.1: Children's and parents' demographic characteristics

	Males (n = 208)	Females (n = 64)	Total sample (n = 272)
Child's Age (M ± Sd)	14.4 ± 2.3	15.2 ± 2.2	14.5 ± 2.3
Family SES (M ± Sd)	52.8 ± 16.7	52.0 ± 17.8	52.6 ± 16.9
Mother's Age (M ± Sd)	45.8 ± 4.9	46.2 ± 5.0	45.8 ± 5.0
Father's Age (M ± Sd)	48.5 ± 5.1	48.1 ± 6.1	48.4 ± 5.4

Note: Sd = Standard deviation; SES = Socio-Economic Status

3.3.1.2 Measures

The sample demographic characteristics were collected together with family socioeconomic status (SES), evaluated through the Four Factor Index of Social Status (Hollingshead, 1975). The SES value is measured through the subjects' education level, employment, marital status and birth assigned sex. The values are grouped in four categories: 0 = very low SES, 10 to 35 = low SES, 40 to 65 = medium SES and more than 70 = high SES.

Emotional-Behavioral

Behavioral and emotional screening was conducted with ASEBA questionnaires.

The ASR/18-69 (Achenbach & Rescorla, 2003) is a 126-item self-report questionnaire for adults assessing emotional and behavioral functioning aspects. Both mothers and fathers completed the form, referring to themselves. In addition to the syndrome scale, the ASR problem items can be scored in two broad groupings of syndromes, "Internalizing", which consider the scores from three syndrome scales: anxious/depressed, withdrawn and somatic complaints. This grouping is defined as Internalizing because it comprises problems that exist mainly within the self. The second grouping, designated as Externalizing, consider the scores from three other syndromes scales: aggressive behavior, rule-breaking behavior and intrusive behavior. The problems comprising the Externalizing grouping mainly involve conflicts with other people and with social mores. Items are rated on a 3-point scale: 0 = Not True, 1 = Somewhat or Sometimes True, 2 = Very True or Often True.

In this study, we took into account the T-scores of the mother Internalizing (M-Int) and Externalizing (M-Ext) Scales, as well as father Internalizing (F-Int) and Externalizing (F-Ext) values.

T-scores are standardized values, displayed in a normal distribution with a mean of 50 and a standard deviation of 10 in absolute values. Psychometric and cross-cultural properties of ASR are given in the relative manual and by Ivanova and colleagues (Achenbach & Rescorla, 2003, Ivanova et al., 2015).

CBCL/6–18 is divided into eight syndrome scales: Anxious/depressed, Withdrawn/depressed, Somatic complaints, Social problems, Thought problems, Attention problems, Rule-breaking behavior, and Aggressive behavior. Like the ASR, the CBCL/6–18 is divided into two major factors: the Internalizing scale (with the anxious/depressed, withdrawn and somatic complaints subscales) and the Externalizing scale, which consists of 35 items and two subscales: rule breaking and aggressive behavior.

In this study, we used the T-score of Internalizing (C-Int) and Externalizing (C-Ext) scales

based on the Italian population (Achenbach & Rescorla, 2001), with a normal distribution (mean = 50, standard deviation = 10). Good internal consistency for Italian versions of the CBCL/6–18 ($\alpha > .78$ for Total Problems and the two broadband scales, and $\alpha > .65$ for most narrow band scales) was reported (Frigerio et al., 2004).

Parenting practices

Parenting practices and family functioning have been investigated with the 'background section' (Family Life Questionnaire, FaLQ) of the parent and adolescent versions of the Development and Well-Being Assessment (DAWBA) diagnostic interview (Goodman et al., 2000). The FaLQ enables the collection of information about the child's and his or her family's life context and parenting practices (Bøe et al., 2014) through a 13-item questionnaire investigating four theoretical scales: Affirmation (four items), Rules (two items), Discipline (four items) and Special Allowances (three items). Participants are asked to indicate how well the descriptions in the questionnaire apply to their child using a 4-point scale: 0 = Not at all, 1 = A little, 2 = A medium amount, 3 = A great deal. It differs from other parenting assessments by measuring the experience of family/parents in relation to a single child and it allows to assess differences in parent–child and sibling–sibling relationships.

Affirmation is defined as behaviors that the parent puts in place to support or help children in various situations or to show them approval and affection and refers to parent-child relationship; examples of Affirmation items are "gets love and affection", "is praised and rewarded", "gets help and support when stressed" and "is liked and respected for who they are". Affirmation subscale has a large convergent validity ($r = .56$, $p < .001$) with Alabama Positive Parenting subscale (APQ, Elgar et al., 2007), one of the most used questionnaire concerning parenting practices.

The Rules scale is defined as the ability to create coherent and shared family rules and to enforce them and it measures structure and organization within the family. Examples of Rules items are "there are clear rules about what they are expected to do and what they are not allowed to do" and "these family rules are applied consistently". Last and colleagues (2012) found that the divergent validity between the FaLQ Rules and the Alabama inconsistent discipline scale (Elgar et al., 2007) was moderate ($r = -.45$, $p < .001$).

Discipline is defined as behaviors by parents in response to and intended to correct misbehavior by the children and it refers to punishment; examples of discipline items are

"told off or corrected for things they do wrong", "physically punished (e.g. a smack or a slap)" and "punished in other ways (e.g. things they like are taken away, grounded, time out)".

Special Allowances refers to various overprotection behaviors as opposed to lack of supervision; hence, it is related to over- and under-involvement from parents. Examples of allowance items are "leads a very protected life" and "spends time by themselves (e.g. with TV, music, games, books)".

Regarding psychometric properties, Last and colleagues (2012) found good internal consistency and test-retest reliability of Affirmation and Rules scales. Discipline subscale had a poor internal consistency but good test-retest reliability, whereas Special Allowance scale had poor internal consistency and moderate test-retest reliability.

3.3.1.3 Data analyses

To describe the sample of children and their parents, frequencies of CBCL and ASR Internalizing and Externalizing Problems scale scores in the clinical range were computed. Based on the ASEBA Multicultural Manual, we considered a clinical range of scores with corresponding T > 63 for the Internalizing and Externalizing Problems scales. Ratings of child and parental psychopathology were computed to describe the sample.

Descriptive analyses were conducted on all of our variables of interest. We analyzed the distribution of variables using absolute cut-offs of skewness, kurtosis and distribution plots.

Correlation analyses

Correlation analyses were conducted to examine possible linear relationships between M-Int, M-Ext, F-Int, F-Ext, C-Int, C-Ext and parenting practices. Association between psychopathology, parenting practices and demographic variables of interest was also analyzed.

Multiple mediation models

Multiple mediation models were built to assess whether the relationship between parents' and children's psychopathology severity was mediated by the quality of parenting practices. We hypothesized that the relationship between parents' psychopathology (M-Int, M-Ext, F-Int, F-Ext) and children's psychopathology (C-Int, C-Ext) could be partly explained by the mediation of parenting practices (Affirmation, Special Allowances, Discipline and Rules).

We performed these analyses using Jamovi software, version 1.0.7.0, implemented in R (The

Jamovi project, R Core Team). The amplitude of the effects was estimated through bootstrapping methodology with a 1000-resampling iterations process to produce robust bootstrapped standard errors (SEs) and 95% confidence intervals (CIs). Demographic variables (gender, SES, and parents' and children's age) were inserted into the models as covariates to control for confounding variables. Two models were built (see Figure 3.1).

Model 1: M-Int, M-Ext, F-Int, F-Ext as predictors (independent variables); Affirmation, Special Allowances, Discipline and Rules (parenting practices) as mediators; and C-Ext as outcome variable (dependent variable).

Model 2: M-Int, M-Ext, F-Int, F-Ext as predictors (independent variables); Affirmation, Special Allowances, Discipline and Rules (parenting practices) as mediators; and C-Int as outcome variable (dependent variable).

The total effect explains the relationship between the considered independent variable (M-Int, M-Ext, F-Int or F-Ext, respectively) and the dependent variable (C-Int or C-Ext, respectively) through a simple regression analysis. In this step, values are not partialized out of mediators' effects.

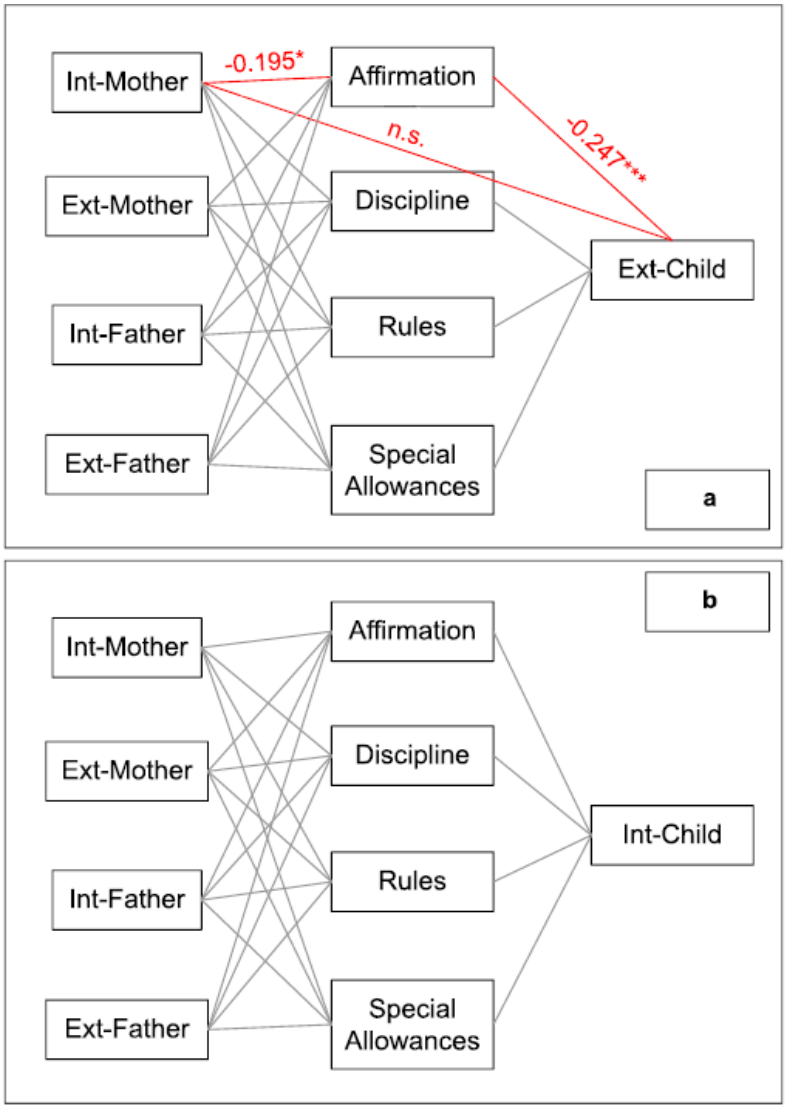
The direct effect explains the association between the considered independent variable (M-Int, M-Ext, F-Int or F-Ext, respectively) on the dependent variable (C-Int or C-Ext, separately) while keeping the other independent variables and mediator values constant. This effect offers an index of association between a parent psychopathology scale and a child psychopathology scale net of sociodemographic, other parent psychopathology variables and mediators' effect.

The indirect effect is a value of the association that passes through the mediator, net of the direct effect between parent and child psychopathology. If the indirect effect is significant, a mediation effect occurs.

Full mediation occurs when direct effect is not significant but single regressions between parent psychopathology and parenting measures, firstly, and parenting measures with children psychopathology, secondly, are significant. Partial mediation occurs when the mediator accounts for some, but not all, of the relationships between the independent variable and the outcome.

Finally, mediation is rejected when either one of the two regressions between independent variable, mediator and dependent variable is not significant.

Figure 3.1: Mediation analyses design



Note: Red line with asterisk: significant parameter; ns: non-significant; *: $p < 0.05$; ***: $p < 0.001$

3.3.2 Results

Table 3.2 provides an overview of frequencies of Internalizing and Externalizing scores in the clinical range assessed by CBCL and ASR. All variables were normally distributed. Distribution Plots indicated no extreme outliers for any of the variables of interest.

Table 3.2: Children's and parents' psychopathology measures

	Internalizing T-values		**Externalizing T-values**	
	(M ± Sd)	**N in clinical range**	**(M ± Sd)**	**N in clinical range**

		(%)		**(%)**
Mothers (n = 272)	55.2 ± 9.6	86 (31.6%)	50.3 ± 8.8	42 (15.4%)
Fathers (n = 242)	53.4 ± 9.5	62 (25.7%)	50.4 ± 8.8	40 (16.5%)
Children (n = 272)	57.0 ± 9.6	101 (37.1%)	53.4 ± 9.0	67 (24.6%)

Correlation analyses

Correlation results are reported in Table 3.3. Pearson linear correlations indicate that both parents' internalizing and externalizing problems are positively related to child psychopathology for internalizing and externalizing problems. Regarding parental measures, Affirmation and Rules scales are negatively related to C-Int and C-Ext scales and to M-Int and M-Ext scales; regarding F-Int, F-Ext scales, this result is significant only for the Affirmation subscale. The parenting Discipline scale is positively related only to C-Ext; Special Allowances in parenting is negatively related only to C-Int.

Table 3.3: Pearson linear correlation coefficients

	C-Ext	C-Int	M-Ext	M-Int	F-Ext	F-Int	Aff.	Rules	Disc.	Spec. All.	SES
C-Int	**.477** ***										
M-Ext	**.377** ***	**.423** ***									
M-Int	**.348** ***	**.441** ***	**.627** ***								

F-Ext	**.209****	**.185****	**.183****	**.206****							
F-Int	**.239*****	**.339*****	**.257*****	**.294*****	**.630*****						
Aff.	**-.359****	**-.190****	**-.228****	**-.293****	**-.210****	**-.164***					
Rules	**-.214****	**-.213****	**-.223****	**-.236****	-.081	-.046	**.452*****				
Disc.	**.205***	.024	.043	-.044	.003	.008	.067	**.139***			
Spec. All.	.065	**-.167****	.098	.099	-.009	.099	.040	.076	**.179****		
SES	-.106	-.076	-.020	-.108	-.019	.000	.041	.078	-.114	.097	
Age	-.102	-.015	-.076	-.066	.048	-.003	-.039	-.070	**-.225****	-.002	-.004

Note: C-Int = Children's Internalizing Problem scale; C-Ext = Children's Externalizing Problem scale; M-Int = Mother's Internalizing Problem scale; M-Ext = Mother's Externalizing Problem scale; F-Int = Father's Internalizing Problem scale; F-Ext = Father's Externalizing Problem scale; Aff. = Affirmation; Disc. = Discipline; Spec. All. = Special Allowances; Gend. = Gender. *$p < .05$; **$p < .01$; ***$p < .001$.

Multiple mediation models

The model results are depicted in Figure 3.1 and Tables 3.4, 3.5 and 3.6. Results from the mediation analysis indicated that M-Int is related to C-Ext through an indirect effect of Affirmation. The occurring relationship was direct: high levels of mother's psychopathology corresponded to high levels of child's symptomatology. As shown in Table 6, the total effect was significant ($p = 0.045$), with a β coefficient of 0.155 (also confirmed by bootstrap percentile CI). Regression results showed a significant ($p = 0.017$) negative effect of M-Int on Affirmation, with a β coefficient of − 0.195; moreover, regression analysis with Affirmation as an independent variable and C-Ext as a dependent variable was significant ($p < 0.001$) with a β coefficient of − 0.247. Specifically, high levels of M-Int were related to low Affirmation,

which in turn was associated with high C-Ext. The direct effect was not significant (p = 0.068); thus, the mediating effect of Affirmation between M-Int and C-Ext was total (Table 3.6). No other significant mediating effects were found in our multiple mediation models.

Table 3.4a: Mediation model, indirect (mediated) effects.

Effect			**β coefficient**	***SE***	**z**	***p***
I.V.	**Mediator**	**D.V.**	**(Bootstrap percentile CI)**			
Mother - Internalizing	Affirmation	Child - Externalizing	**0.048 (0.009; 0.095)**	0.022	2.052	**0.040**
	Discipline		-0.035 (-0.083; 0.008)	0.023	-1.428	0.153
	Rules		0.010 (-0.008; 0.037)	0.012	0.824	0.410
	Special Allow.		0.002 (-0.010; 0.017)	0.006	0.359	0.719
Mother - Externalizing	Affirmation		0.022 (-0.018; 0.065)	0.021	1.134	0.257
	Discipline		0.021 (-0.028; 0.074)	0.025	0.861	0.389
	Rules		0.020 (-0.004; 0.058)	0.017	1.241	0.215
	Special Allow.		-0.001 (-0.012; 0.011)	0.006	-0.154	0.878
Father - Internalizing	Affirmation		-0.009 (-0.054; 0.036)	0.022	-0.381	0.703
	Discipline		-0.004 (-0.037; 0.038)	0.019	-0.180	0.857
	Rules		-0.009 (-0.035; 0.008)	0.011	-0.768	0.442
	Special Allow.		0.004 (-0.012; 0.033)	0.011	0.390	0.697
Father - Externalizing	Affirmation		0.044 (-0.003; 0.101)	0.026	1.777	0.076
	Discipline		0.006 (-0.035; 0.043)	0.020	0.291	0.771
	Rule		0.012 (-0.007; 0.045)	0.013	0.993	0.321

	Special Allow.		-0.004 (-0.027; 0.011)	0.009	-0.418	0.676

Table 3.4b: Mediation model, indirect (mediated) effects.

Effect			β coefficient (CI)	*SE*	z	*p*
I.V.	**Mediator**	**D.V.**				
Mother - Internalizing	Affirmation	Child - Internalizing	-0.009 (-0.044; 0.018)	0.014	-0.584	0.560
	Discipline		0.002 (-0.015; 0.025)	0.010	0.191	0.849
	Rules		0.015 (-0.014; 0.047)	0.015	0.946	0.344
	Special Allow.		0.011 (-0.010; 0.039)	0.012	0.920	0.358
Mother - Externalizing	Affirmation		-0.004 (-0.032; 0.008)	0.010	-0.428	0.669
	Discipline		-0.001 (-0.022; 0.013)	0.008	-0.146	0.884
	Rules		0.028 (0.001; 0.085)	0.021	1.454	0.146
	Special Allow.		-0.004 (-0.029; 0.023)	0.012	-0.335	0.738
Father - Internalizing	Affirmation		0.002 (-0.012; 0.021)	0.008	0.200	0.841
	Discipline		0.001 (-0.010; 0.011)	0.005	0.041	0.967
	Rules		-0.012 (-0.045; 0.0140)	0.014	-0.859	0.390
	Special Allow.		0.021 (-0.020; 0.056)	0.015	1.412	0.158
Father - Externalizing	Affirmation		-0.008 (-0.050; 0.016)	0.015	-0.557	0.578
	Discipline		-0.001 (-0.013; 0.010)	0.005	-0.061	0.951
	Rules		0.017 (-0.010; 0.057)	0.016	1.156	0.248
	Special Allow.		-0.018 (-0.052; 0.001)	0.014	-1.399	0.162

Note: **Table 3.4a:** Parenting style mediating parents' internalizing and externalizing psychopathology (I.V.) and child's externalizing psychopathology (D.V.). Covariates: gender, SES, children's age and parent's age.

Table 3.5: Parenting style mediating parents' internalizing and externalizing psychopathology (I.V.) and child's internalizing psychopathology (D.V.). Covariates: gender, SES, children's age and parent's age. I.V.: Independent Variable; D.V.: Dependent Variable; SES: Socio-Economic Status; Special Allow.: Special Allowances; CI: confidence interval; *SE*: Standard Error; z: Goodman Test value; *p*: probability value.

Table 3.6: Focus on the significant mediating effect of parenting style: Affirmation.

Effect		**β coefficient (Bootstrap percentile CI)**	***SE***	**z**	***p***
Direct	Int-Mother → Ext-Child	0.130 (-0.002; 0.256)	0.066	1.823	0.068
Component	Int-Mother → Affirmation	**-0.195 (-0.069; -0.008)**	0.015	-2.394	**0.017**
	Affirmation → Ext-Child	**-0.247 (-1.771; -0.660)**	0.287	-4.260	**< .001**
Total	Direct + Indirect	**0.155 (0.004; 0.286)**	0.072	2.008	**0.045**

Note: Direct effect, specific indirect effect and total effect of maternal internalizing psychopathology on children's externalizing psychopathology through Affirmation (parenting style). Covariates: gender, SES, children's age and parent's age. SES: Socio-Economic Status; Int: Internalizing; Ext: Externalizing; CI: confidence interval; *SE*: Standard Error; z: Goodman Test value; *p*: probability value.

3.4 Experimental study 2

The study protocols were approved by the Research Ethical Committee of IRCCS Eugenio Medea Scientific Institute and have been performed in accordance with the ethical standards

laid down in the 1964 Declaration of Helsinki and its later amendments. Parents' written informed consent was obtained for all participants.

3.4.1 Methods

3.4.1.1 Sample

The sample included 58 unrelated clinically referred individuals (42 males and 16 females, aged 8.78±2.43 years at first evaluation) who were a subsample of the Cabala-Genesis Project (ReMIND project first and second waves data collections). This unique subsample was evaluated on three different occasions from pre-adolescence to adolescence. At first and second evaluations (T0 and T1), data regarding emotional and behavioral problems were collected using the CBCL/6-18 (Achenbach & Rescorla, 2001). On the third occasion (T2), the assessment included blood drawing for genetic analyses and a magnetic resonance imaging (MRI) session. T1 and T2 were conducted 5.74±1.66 and 7.39±1.65 years after T0, respectively.

Exclusion criteria were diagnoses of Autism Spectrum Disorder or Intellectual Disability, neurological diseases (including epilepsy and traumatic brain injuries), severe sensory and linguistic comprehension deficits.

3.4.1.2 Measures

Socio-economic status

Employment is used as a measure of socio-economic status (SES) coded according to the Hollingshead 9-point scale for occupation (Hollingshead, 1975)

Emotional-Behavioral

Behavioral and emotional screening was conducted with CBCL/6-18 (Achenbach & Rescorla 2001), filled out by parents. CBCL/6–18 is divided into eight syndrome scales: Anxious/depressed, Withdrawn/depressed, Somatic complaints, Social problems, Thought problems, Attention problems, Rule-breaking behavior, and Aggressive behavior. In this study, we employed the attention problems (AP) scale T scores (mean = 50; standard deviation = 10) based on the set of multicultural norms "group 2", which applies to the normative sample of the Italian population as suggested by the multicultural supplement to the ASEBA manual (Achenbach & Rescorla 2007; Ivanova et al., 2007). The AP scale consists of 11 items (e.g., cannot concentrate, cannot pay attention for long, is impulsive or acts without thinking) assessing both inattentive and hyperactive-impulsive symptoms. For

the AP scale, the ASEBA identifies as normal scores below 65, as borderline scores between 65 and 70, and as clinical scores above 70. In the following analyses, we considered the mean score obtained in the AP scale across the three time points, which represents a stable measurement of this behavioral dimension over development.

Diagnoses were evaluated with Kiddie schedule for affective disorders and Schizophrenia for school-age children-present and lifetime version (K-SADS-PL, Kaufman et al., 1997). K-SADS-PL is a semi-structured diagnostic interview created to assess current and past episodes of psychopathology in children and adolescents according to DSM criteria. All subjects in the Cabala-Genesis project (clinically referred sample) were assessed through K-SADS-PL interviews.

The participants' sociodemographic, clinical and cognitive characteristics are reported in Table 3.7.

Table 3.7: Participants sociodemographic, clinical and cognitive characteristics

	Total sample N = 58
T0	
Age (years) [1]	8.78 ± 2.43
SES [1]	45.52 ± 20.17
FSIQ [1]	108.96 ± 15.65
CBCL Attention Problems [1]	64.05 ± 9.80
K-SADS-PL DIAGNOSIS	
ADHD [2]	13 (22.4%)
Any behavioral disorder [2]	10 (17.2%)
Any mood disorder [2]	18 (31.0%)
Any anxiety disorder [2]	28 (48.3%)
Other diagnoses [2]	12 (20.7%)
No current diagnosis [2]	3 (5.2%)
Comorbidities [2]	1 diagnosis = 33 (56.9%) 2 diagnoses = 18 (31.0%)

	3 diagnoses = 4 (6.9%)
T1	
Age (years) [1]	14.52 ± 2.23
CBCL Attention Problems [1]	60.19 ± 7.78
T2	
Age (years) [1]	16.17 ± 2.41
CBCL Attention Problems [1]	58.16 ± 6.43

Note: [1] = Mean ± standard deviation; [2] = N (%); SES = socioeconomic status; FSIQ = full-scale intelligence quotient; K-SADS-PL = Kiddie schedule for affective disorders and schizophrenia for school-age children-present and lifetime version; ADHD = attention deficits/hyperactivity disorder; CBCL = Child Behavior Checklist.

Genotyping

Participants' DNA was obtained from saliva samples collected and extracted using Oragene OG-500 kits (DNA Genotek, Ottawa, Canada). Amplification and sequencing of GRIN2B regions allowed us to type rs2268119 A/T, rs22161128 A/G, rs5796555 -/A, rs1012586 G/C, rs11609779 C/T, rs2192973 G/A Single Nucleotide Polymorphisms (SNPs). Amplifications were performed in 10-μL reactions using JumpStart REDAccuTaq LA DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) and the following protocol: 30 s at 96 ◦C, 35 cycles of 15 s at 94 ◦C/20 s at 58 ◦C/30 s at 68 ◦C, 5 min final elongation time. Sequencing reactions were performed with a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Monza, Italy) and run on ABI Prism 3130xl (Applied Biosystems, Monza, Italy) and 3500AV Genetic Analyzers (Applied Biosystems, Monza, Italy). Table 3.8 shows allelic frequencies and Hardy-Weinberg equilibrium (HWE) for the considered markers. Genotype distributions did not significantly deviate from HWE. No SNPs were therefore excluded from further analyses. The GRIN2B linkage disequilibrium structure (Figure 3.2) shows a squared correlation coefficient between 0.00 and 0.74. Genotypes were grouped into a two-level variable, each level representing the presence or absence of minor frequency alleles.

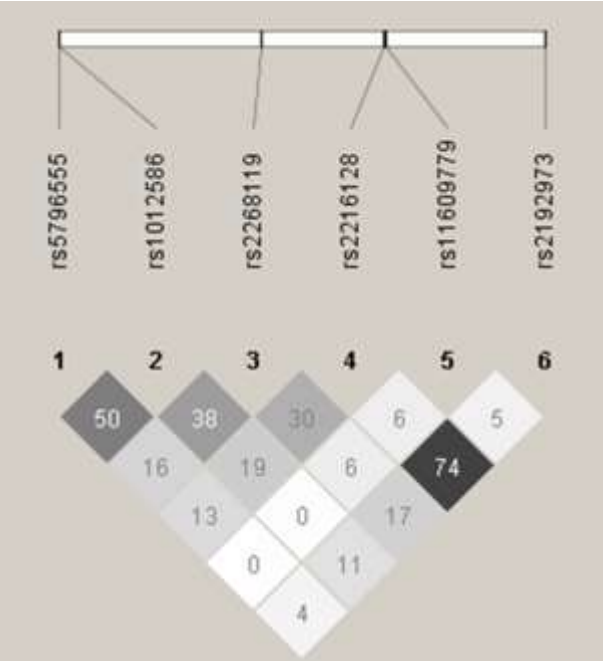
Table 3.8: GRIN2B allele frequencies and Hardy-Weinberg equilibrium's p-values.

GRIN2B SNP	Allele	Frequency [1]	Hardy-Weinberg Equilibrium
rs5796555	- A	0.71 0.29	0.201

rs1012586	G C	0.66 0.34	0.744
rs2268119	A T	0.73 0.27	0.213
rs2216128	A G	0.74 0.26	0.146
rs11609779	C T	0.84 0.16	0.546
rs2192973	G A	0.78 0.22	0.115

Note: SNP: single nucleotide polymorphism. [1] Fraction of the total. - is marker of the allele.

Figure 3.2: GRIN2B linkage disequilibrium. Haploview plot showing pairwise linkage disequilibrium (r2 values) for 6 SNPs of GRIN2B based on the sample's genotypes.



Imaging

MRI data acquisition

Structural MRI data were acquired in the University Hospital of Udine (Udine, Italy) using a Philips Achieva 3.0 Tesla scanner (Philips, Amsterdam, The Netherlands) equipped with an 8-channel head coil for radiofrequency transmission and reception. All images were obtained with a T1-weighted MPRAGE 3D TFE sequence, with the following parameters: echo time =

3.7 ms, repetition time = 8.1 ms, in-plane field of view = 240 × 240 mm^2 , in-plane matrix size = 240 × 240, 190 axial slices with no gap, voxel size = 1 mm^3.

MRI data processing

The MRI images were processed using the open-source Freesurfer software, v5.3.0 (http://surfer.nmr.mgh.harvard.edu/, downloaded on March 8,2017) (Fischl, 2012), which provides an accurate 3D reconstruction of the cerebral cortex. For each subject, starting from the T1-weighted image, Freesurfer performs a brain tissue segmentation and estimates the gray matter/white matter interface, which is used to model the cortical surfaces. In our study, the segmentation output and the reconstructed surfaces were visually inspected and corrected, if necessary, by a trained user. The subject's cortical model was parceled into regions of interest (ROIs) based on the Desikan-Killiany atlas (Desikan et al., 2006) and cortical thickness (CT), cortical surface area (CSA), and gray matter volume (GMV) were estimated at the ROI level and used for the following analyses.

3.4.1.3 Data analyses

General Linear Model (GLM) Analyses

In a set of preliminary GLM analyses based on in-house Matlab scripts (R2018b, The Mathworks, Inc. Natick, MA, USA), we investigated the relation among GRIN2B markers, brain morphology and inattention/hyperactivity.

First, we evaluated the impact of GRIN2B SNPs on neuroanatomical parameters (design #1), GRIN2B SNPs on inattention/hyperactivity (mean CBCL/6-18 AP score over time) (design #2). We then selected the morphological parameters significantly influenced by the GRIN2B SNP/SNPs exerting significant effect on attention/hyperactivity (intersection of results #1 and #2) and investigated their possible influence on the CBCL/6-18 AP variable (design #3).

GRIN2B SNPs and regional morphological parameters were investigated one by one in separate models, i.e., we performed 1116 GLM analyses for design #1 (combination of 62 ROIs, 3 surface-based measures for each ROI and 6 GRIN2B SNPs) and 6 GLM analyses for design #2. In all GLM designs, age and gender were added as covariates to remove their possible contribution to the results. In designs #1 and #3, the total intracranial volume was used as a normalization factor when focusing on GMV, while the total surface area was used when focusing on CSA.

We made inference using double-sided t-tests, where the significance threshold was set to p = 0.05. In the GLM design in #1 analyses, in order to limit false positive rates, a correction for

multiple comparisons (MC) was applied (N = 37, 31 regions in each hemisphere + 6 GRIN2B markers).

The GLM results were examined to detect any joint relationships among GRIN2B SNPs, ROI parameters, and CBCL/6-18 AP score. The mutually related variables were used in the following mediation analysis, with the objective to check whether the causal effect of GRIN2B marker variants on attention/hyperactivity phenotype was mediated (and, if yes, in what proportions) by brain morphology.

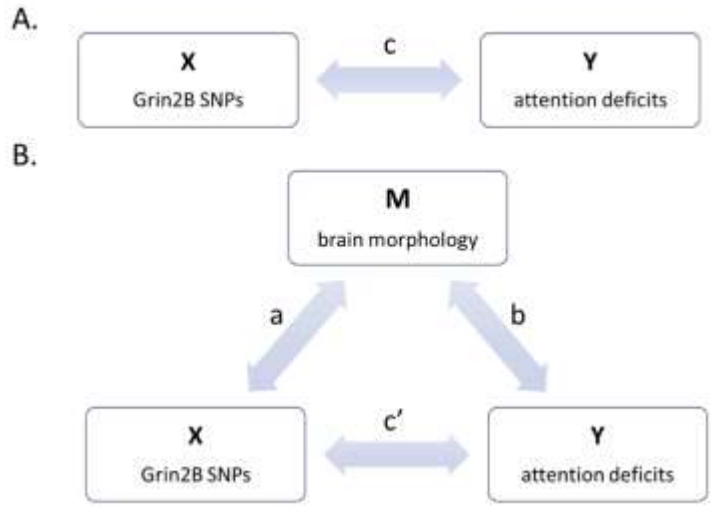
Mediation Analyses

Mediation was assessed using the open-source Bootstrap Regression Analysis of Voxelwise Observations (BRAVO) toolbox (https://sites.google.com/site/bravotoolbox/, downloaded on March 5, 2018) in Matlab. On each triad of selected variables, we designed a simple mediation model, where the GRIN2B SNP was the causal variable X, the CBCL/6-18 AP score the outcome Y, and the ROI morphological parameter the mediator M (Figure 3.3).

Provided that X significantly accounts for variability in both Y (path c) and M (path a), and M accounts for variability in Y when covarying for X (path b), M is mediator of the X-Y relationship if the effect of X on Y substantially decreases when M is entered simultaneously with X as predictor of Y (path c'). Further details on mediation models can be found in Preacher and Hayes (2008).

In our study, we used a mediation regression model with age and gender as covariates. The mediation significance was assessed through a permutation procedure with 5000 iterations. Before running the analyses, the values of X, Y and M were normalized using the Z-score standardization. The strength of model paths, for both observed data and bootstrap distributions, was assessed through Ordinary Least Square regression. Confidence intervals and p-values were then estimated using the bias corrected and accelerated formula described in DiCiccio and Efron (1996). For all model coefficients (a, b, a*b, c, c'), the significance threshold was set to p = 0.05. Multiple comparison corrections were performed if appropriate, based on the number of mediation models applied.

Figure 3.3: Mediation model design. (A) Model of direct effect of X on Y. (B) Mediation model, where X has both direct and indirect (through M) effects on Y.



3.4.2 Results

GLM Analyses

Design #1. GRIN2B Effects on Neuroanatomy

The GLM statistics concerning GRIN2B effects on brain regional morphology are reported in Table 3.9 We detected significant associations between rs5796555-/A marker and regional GMV, and rs2268119A/T and rs2216128T/C markers and regional CSA ($p < 0.05$, MC corrected). On the contrary, no significant effects of GRIN2B markers on CT emerged.

Specifically, the less frequent allele 'A' of marker rs5796555 -/A was associated with GMV deficits in left isthmus of cingulate cortex, left precuneus, right caudal and rostral anterior cingulate cortex, right transverse temporal gyrus and bilateral rostral middle frontal gyrus, inferior parietal gyrus, middle temporal cortex and pars orbitalis. Notably, this effect was highly significant ($p < 0.01$, MC corrected) in regions of the right hemisphere, that is, caudal and rostral anterior cingulate cortex and inferior parietal gyrus. As inferred from Table 3.9, the peak T statistics was observed in the caudal portion of the right anterior cingulate cortex.

We also found a negative association between the genotype carrying the less frequent allele 'T' of marker rs2268119A/T and CSA in left lateral orbitofrontal cortex ($p < 0.05$, MC corrected) and right lateral occipital cortex ($p < 0.01$, MC corrected), and the genotype carrying the minor allele 'C' of marker rs2216128G/C and CSA in the right isthmus of the cingulum ($p < 0.05$, MC corrected). None of the above brain features were affected by gender or age ($p > 0.05$).

Design #2. GRIN2B Association with Attention/Hyperactivity Problems

The GLM analyses assessing the impact of GRIN2B markers on inattention/hyperactivity revealed a significant positive association between the genotype carrying the minor allele 'A' of marker rs5796555-/A and the mean CBCL/6-18 AP score ($T(54) = 2.41$, $p < 0.05$). No influences of gender or age on this score emerged ($p > 0.05$).

Design #3. Neuroanatomy Effects on Attention/Hyperactivity Problems

In view of the results of designs #1 and #2, the neuroanatomy-attention GLM analyses were performed on the only brain morphological parameters influenced by GRIN2B marker rs5796555-/A (Table 9). We found that CBCL/6-18 AP score was inversely proportional to GMV in the left isthmus of the cingulate cortex ($T(54) = 2.67$, $p < 0.05$) and in the right inferior parietal cortex ($T(54) = 2.26$, $p < 0.05$), suggesting a possible role of these regions as mediators of the effect of GRIN2B marker rs5796555-/A on inattention/hyperactivity.

Table 3.9: General Linear Model results of GRIN2B markers effect on brain morphological parameters.

***GRIN2B* SNP**	**Allele**	**FS Feature**	**Brain Region**	**T_1**	***p***	**p_{corr}**

						[2]
rs5796555	'-/A' and 'A/A'	Volume	Left inferior parietal	3.72	< 0.001	< 0.05
			Left isthmus cingulate	3.62	< 0.001	< 0.05
			Left middle temporal	3.90	< 0.001	< 0.05
			Left pars orbitalis	3.72	< 0.001	< 0.05
			Left precuneus	3.78	< 0.001	< 0.05
			Left rostral middle frontal	3.59	< 0.001	< 0.05
			Right caudal ACC	4.32	< 0.0001	< 0.01
			Right inferior parietal	4.14	< 0.001	< 0.01
			Right middle temporal	3.43	0.001	< 0.05
			Right pars orbitalis	3.72	< 0.001	< 0.05
			Right rostral ACC	3.95	< 0.001	< 0.01
			Right rostral middle frontal	3.49	< 0.001	< 0.05
			Right transverse temporal	3.40	0.001	< 0.05
rs2268119	'A/T' and 'T/T'	Area	Left lateral orbitofrontal	3.40	0.001	< 0.05
			Right lateral occipital	3.98	< 0.001	< 0.01
rs2216128	'G/C' and 'C/C'		Right isthmus cingulate	3.42	0.001	< 0.05

SNP: single nucleotide polymorphism; FS: FreeSurfer software; ACC: Anterior cingulate cortex. ROI: region of interest. [1] General Linear Model design: ROI parameter ~ 1 + Gender + Age + GRIN2B SNP. Observations were 58, error degrees of freedom were 54. ROI volumes were normalized using total intracranial volume. ROI surface areas were normalized using total cortical surface area. [2] Level of significance after multiple comparison correction: $p < 0.0016$.

Mediation Analyses

In view of the GLM results, two separate mediation analyses (MA1 and MA2) were performed to investigate the relationship among GRIN2B rs5796555-/A genotype (causal variable X), CBCL/6-18 AP score (outcome Y) and (i) GMV in left isthmus of cingulate cortex (mediator variable M1), (ii) GMV in right inferior parietal cortex (mediator variable M2). No mediation analyses were performed on GRIN2B genotypes or brain features other than those specified due to the absence of the mediation model prerequisites.

The mediation model parameters, 95% confidence intervals (CI) and *p*-values are reported in Table 10. In line with preliminary GLM results, both mediation analyses confirmed a significant total effect of rs5796555-/A genotype on CBCL/6-18 AP score (c = 0.31, $p < 0.05$).

As shown in Figure 3.4, MA1 results confirmed that genotypes carrying the minor allele A of rs5796555-/A were associated with GMV deficits in the left isthmus of cingulate cortex (a = –0.45, $p < 0.001$), and in turn that such deficits (while regressing out rs5796555-/A effect) were linked to CBCL/6-18 AP score (b = –0.25, $p < 0.05$). After inclusion of M1 in the model, the direct effect of rs5796555-/A on CBCL/6-18 AP score was not significant (c' = 0.20, $p = 0.09$), whereas its indirect effect through GMV in the left isthmus of cingulate cortex remained significant (a*b = 0.11, $p < 0.001$). Specifically, 35.89% of the total rs5796555-/A effect on attention was mediated by GMV in this region.

On the contrary, MA2 showed that rs5796555-/A genotypes were linked to GMV in the right inferior parietal gyrus (a = –0.48, $p < 0.001$), but did not confirm a significant influence of the right inferior parietal deficits on attention/hyperactivity problems while controlling for rs5796555-/A contribution (b = –0.18, $p = 0.08$). The absence of a net effect of M2 on Y ruled out the investigation of any mediated effects of this variable. Of note, since only MA1 analysis was successfully conducted, no multiple comparison corrections were performed on MA1 analysis coefficients.

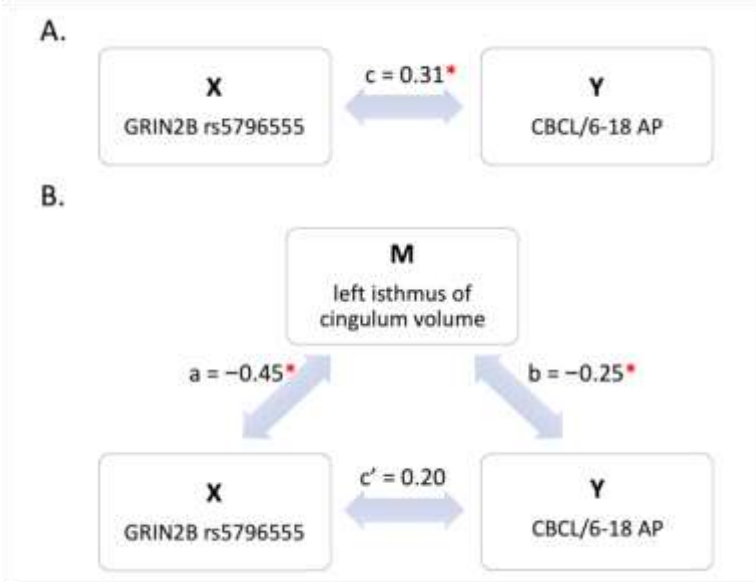
Table 3.10: Summary of mediation results.

Mediation analysis 1	**Mediation analysis 2**

Parameter	Value	95% CI	*p*	Parameter	Value	95% CI	*p*
a	–0.45	[–0.26 0.26]	< 0.001	a	0.48	[–0.26 0.26]	< 0.001
b	–0.25	[–0.27 0.26]	0.037	b	–0.18	[–0.25 0.29]	0.08
c	0.31	[–0.27 0.26]	0.011	c	0.31	[–0.27 0.26]	0.011
a*b	0.11	[–0.04 0.05]	< 0.001	a*b	n.e.	n.e.	n.e.
c’	0.20	[–0.27 0.27]	0.09	c’	n.e.	n.e.	n.e.

Causal variable X: *GRIN2B* marker rs5796555-/A. Outcome variable Y: CBCL/6-18 attention problem score. Mediator variable MA1: left isthmus of cingulate cortex volume. MA2: right inferior parietal cortex. CI: confidence interval. n.e: not evaluated.

Figure 3.4: Mediation analysis 1 (MA1) results. (A) Model of direct effect of X on Y. (B) Mediation model, where X has both direct and indirect (through M) effects on Y. Significant pathways are highlighted with *. CBCL/6-18 AP: Child Behavior Checklist Attention Problem score.



3.5 Discussion

In the first experimental study, we examined the potential role of parenting practices mediating the complex relationship between parents’ and child’s psychopathology in clinically referred subsample of wave 2 ReMIND project. To our knowledge, this was the

first time this relation was investigated in an Italian sample of children with internalizing and externalizing symptomatology.

The results suggested the existence of significant associations between maternal and child psychopathological symptoms through a full mediation of parenting practices. These results are in line with previous research: the association between parental and child psychopathology was well established (Karimzadeh et la., 2017, Vostanis et al., 2006), and similar results regarding parenting mediation were found by Aunola and colleagues (2015).

Specifically, our mediation models results showed that the association between mothers' internalizing symptomatology and children's externalizing psychopathology was fully mediated by Affirmation dimension, a measure of supportive practices and displays of approval and affection by parents towards their offspring. General mother's internalizing psychopathology explained lower levels of affective practices. Those practices could lead, in turn, to an increase in children's maladaptive and externalizing symptoms. On the contrary, our results suggested that positive supportive practices lead to increased functional conduct in the offspring, as highlighted in previous literature (Dishion et al., 2008).

Regarding fathers, our results showed that both father's externalizing and internalizing symptoms are correlated with child psychopathology, confirming recent evidence that underlines the importance of father inclusion and his role in clarifying child symptomatology (Flouri, 2010, Lamb, 2010, Narayanan & Nærde, 2016, Parent et al., 2017).

However, we did not find any effects in the mediation model about father psychopathology and parenting practices. The literature on this topic explains similar findings through the fact that in most families mothers are the primary caregiver, and children may be more influenced by the caregiver who is most involved in their lives (Lewis & Lamb, 2003, Pinquart 2017, Rothbaum & Weisz, 1994). Indeed, only a few papers (Aunola et al., 2015, Vera et al., 2012), found that the relationship between child and father psychopathology is mediated by overprotection.

Further studies, also including genetic and neurobiological variables, would better define the relationship between child's and father's psychopathology and father's practices.

Furthermore, it is important to note that these findings should be considered relevant for all Italian socioeconomic statuses, given the lack of correlation effect between the SES variable and the clinical data in adults and children.

The second experimental study took into consideration risk factors of different nature. In fact, in this case, we performed a preliminary genetic-neuroimaging-behavioral approach study investigating potential causal effects of GRIN2B markers on developmental

attention/hyperactivity problems through neuroanatomy. We genotyped 6 GRIN2B markers and measured brain morphological parameters and inattention/hyperactivity in clinically referred specific subsample of wave 2 ReMIND project followed from childhood to adolescence.

The results confirmed the presence of a causal chain of relationships among the three variables, showing that GRIN2B rs5796555-/A effects on inattention/hyperactivity over time is significantly mediated by volume in the left isthmus of cingulate cortex.

To our knowledge, this is the first study on GRIN2B that considered multiple time measures of behavioral attention/hyperactivity problems. The use of the mean CBCL/6-18 AP score across three time points allowed us to smooth the variability due to different manifestations of this complex behavior from childhood to adolescence. In fact, CBCL/6-18 AP scale assesses both inattentive and hyperactive-impulsive symptoms linked to ADHD, and longitudinal studies suggest that these dimensions may follow separate developmental trajectories and have different manifestations at different ages (Rietveld et al., 2004).

In line with previous studies, we found a significant relation between persistent attention deficits and the GRIN2B gene. Specifically, subjects carrying the minor allele 'A' of GRIN2B rs5796555-/A were characterized by higher CBCL/6-18 AP score. Our findings further confirm the crucial role of GRIN2B in behavioral functions. Given the importance of the Glun2B subunit of NMDA receptor for maturation and plasticity of the central nervous system, it is not surprising that over 60 variants of GRIN2B have been associated with heterogeneous neurodevelopmental and psychiatric disorders (Hu et al., 2016). Previous literature reported evidence of the association of GRIN2B gene variants with attention deficits in general population and patient samples. Of note, in a previous study of our group, Riva and colleagues (2015a) found an association between GRIN2B genotypes and CBCL/6-18 AP score in a general population sample aged 6-11. Dorval and colleagues (2006) investigated inattentive and impulsive symptoms in a sample of ADHD children and found a positive correlation between both symptom classes and nine GRIN2B SNPs. On the same line, a study on attention performance in ADHD patients linked GRIN2B and GRIN2A variants to increased susceptibility to attention problems (Kim et al., 2016).

Our study confirms and strengthens these findings, showing a role of GRIN2B rs5796555-/A in the genetic risk for inattention/hyperactivity traits that remain stable during development.

In the growing imaging-genetics field, just a few studies explored the link between GRIN2B markers and brain morphology. We assessed, for the first time, the influence of 6 GRIN2B SNPs on a set of morphological features. The extraction of regional cortical thickness,

volume and surface area has offered a unique opportunity to delineate the structural brain correlates of GRIN2B variants with high specificity. We found selective associations between GRIN2B markers and morphological features. The brain feature that resulted to be most widely influenced by GRIN2B SNPs was regional gray matter volume (GMV). On the contrary, the cortical thickness feature showed no influences of the GRIN2B markers. The minor allele of marker rs5796555-/A was associated with lower GMV in frontal, parietal and temporal regions. Conversely, the minor allele of marker rs2268119A/T was associated with lower cortical surface area (CSA) in left frontal and right occipital regions, and rs2216128G/C genotypes carrying the minor allele 'C' showed CSA deficits in the right cingulate cortex. Previous studies already showed an association between glutamatergic genes and regional GMV in children neurodevelopmental disorders involving attention deficits at different levels. Probably due to different clinical populations and research methods, literature results are mixed. In patients with obsessive compulsive disorder (OCD), Wu and colleagues (2013) reported GRIN2B SNPs to be associated with total thalamus volume. In another pediatric OCD study, a significant association between left orbitofrontal and right anterior cingulate volumes and GRIN2B SNPs emerged (Arnold et al., 2009). Of note, GRIN2B was linked to left posterior cingulate volume in adolescents with alcohol dependence, one of the disorders most closely related to impulsivity (Dalvie et al., 2016). Since these findings emerged from clinical samples that share only some features with attention deficit syndromes, further investigations are needed to confirm our findings and interpret them in a wider dimensional perspective.

To our knowledge, the only imaging-GRIN2B study that focused on inattention/hyperactivity is a resting state functional MRI (fMRI) study on ADHD children, which showed GRIN2B influence on regional homogeneity in left superior parietal cortex, being part of the attention circuit and with role in inhibition (Kim et al., 2017).

Overall, these results suggest that GRIN2B regulation is not confined to specific brain regions but involves complex brain networks. Indeed, precuneus, cingulate, prefrontal, orbitofrontal, inferior parietal and temporal cortices, which were found to be affected by GRIN2B markers, are included into the default mode network (DMN, Greicius et al., 2003; Ismaylovaet al., 2018). The DMN is a spontaneous resting state network that deactivates during task performance, whose activation has been implicated in attention and, specifically, in exteroceptive and interoceptive attentional orientation (Fransson, 2005, 2006, Gusnard et al., 2001, Leech & Sharp, 2014). The DMN failure to deactivate during tasks might result in attentional intrusions and deficits in performance (Sonuga-Barke & Castellanos, 2007).

Moreover, posterior and rostral cingulate cortex, prefrontal cortex, and inferior parietal lobule are part of the frontoparietal control network (Leech et al., 2011, 2012, Spreng 2012, Vincent et al., 2008), involved in executive control. During tasks demanding direct attention to external information, activity increases in the frontoparietal control network and decreases in DMN (Kelly et al., 2008).

The above evidence supports the hypothesis that GRIN2B effects on brain structure might be interpreted in terms of brain circuitries, especially those that in turn impact on behavioral functions.

In the imaging-behavioral analysis, we deliberately focused on the brain regions that resulted to be affected by GRIN2B rs5796555-/A, which may act as intermediate biological phenotypes in GRIN2B effect on inattention/hyperactivity. The study of the link between other brain features (e.g., regional CSA or CT) and attention/hyperactivity problems went beyond the scope of our study, but could be the subject of future investigations.

Interestingly, we found a negative association between CBCL/6-18 AP score and GMV in the left isthmus of the cingulum and right inferior parietal cortex. The involvement of posterior cingulate cortex and inferior parietal lobule in functional networks of attention control may explain the relation between attention problems and structural abnormalities in these areas and further supports the aforementioned network-based perspective.

In imaging-genetic studies, instead of directly measuring the association between complex behavioral phenotypes and genetics, brain functionality and anatomy might be used as reliable intermediate phenotypes, with a more direct and interpretable relation with genetics.

The results of our preliminary GLM analyses support the hypothesis that regional GMV (in left isthmus of cingulate cortex and right inferior parietal gyrus) might mediate GRIN2B effect on inattention/hyperactivity.

Hence, two separate mediation analyses were performed to investigate the relationship among GRIN2B rs5796555-/A genotype, mean CBCL/6-18 AP score and, as mediator, GMV in left isthmus of cingulate cortex and right inferior parietal cortex.

The failure to verify the second mediation hypothesis suggests that GMV in the right inferior parietal cortex, besides being regulated by GRIN2B rs5796555-/A, does not shape genetic susceptibility for inattention/hyperactivity.

On the contrary, our results suggest that GMV in the left isthmus of the cingulate cortex may play a key role in this mechanism. Indeed, after inclusion of this parameter in the mediation model, the direct effect of GRIN2B rs5796555-/A on CBCL/6-18 AP score became not significant, whereas its indirect effect through GMV in this region emerged to be significant.

Specifically, more than 30% of the GRIN2B rs5796555-/A genotype effect on CBCL/6-18 AP score was mediated by GMV in the left isthmus of cingulate volume. Therefore, we believe that this region might play a relevant role in translating GRIN2B variation to the complex attention phenotype.

The two studies presented suffered from several limitations. In the first study, multiple measures were obtained by the same informants (i.e. parents) and this could lead to shared method variance and inflate the estimates of results. Further studies regarding internalizing and externalizing psychopathology reported by multiple informants are needed. Moreover, since parental reports regarding their parenting practices and their family functioning are often biased as parents tend to give socially desirable answers and avoid reporting problematic behaviors to a third party (Fransson, 2005, 2006), it would be desirable to include observational assessments around parenting functioning.

In the first study, a genetic characterization was not included, whilst in the second study environment was not taken into account. Future research is needed to disentangle environmental versus genetic influences on familial psychopathology transmission and gene-environment interactions, which are implicated in the development of psychopathological complex behaviors. We should investigate the interactions between genes and the environment through epigenetics.

Indeed, specifically regarding the second study, the sample size was limited. The MRI acquisitions required a high level of patients' compliance, which was not always achieved due to the young age and clinical characteristics of our sample. The use of a modest sample has limited the statistical power and the reliability of the emerged imaging-genetic-behavioral associations, which need to be reproduced on larger, independent samples. Moreover, the integration of genetic, neuroimaging, and psychopathologic information enhanced the possible sources of error. Larger sample size replications are needed in order to minimize the risk of false positive results. Regarding the measurement of attention/hyperactivity problems, we used the CBCL/6-18 "Attention Problems" subscale, fulfilled by the participants' caregivers. This scale evaluates both inattention and impulsivity from a hetero-referred point of view. In the future, it is desirable to integrate the measurement of these traits with neuropsychological tasks or clinical measures from different raters, and to disentangle the contributions of inattention and hyperactivity problems. A final, intrinsic limitation of our study concerns the focus on a specific gene of the glutamatergic pathway. This choice was driven by growing evidence from genetic studies, suggesting a role of GRIN2B in attention

deficits/hyperactivity traits. Given the increasing interest in polygenic risk factors, future study extensions should additionally consider the effect of other genes implicated in this behavior. We should investigate the interactions between genes, also known as “epistasis”.

In both studies, we used a cross-sectional design (in the case of the second study, we had three evaluation of psychopathological trait, but we decided to use the mean of the three time point in order to smooth the variability due to different manifestations of this complex behavior), so our findings provide only a static view of the data: for first experimental study, the mediation model’s causal nature could be better addressed in further longitudinal studies. Therefore, first study results do not provide information on the onset of, progress of or changes in the symptoms over time. For these reasons, it is not possible to provide any certainty on the direction of effects. The situation is different for the second experimental studies, in which the different nature of the variables permits to theoretically account for this relation. Moreover, it is worth mentioning that our research protocols included children and adolescents with emotional and behavioral difficulties but not comparison subjects.

3.6 Conclusions

Both the studies presented used mediation models to evaluate the relationship between risk factors of very different nature and psychopathological traits in clinically referred wave 2 ReMIND project sample. Thanks to the first study, we observed that positive parenting practices, in particular affirmation, have a relevant role in mediating the link between mother and child symptomatology presentation. This is particularly relevant when programming interventions and represents an implication to be taken into account in clinical and educational areas. Our second experimental study highlighted that the complex relationship between genetic makeup and psychopathological traits might be mediated by neural structures, specifically, we focused on volumetry, which is closely related to GRIN2B variations and can act as intermediate phenotype between genetics and inattention/hyperactivity behaviors.

There is great need for longitudinal studies which take into account risk and resilience factors of different nature and enable to better delineate the genetic, neuronal and environmental mechanisms contributing to developmental risk pathways: the results of these kink of studies would have important implications for effective prevention, identification and treatment of early-onset psychiatric disorders.

Chapter 4

The role of (epi)genetics: a link between genetic and environment factors in understanding internalizing and externalizing clusters

4.1 Introduction

Recently, the literature started to focus on the study of psychopathological traits in adolescence, a very delicate period of life, in which the individual faces up to several changes in terms of biological development and new environmental requests.

Worldwide research results depict early adolescence as a challenging moment, which is in many cases characterized by a decrease in psychological well-being. This impact on mental health can also persist and affect youths' future development (Muratori et al., 2021). The percentage of youth that experience emotional and behavioral problems in Italy is up to 18% (Frigerio et al., 2009, Gritti et al., 2014, Smorti et al., 2019).

Given that psychopathological manifestations seem to span along constructs, reflecting a continuous rather than a dichotomy between health and illness, and between one diagnosis and another, and that a wide range pattern of psychopathological manifestations cannot be associated with a specific diagnosis of mental disorder, much research in this field focuses on psychopathological traits rather than on psychiatric categorical diagnosis.

In neurodevelopmental psychopathology, the use of the terms "internalizing" and "externalizing" traits is well established to indicate class of psychopathological manifestations, symptoms and behaviors which may be found trans-diagnostically. Internalizing problems refer to inwardly focused negative behaviors such as anxiety, depression, and somatic symptoms, while externalizing problems refer to outwardly focused negative behaviors such as hyperactivity, aggression, disruptive conduct, and substance use (Achenbach and Rescorla, 2001, Tien et al., 2020). There are high rates of overlap between internalizing and externalizing problems. Very recent data from the New Zealand Dunedin Multidisciplinary Health and Development Study, for example, show that less than 15% of participating individuals diagnosed with externalizing or internalizing disorders showed only homotypic symptomatology (Caspi et al., 2020).

Both internalizing and externalizing disorders appear to increase when children enter early adolescence. Internalizing and externalizing behaviors during childhood are predictive of later negative adolescent and adult behavioral, emotional, cognitive, and physical health outcomes, including increased risk for aggression and violence, substance abuse, depression,

anxiety disorders, lowered academic competence, and increased long-term mortality risk in adulthood (Tien et al., 2020).

In recent years, the use of bottom up approaches and machine learning (ML), as methods to approach psychopathological traits complexity has increased. ML techniques automatically learn patterns from big data and are not restricted by data structure.

The psychopathological literature in this area is at a very premature stage, but it seems a promising field.

Some studies focused on identifying behavioral patterns presentation within specific disorders and disabilities (Grazioli et al., 2021, Price et al., 2022, Schraegle et al., 2022).

In a study conducted with the aim of addressing interrater reliability problems regarding categorical diagnoses, the authors used a machine learning methodology to estimate potential syndromes in psychopathology, based on people's open-ended narratives about their lived experiences with mental illness (Ghosh et al., 2022). Clustering was performed on data provided by 10,933 patients with at least one diagnosis of mental health disorder, and they found 4 clusters of which approximated the following DSM-5 categories: eating disorders, obsessive–compulsive and related, depressive disorders, anxiety disorders disorders.

Very few studies applied cluster analysis to the scope of evaluating psychopathological traits in adolescence from the general population.

Amendola and colleagues (2021) evaluated the presence of clusters of symptoms within the internalizing area of psychopathology in an Italian community based sample of 1127 children and adolescents, aged 8–16 years. The four clusters identified were labeled as follows: "high somatic symptoms and average depression/anxiety", "high somatic symptoms and high depression/anxiety", "average somatic symptoms and above average depression/anxiety", "low somatic symptoms and low depression/anxiety".

Muratori and colleagues analyzed the whole spectrum of psychopathology using these methods with Strengths and Difficulties Questionnaire (SDQ; Goodman, 1997) in an Italian sample of 2959 preadolescence (from 10 to 14 years) from the general population. They found 4 clusters of psychopathological traits presentation, two of them characterized by low levels of psychopathology (74.5% of the total sample) and two characterized by "predominantly internalizing" of "predominantly externalizing" symptoms (19% and 6.5% of the total sample, respectively).

In this chapter, a study aimed to analyze the presence of different psychopathological outcomes in Wave 2 clinically referred ReMIND Project subsample is presented.

To reach our aim, we applied an unsupervised machine learning (ML) algorithm to evaluate the presence of specific clusters of symptoms in both externalizing and internalizing areas measured with the Child Behavior Checklist/6-18 (Achenbach & Rescorla, 2001).

This will allow us to identify, with a bottom-up strategy, groups of patients with different psychopathological traits outcomes. In fact, despite the fact that initially the whole sample was composed of help seeking children, we expect to find different clusters characterized by different expressions of internalizing and externalizing symptoms severity.

Than, the second goal was to test whether the identified clusters of adolescents differ for the presence of environmental risk factors (i.e., presence of stressful life events, of pre- and postnatal risk factors, socioeconomic and demographic characteristics) and specific epigenetic profiles (specifically, DNA methylation)

Moving from a fully data driven approach to a supervised prediction model, we used a further ML algorithm - a decision tree classifier - to evaluate the role of environmental risk factors (i.e., presence of stressful life events, of pre- and postnatal risk factors, socioeconomic and demographic characteristics) and epigenetic profiles (specifically, DNA methylation) in the causation of psychopathological traits clusters

It is well established the need to account for genetic and environmental risk factors when trying to understand complex phenotypes such as psychopathological traits (Hirschhorn et al., 2002, Nees et al., 2021, Pinto et al., 2015, State and Thapar, 2015).

In literature, environmental risk factors comprehends adverse experience that may occur pre- and perinatally.

Prenatal exposure to maternal alcohol, smoking, and drugs use during pregnancy, pregnancy-related and birth complications (e.g., preeclampsia, maternal obesity, maternal diabetes, maternal hypertensive disorder, maternal infection/inflammation, maternal exposure to heavy metals or other neurotoxicants, cesarean section, perinatal hypoxia, preterm birth) have been associated with increased risk of psychopathological behaviors and disorders in children (Giannopoulou et al., 2018, Modabbernia et al., 2017, Monk et al., 2019, Rutter and Azis-Clauson; 2015, Tien et al., 2020;).

Later in life stressful experiences continue to have a great impact on mental health: adverse childhood experiences (ACEs) have been widely associated with long term impact on individuals physical and mental health, with a significantly increased risk for sexual risk

taking behaviors, mental ill health, problematic alcohol use, drug use and interpersonal and self-directed violence (Hughes et al., 2017). ACEs includes childhood physical or sexual abuse, household of substance abuse, criminality or mental illness, exposure to domestic violence, emotional, psychological or verbal violence, divorce or separation of parents, neglect, familiar financial problems or conflicts, bullying, death of a parent or a close relative or friend, separation from family, serious childhood illness or injury.

An important potential source of complexity in the study of risk factors for psychopathology is the gene–environment interaction. This occurs when the relation between environmental risk factors and a phenotype is conditional on individual differences in genetics, or, on the other side, when the relation between individual genotype and a phenotype is conditional on environmental experiences (Pinto et al., 2015).

Epigenetics is defined as different mechanisms that explain how environmental experiences, which an individual may have faced pre birth as well as during childhood, might influence the development of psychopathology later in life. In fact, through epigenetic mechanisms, environmental adverse events influence modulation of DNA transcriptional activity (Hyman, 2009). Epigenetics is defined as chemical modifications of DNA, or of the structural regulatory proteins bound to it, that alter DNA transcription and protein production, without changing nucleotide sequences (Provenzi et al., 2018). DNA methylation, which is also responsible for cell differentiation, is the most studied of these mechanisms in psychopathology: it works through the binding of a methyl group to specific 5′-cytosine guanine-3′ dinucleotides (i.e., CpG sites) (Provenzi et al., 2018).

The presence of altered methylation profiles, as a consequence of environmental events, has been seen in both animal and human studies. Animal studies found that environmental manipulation is associated with long term effects in behaviors, in particular the ones related to stress responses, and have associated alterations in neurotransmitter functioning and structural brain changes (Champagne et al. 2008, Meaney 2010, Stevens et al. 2009). These results have been also replicate in human studies: there is a consistent amount of studies that documented altered DNA methylation of various genes in individuals exposed to stressful adverse experiences during prenatal (e.g., maternal depression) and postnatal life (e.g., childhood abuse) (Beach et al., 2011, Maud et al., 2018, Provenzi et al., 2018, Thaler 2014).

4.2 Aim of the chapter

The aim of the experimental study presented in this chapter is to examine the hypothesis that different groups of adolescents exist within our clinically referred sample, that show specific

profiles on Child Behavior Checklist/6-18 and that also present distinct environmental and epigenetic risk factors. Our first goal was therefore to identify separate groups of patients by analyzing externalizing and internalizing traits on Child Behavior Checklist/6-18 with an unsupervised machine learning algorithm. The second goal was to test whether the identified clusters of adolescents differ for the presence of environmental risk factors (i.e., presence of stressful life events, of pre- and postnatal risk factors, socioeconomic and demographic characteristics) and specific epigenetic profiles (specifically, DNA methylation)

Subsequently, moving to a supervised algorithm - a decision tree classifier - we evaluated the role of environmental risk factors (i.e., presence of stressful life events, of pre- and postnatal risk factors, socioeconomic and demographic characteristics) and epigenetic profiles (specifically, DNA methylation) in the causation of psychopathological traits clusters.

4.3 Experimental study

The study protocols were approved by the Research Ethical Committee of IRCCS Eugenio Medea Scientific Institute and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Parents' written informed consent was obtained for all participants.

4.3.1 Methods

4.3.1.1 Sample

The sample included 205 clinically referred individuals who were a subsample of the Cabala-Genesis Project (ReMIND project second wave data collection). All the participants have been referred during childhood to the Eugenio Medea Scientific Institute for emotional and behavioral problems.

The presence of neuropsychiatric diagnosis was evaluated at Wave 1 with Kiddie schedule for affective disorders and Schizophrenia for school-age children—present and lifetime version (K-SADS-PL, Kaufman et al., 1997) and confirmed by an expert clinician. In the subsample taken into consideration in this work, child neuropsychiatric diagnoses were: anxiety disorders 43.90%, attention deficit hyperactivity disorder 31.71%; mood disorders 12.20%; oppositional defiant disorder or conduct disorder 4.88%; one subject presented obsessive compulsive disorder, and 6.83% of the sample did not receive any categorical diagnosis.

Exclusion criteria were: diagnoses of autism spectrum disorder or intellectual disability, neurological diseases (including epilepsy and traumatic brain injuries), severe sensory and linguistic comprehension deficits.

4.3.1.2 Measures

Socio-demographics

Age, birth assigned sex, and familiar socioeconomic status (SES) were collected. SES was coded according to the Hollingshead scale (Hollingshead, 1975) from parental employment.

Emotional-Behavioral

Information regarding psychopathological symptoms was collected through the Child Behavior Checklist for Ages 6-18 (CBCL/6-18) (Achenbach & Rescorla, 2001), from the Achenbach System of Empirically Based Assessment (ASEBA), filled out by parents. The questionnaire allows to screen for a wide range of emotional, behavioral and social problems in children and adolescents aged from 6 to 18. It gives back 8 empirically based syndromic scales (i.e., Anxious/depressed, Withdrawn/depressed, Somatic complaints, Social problems, Thought problems, Attention problems, Rule-breaking behavior, and Aggressive behavior) and 3 summary scales based on the previous mentioned syndromic scales (i.e., Total problems scale, Internalizing problem scale which takes into account Anxious/depressed, Withdrawn/depressed, and Somatic complaints scales, and Externalizing problems scale which takes into account Rule-breaking behavior, and Aggressive behavior scales). The presence of homogeneous clusters of psychopathological symptoms have been evaluated using T scores of Internalizing and Externalizing scales.

Environmental risk factors

- *Stressful life events (SLEs)*: the presence of SLE was evaluated through parents reports with DAWBA interview (Goodman et al., 2000): the interview background section list the following SLE: to be in a serious accident or to be badly hurt in an accident; to have a serious illness which required a stay in hospital; the death of a parent, sibling or close friend; the end of a close friendships; to have the family faced a major financial crisis (e.g., losing the equivalent of three months income); any other stressful events affecting the child or the family. The presence of at least one SLE has been considered as an environmental risk factor.

- *Perinatal risk factors*: The presence of pre- and perinatal risk factors has been evaluated: with an ad hoc questionnaire filled out by parents. The questionnaire asks for the presence of viral, parasitic or bacterial infections during pregnancy (e.g., rubella, syphilis, flu, rubella, toxoplasma gondii, herpes simplex virus - type 2, borna disease). Moreover, the experience of obstetric complications and fetal growth problems has been evaluated (e.g., RH factor incompatibility, pre-eclampsia, threat of abortion, premature rupture of membranes, labor > 24 hours or "precipitous", twin birth - simple or complicated, prolapse or rupture of the umbilical cord or kinking of the cord umbilical around the newborn's neck, premature birth with duration of gestation < 37 weeks or delayed delivery with duration of gestation > 42 weeks, caesarean section - complicated or urgent, breech or abnormal presentation, use of forceps or other delivery tools, birth weight < 2 kg, incubator / resuscitation / "blue baby", newborn with gross physical abnormalities). The presence of at least one perinatal risk factor has been considered as an environmental risk factor.

Epigenetic measures

DNA methylation levels were obtained from saliva samples, with non-invasive procedures (for details regarding procedures see **Appendix**). The methylation status of specific portions of the BDNF, FKBP5, IGF2, and OXTR genes were assessed with PCR amplification of bisulfite-treated DNA followed by next-generation sequencing. Specifically, we analyzed:

- Brain-derived neurotrophic factor (BDNF) intron 1 (chr11:27723077-27723244, 11 CpGs);
- FK506-binding protein 5 gene (FKBP5) intron 7 (chr6:35558405-35558550, 3 CpGs);
- Insulin-like growth factor-2 (IGF2) differentially methylated region (chr11:2169373-2169658, 5 CpGs);
- three regions of Oxytocin receptor (OXTR): promoter (chr3:8811488-8811837, 7 of 9 CpGs analyzed), intron 1 (chr3:8810654-8810919, 13 CpGs), and exon 3 (chr3:8809340-8809530, 15 CpGs).

BDNF is a neurotrophin associated with different aspects of neural development and differentiation, such as survival of nerve cells, neurite outgrowth, and synaptic plasticity. Specifically, BDNF is implicated in the regulation of dopaminergic, cholinergic and serotonergic neurons (Zheleznyakova et al., 2016). Increased BDNF methylation have been found to be an important indicator of the impact of early developmental stress (Roth et al., 2011), and to be associated with the presence of both internalizing and externalizing

symptoms, such as suicidality risk and borderline personality disorder (Perroud et al., 2013). Altered BDNF promoter methylation is associated with impulse-dysregulation and mood instability in patients with bulimia nervosa (Thaler 2014).

FKBP5 is a regulator of glucocorticoid receptor sensitivity (Binder 2009). Glucocorticoids are responsible for the physiologic stress response via corticosteroid-activated glucocorticoid receptors. Impaired glucocorticoids signaling, leading to partial glucocorticoid resistance, is one of the biological abnormalities most observed in mood disorders and anxiety disorders (Binder 2009; Pariante and Miller, 2001) Together with serotonin-transporter and catechol-O-methyl-transferase, FKBP5 is responsible for the 30% genetic influence on post traumatic stress disorder (PTSD) (Young 2017). Methylation in this gene has been associated with PTSD and major depression (Menke et al., 2013, Mehta et al., 2011), in interaction with the presence of early trauma; Klengel and colleagues found that this relationship seems to be dependent from allele-specific environmentally dependent changes in methylation of specific FKBP5 sites.

Insulin-like growth factor-2 (IGF2) gene provides information for an important fetal hormone that plays an important role in pre-birth brain development, but also mature mammalian brains (Pardo et al., 2019). Altered IGF2 expression has been linked to anxious and depressive symptoms. The presence of maternal anxiety during pregnancy has been associated with changes in DNA methylation of the IGF2 gene in cord blood mononuclear cells at birth (Mansell et al. 2016, Vangeel et al. 2015), and with changes in the IGF2 mRNA levels in the placental tissue (Mina et al. 2015). There is evidence of patients with PTSD that, even years after trauma, preserved alterations in stress response pattern, including IGF2 genes in peripheral blood (Zieker et al. 2007). IGF2 is also expressed in the human hippocampus, a brain region implicated in depression: in adult monozygotic twins, variability in the regulation of IGF2 expression is correlated with the status of depression (Cordova-Palomera et al. 2015).

Oxytocin (OXT) is a neuropeptide and hormone implicated in prosocial and emotional functioning (Shamay-Tsoory & Young). OXT plays a crucial role in enhancing mother-child bonding and in moderating postpartum stress response: higher OXT levels are associated with enhanced empathy and decreased plasma levels of stress hormones in the mothers (Maud et al., 2018; Swain et al., 2008; Heinrichs et al., 2001). There are currently few studies investigating methylation in OXT receptor (OXTR) sites, but preliminary results showed some associations between the presence of perinatal stress indicators and methylation levels of cord blood oxytocin receptor (Unternaehrer et al., 2016), and, during childhood, between

methylation levels of OXT sites and social anxiety (Ziegler et al., 2015) and autism (Elagoz et al.,2016). Maud and colleagues (2018) suggested that increased methylation in the OXTR (which may indicate reduced expression) is implicated in impairments in social, cognitive and emotional functioning, whilst decreased methylation may have a role in impairments related to mood and anxiety disorders.

Paired ends reads from each sample were independently aligned to all the reference sequences by a parallel striped Smith-Waterman algorithm (Smith, 1981). Only paired reads that aligned coherently to the same reference sequence were retained. At each CpG site in each sequence, the 4 base frequencies were evaluated and reported along with the C→T percentage.

Methylation data, grouped in percentages and reads were screened and pruned based on the number of reads for each subject and each gene, not taking into account all the values below 100 reads count.

4.3.1.3 Data analyses

Statistical analyses were performed using R statistical software (R core Team, 2021, version 4.1.0) with the additional "mclust" package for Gaussian mixture modeling and the decision tree algorithm was implemented using the 'caTools' (Tuszynski, 2021), 'RWeka' (Hornik et al., 2009), and 'caret' (Kuhn, 2020) toolboxes. The alpha level was set to 0.05 for all analyses.

First, descriptive statistics were preliminarily computed to check the distribution of variables in the whole sample.

Unsupervised ML: cluster analysis on clinical measures

The presence of homogeneous clusters of psychopathological outcomes were evaluated using an unsupervised algorithm, the finite mixture model (FMM) implemented on the T scores of CBCL/6-18 Internalizing and Externalizing scales (Achenbach & Rescorla, 2001). Models estimating solutions of two or more clusters were compared using the Bayesian information criterion (BIC), with the best model having a BIC value closer to 0 (Scrucca et al., 2016). The best model was selected based on the BIC.

Each subject was then assigned to their his/her best probability cluster. Each cluster was characterized by estimating its parameters (i.e., subgroup mean and standard deviation) and the socio demographic and clinical characteristics associated with the clusters of subjects.

χ^2 and t tests were performed to analyze possible differences in psychopathological, sociodemographic and methylation characteristics between the two identified clusters and alpha level corrected for multiple comparison using Bonferroni correction.

Supervised ML: decision tree classifier

To evaluate the role of environmental risk factors (i.e., presence of SLEs, of pre- and postnatal risk factors, socioeconomic and demographic characteristics) and epigenetic profiles in the causation of psychopathological traits clusters, we implemented a decision tree classifier. Decision tree algorithm uses "information gain", which is a measure linked to the reduction of entropy: each splitting condition aims to reach a higher amount of information gain (Hastie et al. 2017). The decision tree is a flowchart like structure starting with the full dataset on the top of a series of splitting in nodes. At each node the observations satisfying the splitting condition are assigned to the left branch and the others to the right branch (Hastie et al. 2017). In the last node, the so-called "leaf" (Patel and Prajapati, 2018), the most frequently observed class is considered as a prediction (Hornik et al., 2009).

4.3.2 Results

After performing data cleaning, in which subjects with more than 50% of missing data were excluded from the analyses, 200 participants composed the final sample (76% males, aged 14.45 ± 2.16 years). The results displaying the sample characteristics are depicted in Table 1.

Table 1: *Descriptive statistics: variables distribution in the whole sample*

Variable	**Mean ± SD**	**Min**	**Max**
Age	14.45 ± 2.16	10	20
Sex (Female:Male)	47:153	\	\
SES	48.95 ±19.39	0	90
Clinical variables			
Internalizing	57.1 ± 9.02	34.0	78.0
Externalizing	53.2 ± 9.05	34.0	86.0
Environmental risk		**Absent (% of subjects)**	**Present (% of subjects)**
SLEs		58%	42%

Perinatal risk factors		56%	44%

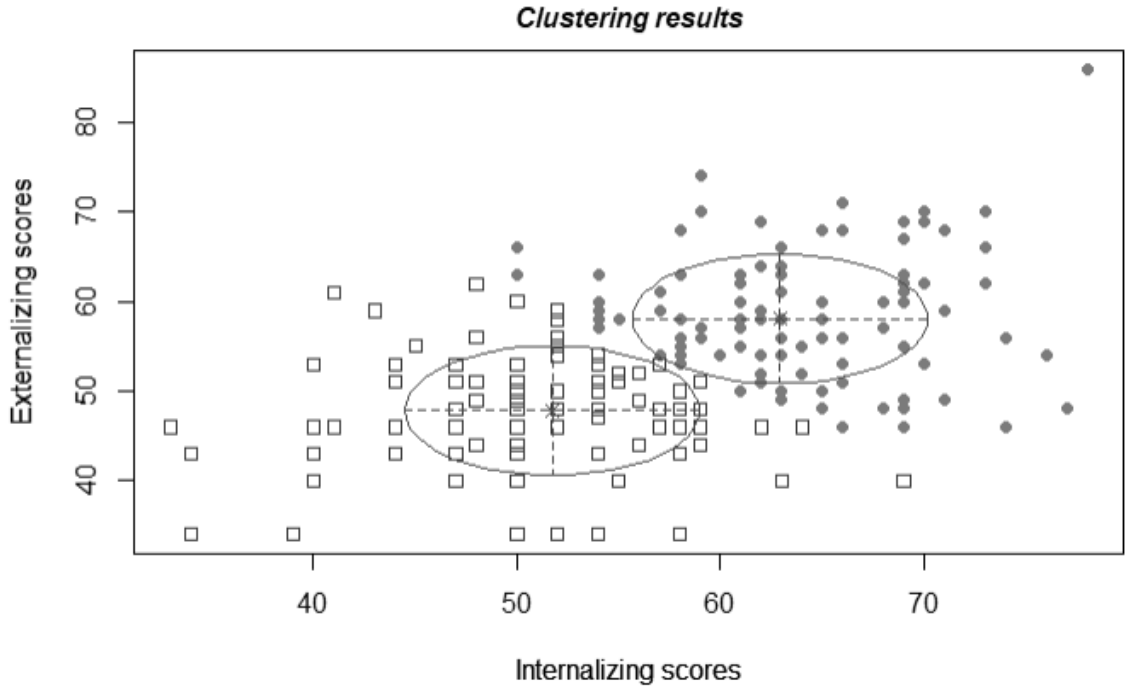
Notes: SD = standard deviation; SES = socioeconomic Status; SLEs = stressful life events

The characteristics of methylation variables are shown in Table 1a in the Appendix section.

Unsupervised ML: cluster analysis on clinical measures results

The results of the analysis are shown in Figure 4.1. The best model (BIC=-2887.49, log-likelihood=-1427.85) suggested the presence of two clusters. The clusters' estimated results are summarized in Table 4.2. The "Low severity" cluster (51% of sample's subjects) was characterized by the presence of mean subclinical values of both Internalizing and Externalizing symptoms; the "High severity" cluster (49% of sample's subjects), instead, was characterized by high mean psychopathology in both areas

Figure 4.1: Cluster analysis results



Note: Squares represent participants belonging to the "Low severity" cluster, whereas circles represent participants belonging to the "High severity" cluster. Internalizing and externalizing symptoms scores are expressed in t scores, which have a mean of 50 and a deviation standard of 10.

We performed χ^2 and t tests to analyze possible differences in psychopathological, sociodemographic and methylation characteristics between the two identified clusters. The

results are shown in Table 4.2. All t tests p values are corrected for multiple comparisons. The two clusters presented peculiar characteristics: in the "High severity", compared to the "Low severity" cluster, mean values of Internalizing and Externalizing psychopathology were significantly higher and perinatal risk factors were present in a significantly higher proportion. Moreover, in the "Low severity", compared to the "High severity" cluster, mean values of methylation were significantly higher in BDNF Cpgs 4 and 5, IGF2 Cpg 2, and OXTRPR Cpg 5. No other significant differences were found.

Table 4.2: Characterization of the two clusters

	Cluster 1 "Low severity"	**Cluster 2 "High severity"**	**Statistical value**	***p***
N (%)	102 (51%)	98 (49%)		
Age (Mean ± SD)	14.5 ± 2.02	14.4 ± 2.31	-0.59[a]	0.554
Sex (Female:Male)	76:26	77:21	0.45[b]	0.498
SES (Mean ± SD)	50.8 ± 20.3	47.0 ± 18.3	-1.37[a]	0.172
Clinical variables (Mean ± SD)				
Internalizing	51.0 ± 6.91	63.7 ± 5.97	13.97[a]	**<0.001**
Externalizing	47.1 ± 6.51	58.9 ± 7.10	12.24[a]	**<0.001**
Environmental risk				
SLEs	Absent: 68% Present: 37%	Absent: 52% Present: 48%	1.93[b]	0.165
Perinatal risk factors	Absent: 54% Present: 46%	Absent: 34% Present: 66%	7.51[b]	**0.006**
Methylation level (%) (Mean ± SD)				
BDNFCpg1	1.71 ± 0.51	1.65 ± 0.44	-0.81[a]	0.420
BDNFCpg2	0.40 ± 0.32	0.41 ± 0.18	0.14[a]	0.889
BDNFCpg3	0.40 ± 0.20	0.42 ± 0.17	-0.76[a]	0.449
BDNFCpg4	0.33 ± 0.14	0.28 ± 0.17	2.19[a]	**0.030**

BDNFCpg5	0.54 ± 0.25	0.46 ± 0.16	-2.90[a]	**0.004**
BDNFCpg6	0.47 ± 0.17	0.46 ± 0.21	-0.35[a]	0.727
BDNFCpg7	0.53 ± 0.41	0.46 ± 0.18	-1.52[a]	0.131
BDNFCpg8	0.79 ± 0.36	0.77 ± 0.25	-0.47[a]	0.640
BDNFCpg9	0.63 ± 0.27	0.65 ± 0.27	0.59[a]	0.560
BDNFCpg10	0.83 ± 0.29	0.83 ± 0.29	-0.07[a]	0.944
BDNFCpg11	0.59 ± 0.19	0.51 ± 0.15	-0.45[a]	0.660
FKBP5Cpg1	72.38 ± 4.66	72.32 ± 5.59	-0.08[a]	0.935
FKBP5Cpg2	92.87 ± 3.81	92.76 ± 3.95	-0.20[a]	0.844
FKBP5Cpg3	90.22 ± 4.59	89.89 ± 5.03	-0.51[a]	0.614
IGF2Cpg1	49.58 ± 5.73	48.95 ± 5.04	-0.83[a]	0.409
IGF2Cpg2	39.05 ± 3.86	37.65 ± 5.29	-2.14[a]	**0.034**
IGF2Cpg3	40.42 ± 4.60	39.47 ± 4.67	-1.44[a]	0.152
IGF2Cpg4	34.61 ± 4.92	33.48 ± 5.16	-1.58[a]	0.115
IGF2Cpg5	0.08 ± 0.05	0.08 ± 0.05	0.20[a]	0.840
OXTRE3Cpg1	7.94 ± 3.38	7.93 ± 3.10	-0.03[a]	0.980
OXTRE3Cpg2	6.56 ± 3.01	6.44 ± 2.87	-0.31[a]	0.759
OXTRE3Cpg3	4.65 ± 2.59	4.70 ± 2.14	0.15[a]	0.884
OXTRE3Cpg4	4.48 ± 2.83	4.56 ± 2.42	0.22[a]	0.826
OXTRE3Cpg5	2.01 ± 1.42	1.98 ± 1.19	-0.20[a]	0.840
OXTRE3Cpg6	8.30 ± 3.72	8.49 ± 3.98	0.33[a]	0.747
OXTRE3Cpg7	5.86 ± 3.12	5.93 ± 2.96	0.16[a]	0.872
OXTRE3Cpg8	2.95 ± 1.93	3.12 ± 1.89	0.62[a]	0.535
OXTRE3Cpg9	5.24 ± 3.02	5.40 ± 2.85	0.40[a]	0.690
OXTRE3Cpg10	5.80 ± 3.16	5.81 ± 2.92	0.02[a]	0.981
OXTRE3Cpg11	5.67 ± 3.51	5.84 ± 3.34	0.36[a]	0.718
OXTRE3Cpg12	3.04 ± 2.24	3.09 ± 2.01	0.15[a]	0.877

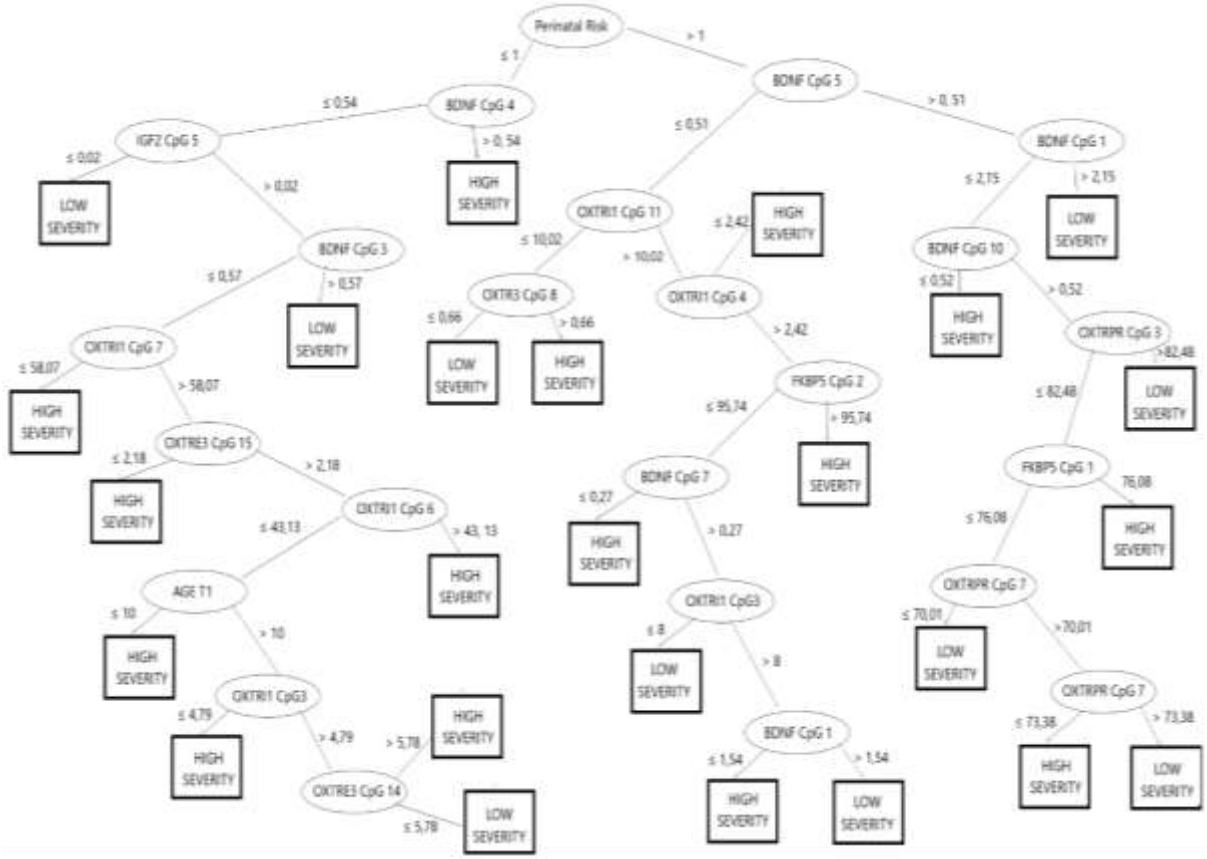
OXTRE3Cpg13	4.82 ± 3.43	5.10 ± 3.15	0.58[a]	0.558
OXTRE3Cpg14	3.02 ± 2.09	2.93 ± 1.98	-0.30[a]	0.764
OXTRE3Cpg15	4.26 ± 2.51	3.40 ± 2.33	-0.77[a]	0.440
OXTRI1Cpg1	1.76 ± 0.73	1.69 ± 0.81	-0.69[a]	0.492
OXTRI1Cpg2	2.76 ± 0.94	2.73 ± 1.10	-0.23[a]	0.822
OXTRI1Cpg3	7.36 ± 2.04	7.15 ± 2.09	-0.71[a]	0.476
OXTRI1Cpg4	2.92 ± 1.71	2.75 ± 1.06	-0.81[a]	0.417
OXTRI1Cpg5	36.33 ± 4.87	35.89 ± 4.97	-0.64[a]	0.522
OXTRI1Cpg6	38.70 ± 5.60	38.29 ± 5.58	-0.53[a]	0.597
OXTRI1Cpg7	62.71 ± 4.91	61.45 ± 5.17	-1.77[a]	0.078
OXTRI1Cpg8	43.77 ± 4.57	43.25 ± 5.24	-0.76[a]	0.446
OXTRI1Cpg9	23.43 ± 5.00	23.32 ± 4.79	-0.16[a]	0.868
OXTRI1Cpg10	8.55 ± 2.58	7.97 ± 2.25	-1.68[a]	0.093
OXTRI1Cpg11	10.92 ± 2.99	10.44 ± 3.00	-1.14[a]	0.257
OXTRI1Cpg12	12.37 ± 3.97	11.77 ± 3.15	-1.17[a]	0.243
OXTRI1Cpg13	13.40 ± 3.45	13.03 ± 3.26	-0.79[a]	0.430
OXTRPRCpg1	91.63 ± 2.29	91.05 ± 2.20	-1.80[a]	0.072
OXTRPRCpg2	78.71 ± 4.09	78.39 ± 4.68	-0.50[a]	0.615
OXTRPRCpg3	79.64 ± 4.13	78.87 ± 3.94	-1.36[a]	0.175
OXTRPRCpg4	64.66 ± 5.94	63.93 ± 4.64	-0.97[a]	0.335
OXTRPRCpg5	85.13 ± 2.81	84.34 ± 2.65	-2.01[a]	**0.044**
OXTRPRCpg6	47.53 ± 5.07	46.42 ± 4.96	-1.57[a]	0.118
OXTRPRCpg7	71.09 ± 4.92	70.19 ± 3.78	-1.46[a]	0.147

Notes: [a] = Bonferroni-adjusted t test; [b] = χ^2; BDNF = Brain-derived neurotrophic factor; FKBP5 = FK506-binding protein 5 gene; IGF2 = Insulin-like growth factor-2; OXTRE3 = Oxytocin receptor exon 3; OXTRI1 = Oxytocin receptor intron 1; OXTRPR = Oxytocin receptor promoter; SD = standard deviation; SES = socioeconomic status; SLEs = stressful life events.

Supervised ML: decision tree classifier results

The decision tree classifier results are depicted in Figure 4.2. The classifier showed an accuracy of 97% (95% confidence interval = 93-99%); this result was significant (NIR = 0.52; $p < 0.001$). The model had a 99% specificity and a 94% sensitivity. Note that the most discriminant information is represented by the presence versus the absence of perinatal risk factors, followed by methylation levels in BDNF Cpg 4 and 5. Indeed, these variables presented significantly different values in the two clusters.

Figure 4.2: Decision tree results



4.4 Discussion

Adolescence is a very important and delicate period of life, in which everyone experiences changes in several areas. The crucial changes due to biological development and the sudden adaptations that new environments request are often sources for individual difficulties and moments of crisis. It is not by chance that both internalizing and externalizing behaviors appear to increase when children enter early adolescence.

In this work we aimed to understand the course of internalizing and externalizing traits in a sample of preadolescents and adolescents which were clinically referred during childhood for

different mental health issues. To do so, we implemented an unsupervised and interpretable ML on Internalizing and Externalizing scores of CBCL/6-18 using a cluster analysis method. Then, moving to a supervised algorithm, with a decision tree classifier we evaluated the role of environmental risk factors (i.e., presence of stressful life events, of pre- and postnatal risk factors, socioeconomic and demographic characteristics) and epigenetic profiles (specifically, DNA methylation) in the causation of the identified psychopathological traits clusters.

We used a FMM on the T scores of CBCL/6-18 Internalizing and Externalizing scales (Achenbach & Rescorla, 2001) to identify homogeneous clusters of psychopathology which take into account both symptomatological areas. We identified, with this bottom-up strategy, two different groups of subjects -LOW and HIGH. Participants in the LOW cluster presented CBCL scores in the typical range, whereas subjects in the HIGH cluster reported higher externalizing scores tied with internalizing difficulties situated in the borderline range for clinical severity. We think it is extremely important to notice that approximately one half of the sample belongs to the HIGH cluster, suggesting that adolescence remains a critical period for individuals which experienced difficulties during childhood.

We considered psychopathology as continuous, taking into consideration a group of adolescents without a categorical diagnosis but with a clinical history of psychological difficulties. Moreover, thanks to cluster analysis methods, we evaluate the presence of internalizing and externalizing problems jointly, differentiating our participants into two groups with a data guided methodology. To our knowledge, this is the first time this statistical model has been applied with the aim of evaluating the presence of different outcomes in psychopathological traits, without a specific categorical diagnosis, on CBCL scores in adolescence. We decide to evaluate psychopathology as traits in order to have a wider view of the complexity that characterize psychiatry, especially during developmental age, when frequently, as also evidenced by our results, the presences of internalizing and externalizing symptoms are associated.

The literature exploring psychopathological traits in adolescence with machine learning methodologies is a very early stage. There are two previous studies conducted in Italy.

Amendola and colleagues (2021) evaluated the presence of clusters of symptoms focusing only on the internalizing area of psychopathology. They evaluated an Italian community-based sample of 1127 children and adolescents, aged 8–16 years. Muratori and colleagues analyzed the whole spectrum of psychopathology, using machine learning methods, but measuring psychopathology with a different instrument: the Strengths and Difficulties

Questionnaire (SDQ; Goodman, 1997). They analyzed an Italian sample of 2959 preadolescence (from 10 to 14 years) from the general population. They found 4 clusters of psychopathological traits presentation, two of them characterized by low levels of psychopathology (74.5% of the total sample) and two characterized by "predominantly internalizing" of "predominantly externalizing" symptoms (19% and 6.5% of the total sample, respectively). Given the differences in the methodologies and that our sample was composed of adolescents that were help seeking for mental health problems children, we think that these results are in line with ours. These results highlight the importance of adolescence as a very delicate period of life, in which the individual faces up to several changes in terms of biological development and new environmental requests. It is a challenging moment, which is in many cases characterized by a decrease in psychological well-being. This impact on mental health can also persist and affect youths' future development (Muratori et al., 2021).

Moreover, for the first time, we evaluated whether these clusters are characterized by differences in the exposure to prenatal adversities, stressful life events, and methylation of DNA in candidate genes involved in psychopathology. The majority of studies in this field, took into consideration groups of patients with or without a psychiatric disorder and examined their different exposure to risk factors.

The two clusters differed in exposure to environmental risk factors and methylation of BDNF, IGF2, and OXTRPR specific Cpgs. Conversely, it is important to note that the two clusters were similar for BAS, age, and SES, possibly confounding factors as frequently associated with differences in psychopathology expression (Muratori et al., 2021; Nobile et al., 2007).

With respect to exposure to environmental risk factors, the HIGH cluster presented a significantly greater number of perinatal complications, and a slightly higher number of SLEs. The relationship between perinatal risk factors and psychopathology has been previously investigated, and similar results have been identified for both internalizing and externalizing behaviors. Orri and colleagues (2020) analyzed the relationship between exposure to perinatal adversity and later risk of suicide attempt. Their results are in line with ours, in fact they found that subjects with greater number of pre and perinatal risks showed and higher risk for suicidal attempt by early adulthood, compared with individuals in the "No perinatal risk" profile. Another study conducted focusing on externalizing area (Roigé-Castellví et al., 2021), evidenced that gestational diabetes, instrumental delivery, family

history of psychopathology, and maternal attentional deficit and hyperactivity phenotype were associated with the presence of the same disorder in children.

LOW and HIGH clusters also differed significantly for percentage of methylation at Cpgs 4 and 5 of BDNF intron 1 (chr11:27723077-27723244), Cpg2 of IGF2 differentially methylated region (chr11:2169373-2169658), and Cpg5 of OXT promoter (chr3:8811488-8811837), with subject in HIGH cluster having lower methylation in all Cpgs.

BDNF, as the most abundant neurotrophin, plays a crucial role in regulating neurogenesis and neurodevelopment, synaptic plasticity, and connectivity throughout life (Barnett Burns et al., 2018). Previous studies found BDNF to be implicated in dopaminergic, cholinergic and serotonergic regulation, with an identified association between its altered methylation profiles and the presence of both internalizing and externalizing symptoms (Perroud et al., 2013).

Our results evidenced a decrease of methylation in BDNF Cpgs 4 and 5, whilst the majority of the studies evaluating BDNF methylation in relation with the presence of psychopathology, found an increment of DNA methylation (Perroud et al., 2013, Thaler et al., 2014). Regarding factors that may explain this difference, it is worthy to note that, differently from us, the majority of the studies evaluating this relation focused on adult samples with specific mental disorder diagnosis (see, Chen et al., 2010, Mill et al., 2008, Perroud et al., 2013, Thaler et al., 2014, Toledo-Rodriguez et al., 2010). DNA methylation varies over lifespan and it shows some peculiarities during adolescence (Dadds et al., 2014). DNA methylation is most active during prenatal and neonatal periods and becomes stable in adulthood (Goodman et al., 2019). Even though adolescence has been understood to be a period of increased alteration in methylation (Alisch et al., 2017, Goodman et al., 2019), studies during this developmental period are more scarce compared to those conducted in early childhood and later adulthood.

Moreover, some evidence from animal and human studies suggested that the effect of life stressor can lead to mixed results, with both incremental and decremental expression of the gene associated with different psychiatric disorders (Boulle et al., 2012).

IGF2 is abundantly expressed during pregnancy and in the brain, and alteration in its pathways have been associated with fetal and infant growth and with the exposure to pre birth risk factors (Bouwland-Both et al., 2013, Mansell et al., 2016, Rijlaarsdam et al., 2017, Vangeel et al., 2015). Even though the literature is at an early stage in the interpretation of the relationship between methylation of IGF2 and augmented risk of psychiatric symptoms and

disorders (Pardo et al., 2019, Pai et al., 2019), we can state that our results are in line with previous findings, associating especially internalizing disorders with decremented or altered methylation of this gene (Chen et al., 2014, Cordova-Palomera et al., 2009, Vangeel et al., 2015, Zieker et al., 2007).

As for BDNF studies, there is paucity of specific studies evaluating the relationship between methylation of IGF2 and the presence of psychopathology in children and adolescents and the results are difficult to compare because of different methodologies. In a study recruiting 120 children, Goodman and colleagues (2019) found that the presence of externalizing symptoms was related to higher methylation, whilst internalizing symptoms were related to lower methylation of the gene.

Oxytocin have been found to play a crucial role in parturition and lactation, as well as in affiliative/prosocial behavior and consequently in social skills and cognition related to empathy (Dadds et al., 2014). For these reasons, alteration in methylation of OXTR have been associated with autistic spectrum disorder traits, callous-unemotional traits, depression (Behnia et al., 2015, Kumsta et al., 2013, Liu et al., 2021, Simons et al., 2017).

Also, in the case of this gene, the literature is more abundant in adult studies and they found both increased (Gouin et al., 2017) and decreased (Reiner et al., 2015) methylation of OXTR to be associated with psychopathology. The majority of the studies conducted on developmental age focused on autistic spectrum disorders; epidemiological and clinical studies have shown that the relation between maternal diabetes and preterm births and autistic spectrum disorder development was potentially mediated by OXTR methylation (Behnia et al., 2015, Liu et al., 2021). In young male children, aged from 4 to 16 years, methylation of OXTR characterizes the early signs of psychopathy: high levels of callous unemotional traits were associated with methylation of the promoter region of the OXTR gene. This association was more evident for adolescents (Dadds et al., 2014).

Our results, in line with previous findings (Alisch et al., 2017; Goodman et al., 2019), suggested that adolescence is a sensitive age for the study of DNA methylation in relation with psychopathology. We think it is essential to interpret these results with caution because the literature regarding methylation of DNA and psychopathology is extremely heterogeneous in terms of different methodologies involved in the evaluation of all variables involved. Regarding the study of methylation, we noted that different studies took into

consideration different segments of the genes, and that methylation has been evaluated with different methodologies in different biological tissues, in pre and post mortem samples. For example, in the present study we analyzed exactly the same gene region of Vangeel and colleagues (2015), but in different tissues; another example of differences in methodologies is the use of factor analysis to reduce methylation variables (Rijlaarsdam et al., 2017, Simons et al., 2017) we did not use.

Then, after characterizing our clusters, we moved to an interpretable supervised decision tree classifier to understand the role that age, BAS, SES, SLEs, pre- and perinatal adversities and epigenetics might have had in explaining the differences between adolescents belonging to the HIGH versus the LOW one. Machine learning methods have been widely used in recent years in psychiatric research to understand and predict the complexity of multifactorial diseases (Badillo et al., 2020; Grazioli et al., 2022). Our decision tree analysis reached a very good accuracy (i.e., 97%), with 99% specificity and a 94% sensitivity. The variable which mostly discriminated between the two clusters was the presence of perinatal risk factors, followed by percentages of methylation in BDNF CpGs 4 and 5. These might have been expected because these variables presented significantly different values in the two clusters. The decision tree classifier results enabled us to better understand the relationships interworking within the identified risk factors. Indeed, the presence of more than one perinatal risk factors resulted in influencing the belonging to the HIGH cluster only when associated with high methylation of BDNF CpG5 and low methylation of BDNF CpGs 1 and 10. When the subjects have lower methylation of BDNF CpG5, it is important to consider epigenetics of the OXTR gene in order to understand the presence of psychopathology during adolescence. Following the opposite path, it was possible to notice that, for the presence of only 1 or no perinatal risk factors, the most discriminant condition for belonging to the HIGH cluster was to have higher methylation in BDNF Cpg4. Otherwise, for a lower percentage of methylation in BDNF Cpg4, the path followed and the discriminant were the percentage of methylation in Cpgs of IGF2, BDNF and OXTR genes.

It is curious to notice that, apart from perinatal risk factors, the only non-epigenetic variable represented in the decision tree is the variable age. Adverse life events and socioeconomic and demographic characteristics, which were included into the model and literature recognized as risk factors for psychopathology, were not discriminant in for belonging to the HIGH vs LOW cluster.

The present study suffered from some limitations. First, as we decide to evaluate psychopathology as traits, in order to have a wider view of the complexity that characterize psychiatry, the presence of the identified risk factors remains nonspecific.

We were interested in evaluating psychopathological traits outcomes in a sample of adolescents, who were clinically referred children, regardless of the categorical diagnosis. We think that approaching psychopathology as a continuous is essential, especially during adolescence, which is characterized by mutation of psychopathology phenotypes. Anyway, it might be important to replicate the results in a clinical sample, taking into consideration specific psychiatric disorders, if the interest is to identify specific risk factors.

Our methylation analysis suffered from some limitations because not all the confounding factors related to methylation have been considered in the present study. Even though genotypes have been associated with differences in methylation (Boulle et al., 2012, Goodman et al., 2018, Mill et al., 2008), we did not consider it in our analysis.

We think that the cross-sectional nature of this evaluation is a limitation when studying methylation, a phenomenon known to be variable during ages. To evaluate the association we found in a longitudinal study might help to better understand the importance of epigenetics in the predisposition to psychopathological traits.

4.5 Conclusions

We identified, regardless of the categorical diagnosis, two possible outcomes in a clinically referred sample of children. In fact, through a data guided approach, we evaluated the presence during adolescence of different clusters of presentation of internalizing and externalizing behaviors using CBCL/6-18 scores. We found that one half of the sample belongs to the HIGH cluster, suggesting that adolescence remains a critical period for individuals which experienced difficulties during childhood.

To our knowledge, this is the first time this statistical model has been applied with the aim of evaluating the presence of different outcomes in psychopathological traits on CBCL scores in adolescence.

Moreover, we evaluated whether these clusters are characterized by differences in the exposure to prenatal adversities, stressful life events, and methylation of DNA in candidate genes involved in psychopathology. The presence of peculiar methylation patterns of IGF2, BDNF and OXTR in the HIGH subgroup of adolescents, part of a previously child-referred sample, is in line with previous results. To our knowledge this is the first time this evaluation has been conducted with an adolescent sample and taking into consideration both

internalizing and externalizing areas. The fact that this subgroup is also characterized by higher frequency of perinatal risk factors, suggested to us that our data confirm the presence of a strong association between adverse events and DNA methylation.

The results of the decision tree classifier helped in the identification of future research directions in this field, underlying the importance of both early environmental experience and epigenetics in causing presence of psychopathological traits during adolescence.

Chapter 5

Psychopathological developmental traits trajectories: third wave preliminary results, future directions and conclusions

5.1 ReMIND Project: Psychopathological developmental trajectories

My thesis "Multi neuro-functional biomarkers for monitoring developmental trajectories in early onset psychopathology" work is part of a larger longitudinal project called *"ReMIND - REal Matters IN Developmental psychopathology. A 15 years follow-up study of risk and resilience factors and outcomes from childhood to adulthood"*, founded by the Italian Ministry of Health, which takes place at IRCCS Eugenio Medea - Associazione La Nostra Famiglia in Bosisio Parini (Lecco).

As previously mentioned, the ReMIND project's primary aims are to achieve a better understanding of the heterogeneity of neuropsychiatric conditions and to identify and characterize children which are most in need of intervention. To reach these goals, the ReMIND project recruits young adults which have already been evaluated during childhood and adolescence throughout a 20 years of age range (2003-2023).

Specificity regarding the sample recruitment and the first and second evaluation of the ReMIND project are reported in Chapter 1. The third wave evaluation is still ongoing, the recruitment of participants is going to end in 2023 and the data analysis that will allow to integrate all the information collected is going to be done subsequently.

Anyway, thanks to the online platform we implemented for data collection, we are able to conduct preliminary analysis of psychological traits evaluated through three time points, from preadolescence to adulthood.

5.2 Aim of the Chapter: third wave preliminary analysis

The aim we pursue with the preliminary analysis I'm going to present is to identify specific clusters of symptom trajectories in internalizing and externalizing areas and evaluate their different exposure to specific risk factors.

To do so, we considered the data collected longitudinally thanks to the ReMIND project: we evaluated psychopathology at three time points and implemented a machine learning algorithm, specifically an unsupervised cluster analysis on the data collected during the second and the third evaluation, to estimate their trajectories through time. Then, we evaluated whether belonging to a specific cluster was associated with sociodemographic

characteristics, presence of environmental risk factors (i.e., perinatal complications and stressful life events), psychopathological symptoms measured at T0.

5.3 Experimental study

The study protocols were approved by the Research Ethical Committee of IRCCS Eugenio Medea Scientific Institute and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Parents' written informed consent was obtained for all participants.

5.3.1 Methods

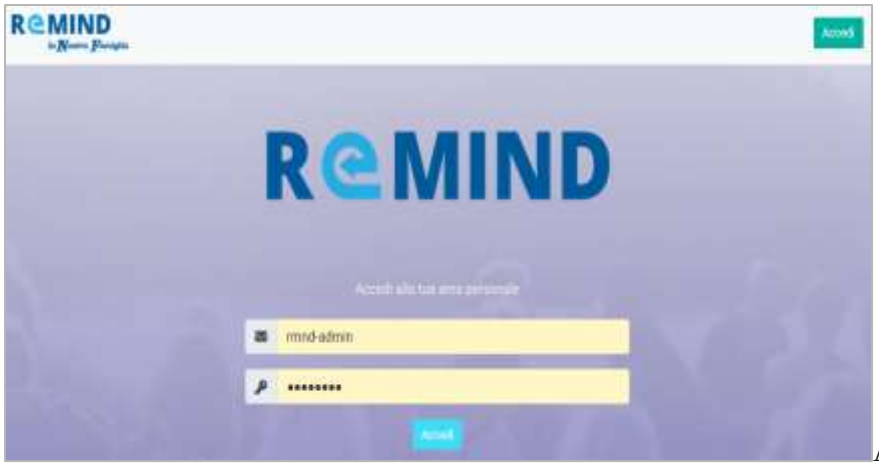
5.3.1.1 Sample

The sample included in this preliminary study is composed of all the participants involved in the ReMIND Project that already completed the Third wave psychopathological and environmental evaluation in September 2022. Specifically, 96 participants are included, 44 of them belong to the clinically referred Cabala-Genesis project subsample (46% of the total sample) and 52 belong to the general population Prisma project subsample (54% of the total sample). The previous evaluations were conducted when this subsample presented mean age of 11 and 16 years (specifically, mean years ± standard deviation were 11±2 and 16±2 for Wave 1 and Wave 2, respectively). At third evaluation our sample of young adults aged 26±4 years.

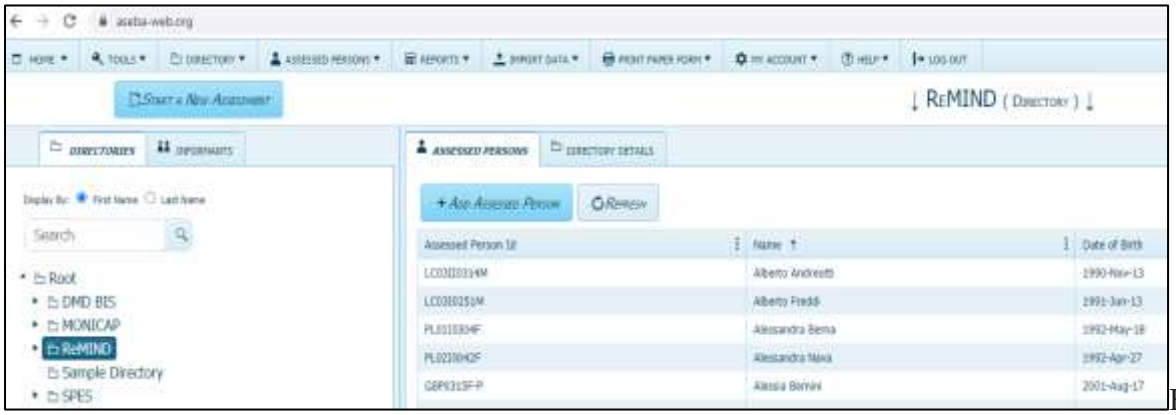
5.3.1.2 Measures

Socio demographic, environmental stress and emotional behavioral information at Wave 3 were collected through the ReMIND project online platform we implemented. Our platform enables us to collect data directly or, thanks to its possibility to communicate with other online platforms, from the ASEBA questionnaires (see figure 5.1.B). Moreover, the ReMIND platform can aggregate information, collected with different instruments, and create datasets which facilitate data analysis.

Figure 5.1



A



B

Socio-demographics

Age, birth assigned sex, and familiar socioeconomic status (SES) were collected. SES was coded according to the Hollingshead scale (Hollingshead, 1975) from parental employment.

Emotional-Behavioral

Information regarding psychopathological symptoms was collected at wave 1 and wave 2 through the Child Behavior Checklist for Ages 6-18 (CBCL/6-18) (Achenbach & Rescorla, 2001), from the Achenbach System of Empirically Based Assessment (ASEBA), filled out by parents. The questionnaire allows to screen for a wide range of emotional, behavioral and social problems in children and adolescents aged from 6 to 18. It gives back 8 empirically based syndromic scales (i.e., Anxious/depressed, Withdrawn/depressed, Somatic complaints, Social problems, Thought problems, Attention problems, Rule-breaking behavior, and Aggressive behavior) and 3 summary scales based on the previous mentioned syndromic scales (i.e., Total problems scale, Internalizing problem scale which takes into account Anxious/depressed, Withdrawn/depressed, and Somatic complaints scales, and Externalizing

problems scale which takes into account Rule-breaking behavior, and Aggressive behavior scales).

At wave 3 we used the Adult Self Report (ASR/18-69, Achenbach & Rescorla, 2003) filled out by participants themselves. The ASR is a 126-item self-report questionnaire, from the ASEBA, for adults assessing emotional and behavioral functioning aspects. It gives back 8 empirically based syndromic scales (i.e., Anxious/depressed, Withdrawn, Somatic complaints, Thought problems, Attention problems, Aggressive behavior, Rule-breaking behavior, Intrusive) and 3 summary scales based on the previous mentioned syndromic scales (i.e., Total problems scale, Internalizing problem scale which takes into account Anxious/depressed, Withdrawn, and Somatic complaints scales, and Externalizing problems scale which takes into account Aggressive behavior scales, Rule-breaking behavior, and Intrusive).

Symptom trajectories evaluated as homogeneous clusters have been evaluated using T scores of Anxious/depressed, Withdrawn, Somatic complaints, Aggressive behavior, Rule-breaking behavior subscales measured at wave 2 and 3. Internalizing and Externalizing scales measured at wave 1 were used as predictors of the belongs to a specific cluster.

Environmental risk factors

Stressful life events (SLEs)

The presence of SLE was evaluated through the online platform. The following SLEs were collected

1. parent had separated or broken up in a steady relationship;
2. parent had a serious illness which required a stay in hospital;
3. subject had separated or broken up in a steady relationship;
4. subject had a serious illness which required a stay in hospital;
5. subject had been in a serious accident or badly hurt in an accident;
6. parent death;
7. brother or sister death;
8. grandparent death
9. subject close friend death;
10. pet death;
11. subject had broken off close friendship;
12. subject had broken off affective relationship;
13. family had a major financial crisis;

14. parent had a problem with the police involving a court appearance;
15. subject had a problem with the police involving a court appearance;
16. subject had a pregnancy or abortion;
17. subject became parent;
18. subject had been repetitively humiliated, bullied, or physically assaulted
19. subject had been a victim of sexual harassment

Perinatal risk factors

The presence of pre- and perinatal risk factors has been evaluated: with an ad hoc questionnaire filled out by the subjects. The questionnaire asks for the presence of threat of abortion, twin birth - simple or complicated, incubator / resuscitation / "blue baby", maternal use of drugs, alcohol or tobacco during pregnancy, duration of gestation and birth weight.

5.3.1.3 Data analyses

Statistical analyses were performed using R statistical software (R core Team, 2021, version 4.1.0) with the additional "mclust" package for Gaussian mixture modeling. Variables associated with clusters belonging were tested with non-parametric ANOVA. The alpha level was set to 0.05 for all analyses. First, descriptive statistics were preliminarily computed to check the distribution of variables in the whole sample.

The presence of psychopathological traits were evaluated using an unsupervised algorithm, the Multivariate Finite Mixture Model (MFMM) implemented on the T scores of Anxious/depressed, Withdrawn, Somatic complaints, Aggressive behavior, Rule-breaking behavior subscales measured at wave 2 and 3.

Models estimating solutions of two or more clusters were compared using the Bayesian information criterion (BIC), with the best model having a BIC value closer to 0 (Scrucca et al., 2016). The best model was selected based on the BIC.

Each subject was then assigned to their his/her best probability cluster.

Differences between the identified clusters regarding sociodemographic variables, environmental risk exposure and preadolescence psychopathology (measured with Internalizing and Externalizing problem scales of CBCL/6-18 at wave 0) were tested with non parametric ANOVA.

5.3.2 Results

The best model suggested the presence of three trajectories/clusters for Anxious/depressed ("Borderline" which comprehends individuals characterized by stable above mean T scores, "Low" which comprehends individuals characterized by stable below mean T scores, and "Low to high" with comprehends individuals characterized by a worsening of symptoms during adulthood) two for Withdrawn ("Borderline" which comprehends individuals characterized by stable above mean T scores, and "Low" which comprehends individuals characterized by stable below mean T scores) and three for Somatic complaints subscale (Borderline" which comprehends individuals characterized by stable above mean T scores, "Low" which comprehends individuals characterized by stable below mean T scores, and "High to low" with comprehends individuals characterized by an improvement of symptoms during adulthood). 23% of the sample belonged to clusters presenting stable clinical symptoms of anxiety, depression and somatic problems. Traits trajectories for internalizing area are depicted in Figure 5.1.a, b and c.

MFMM did not highlight any cluster in the externalizing subscales (i.e., Aggressive and Rule Breaking), hence, the subjects trajectories were considered homogeneous.

Figure 5.1.a Anxious/depressed subscale scores

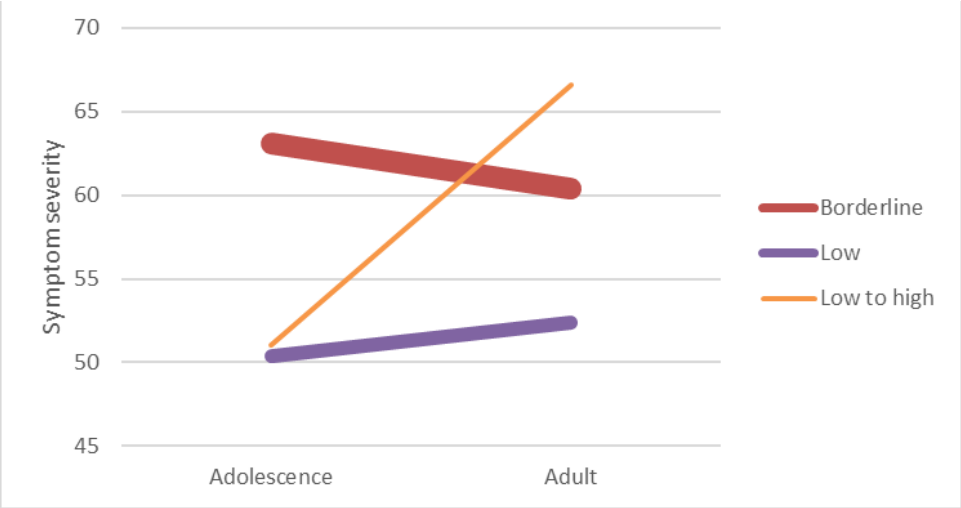
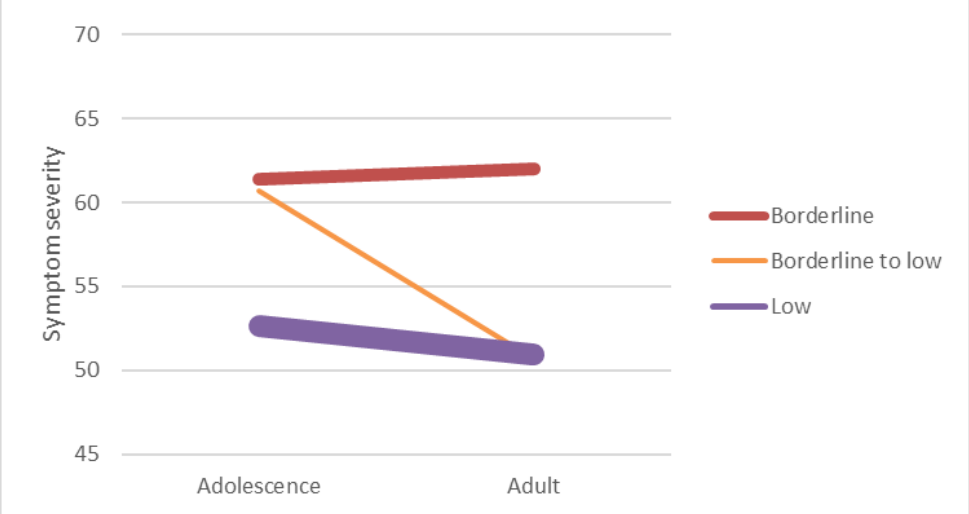


Figure 5.1.b Withdrawn subscale scores

Figure 5.1.c Somatic complaints subscale scores



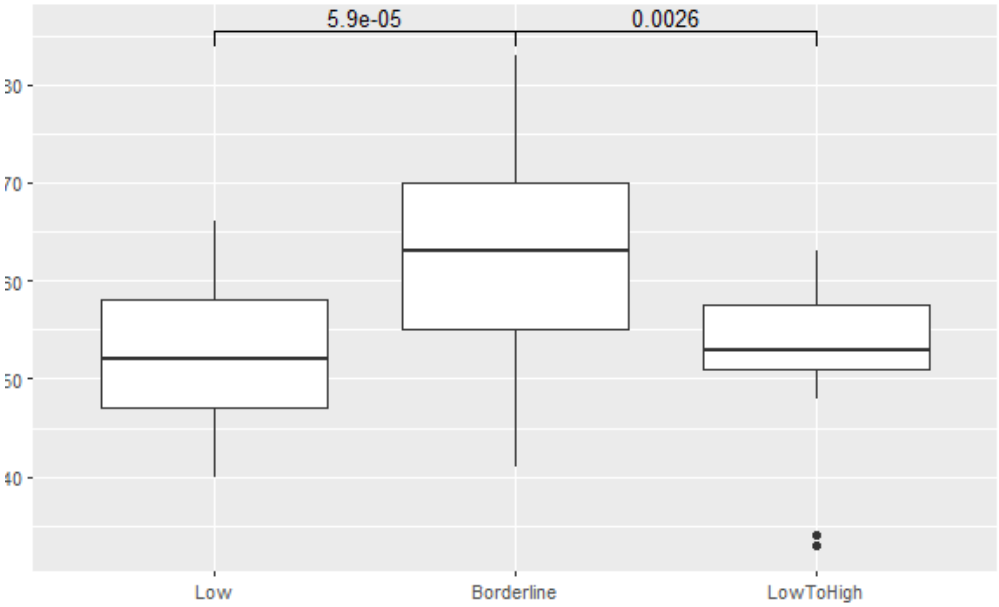
The psychopathological trajectories identified with the cluster analysis did not show any association with socio demographic variables, perinatal risk factors and SLEs.

Psychopathological traits at Wave 0 resulted as the best predictor of clusters belonging. In fact, belonging to the "Borderline" Anxious/depressed cluster was associated with the presence of both high internalizing and externalizing symptoms during preadolescence (see Figure 5.2.a and b).

Belonging to the "Borderline" Withdrawn cluster was associated with the presence of high externalizing symptoms during preadolescence. The association between the "Borderline" Withdrawn cluster and the presence of high internalizing symptoms during preadolescence resulted as a trend (n.s, $p > .005$., see Figure 5.3.a and b).

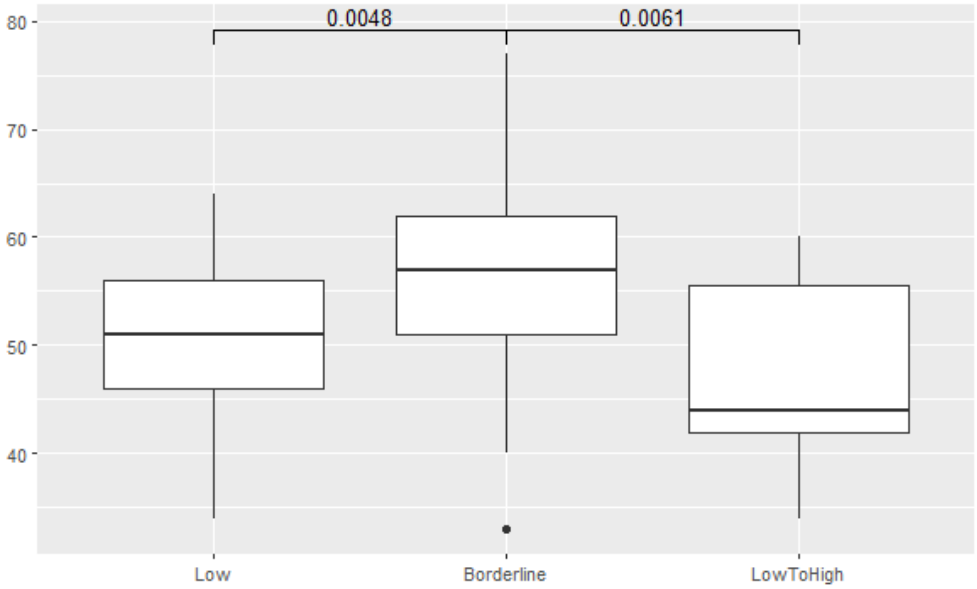
Belonging to the "Borderline" Somatic complaints cluster was associated with the presence of high externalizing symptoms during preadolescence. The presence of high internalizing symptoms during preadolescence was associated with both the belonging to the "Borderline" and the "High to low" Somatic complaints clusters (see Figure 5.4.a and b)

Figure 5.2.a - relationship between internalizing symptoms during preadolescence and Anxious/depressed trajectories from adolescence to adulthood



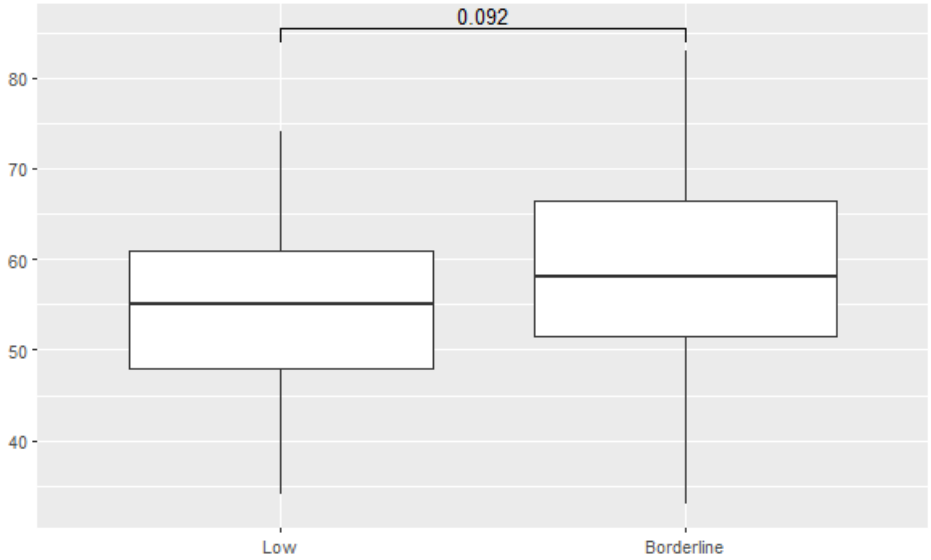
Note, on the x axis the T scores for Internalizing subscale scores at wave 1 are reported; on the y axis the clusters evaluated on Anxious/depressed subscale scores at wave 2 and 3 are reported

Figure 5.2.b - relationship between externalizing symptoms during preadolescence and Anxious/depressed trajectories from adolescence to adulthood



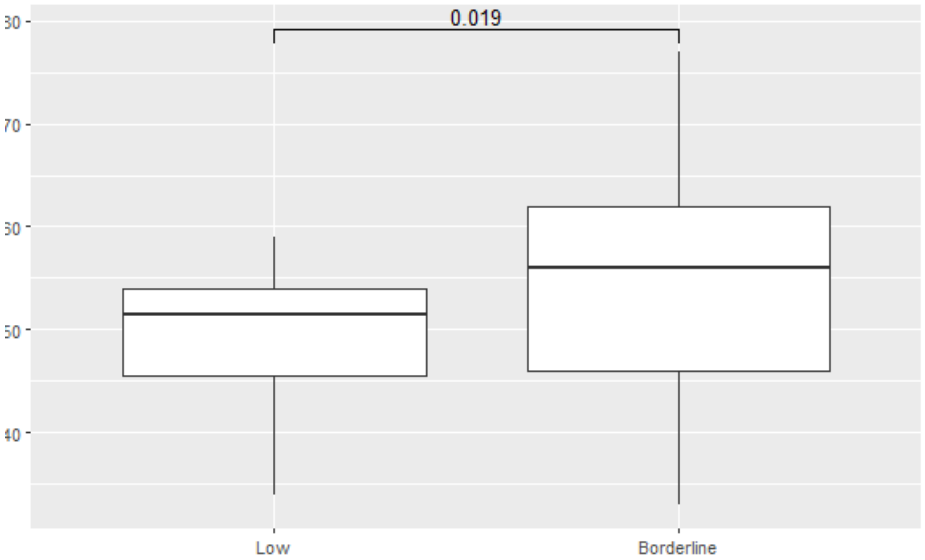
Note, on the x axis the T scores for Externalizing subscale scores at wave 1 are reported; on the y axis the clusters evaluated on Anxious/depressed subscale scores at wave 2 and 3 are reported

Figure 5.3.a - relationship between internalizing symptoms during preadolescence and Withdrawn trajectories from adolescence to adulthood



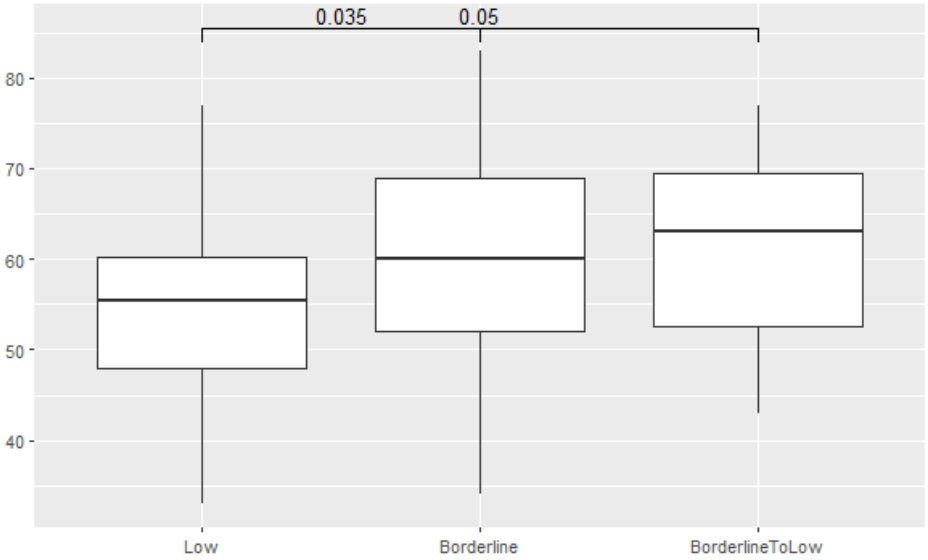
Note, on the x axis the T scores for Internalizing subscale scores at wave 1 are reported; on the y axis the clusters evaluated on Withdrawn subscale scores at wave 2 and 3 are reported

Figure 5.3.b - relationship between externalizing symptoms during preadolescence and Withdrawn trajectories from adolescence to adulthood



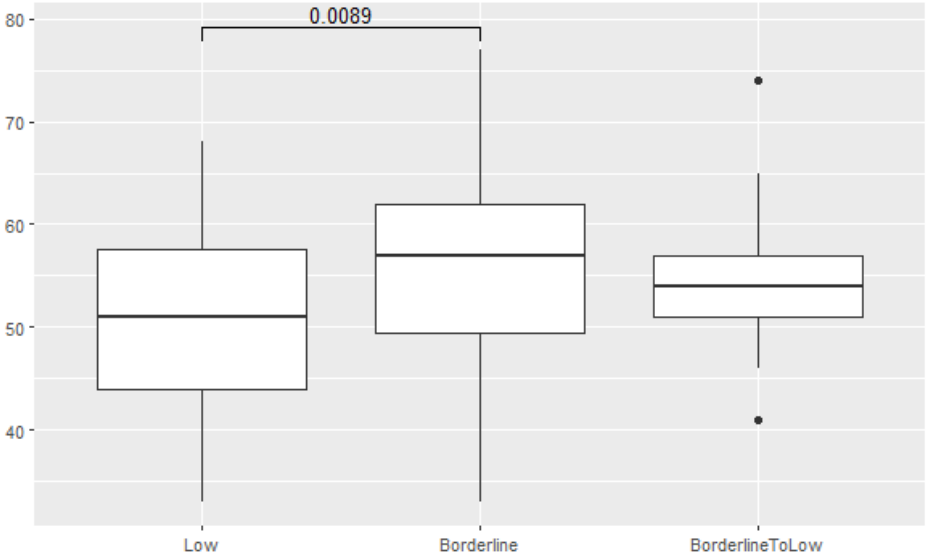
Note, on the x axis the T scores for Externalizing subscale scores at wave 1 are reported; on the y axis the clusters evaluated on Withdrawn subscale scores at wave 2 and 3 are reported

Figure 5.4.a - relationship between internalizing symptoms during preadolescence and Somatic complaints trajectories from adolescence to adulthood



Note, on the x axis the T scores for Internalizing subscale scores at wave 1 are reported; on the y axis the clusters evaluated on Somatic complaints subscale scores at wave 2 and 3 are reported

Figure 5.4.b - relationship between externalizing symptoms during preadolescence and Somatic complaints trajectories from adolescence to adulthood



Note, on the x axis the T scores for Externalizing subscale scores at wave 1 are reported; on the y axis the clusters evaluated on Somatic complaints subscale scores at wave 2 and 3 are reported

5.4 Discussion

The aims of my research work, "*Multi neuro-functional biomarkers for monitoring developmental trajectories in early onset psychopathology*", was to better understand the course of psychopathological traits, in order to identify earlier those children more in need for interventions, and to understand the role of different risk factors in shaping these trajectories with the scope of ameliorate prevention and treatment in mental health.

My first aim was pursued thanks to the possibility of measuring psychopathological traits at different time points from childhood to early adulthood. A unique sample, composed of individuals belonging to a general population and a help seeking children subsamples, was followed within a 20-year life span. My thesis work, in fact, is part of the "*ReMIND project - REal Matters IN Developmental psychopathology. A 15-year follow-up study of risk and resilience factors and outcomes from childhood to adulthood*", founded by the Italian Ministry of Health, which takes place at IRCCS Eugenio Medea - Associazione La Nostra Famiglia in Bosisio Parini (Lecco).

In experimental studies presented in Chapters 2 and 4, I focused on the identification of psychopathological traits with *bottom-up* approaches in cross sectional studies, in order to evaluate the presence of internalizing and externalizing problems in developmental ages. Specifically, latent class analysis and cluster analysis methodologies have been used to identify individuals expressing higher levels of psychopathology, regardless of categorical diagnosis, taking into account a wide span of symptoms thanks to the ASEBA questionnaires. Special attention has been paid to the presence of emotional/behavioral dysregulation, as a specific risk factor for worse psychopathological developmental trajectory. In experimental studies presented in chapter 2, using a person-centered statistical approach on data from the first wave of ReMIND project, four different psychopathological profiles were identified in a large sample including both referred and general-population children and adolescents. Membership in these groups appears to be related to different positive and negative outcomes in terms of psychopathological diagnosis. In particular, our results underline the presence of a profile characterized by severe emotional and behavioral dysregulation, which is mostly associated with the presence of multiple diagnoses. Emotional/behavioral dysregulation reflects both failure of cognitive control and high intensity of arousal (Soloff et al., 2015), which cause higher sensitivity to emotional arousal, slower return to baseline activation, and deficits in coping strategies. Our results were in line with previous studies which found an association between emotional/behavioral dysregulation and poor outcomes of mental health

problems and the presence of different comorbidities within both externalizing (e.g., oppositional defiant disorder) and internalizing (e.g., depression, dysthymia) disorders (Shaw et al., 2014, Wang et al., 2018). In fact, especially in children and adolescents, emotional/behavioral dysregulation is responsible for low tolerance to frustration, impatience, easy anger and excessive emotional excitement (Biederman et al., 2012). With *bottom-up* strategies we found a dysregulated profile in both children and adult samples and these results suggest that a trait characterized by simultaneous internalizing and externalizing problems, compromising the emotional and behavioral functioning of individuals, is remarkable through development. We replicated LCA on adult samples and counterparts of the DYS profile were found in adult population samples from 10 very diverse societies. The prevalence ranged from 6.1% to 12.7%, with an omnicultural mean of 9.2%.

In the study presented in chapter 4, the first aim was to identify, regardless of the categorical diagnoses, possible outcomes in a clinically referred sample of children. Indeed, through a data guided approach, we evaluated the presence during adolescence of two different clusters of presentation of internalizing and externalizing behaviors using CBCL/6-18 scores. We found that one half of the sample belongs to the HIGH cluster, which presents both high internalizing and externalizing symptoms, suggesting that adolescence remains a critical period for individuals which experienced difficulties during childhood. The literature exploring psychopathological traits in adolescence with machine learning methodologies is a very early stage. Amendola and colleagues (2021) evaluated the presence of clusters of symptoms focusing only on the internalizing area of psychopathology. They evaluated an Italian community-based sample of 1127 children and adolescents, aged 8–16 years. Muratori and colleagues analyzed the whole spectrum of psychopathology using these methods with Strengths and Difficulties Questionnaire (SDQ; Goodman, 1997) in an Italian sample of 2959 preadolescence (from 10 to 14 years) from the general population. They found 4 clusters of psychopathological traits presentation, two of them characterized by low levels of psychopathology (74.5% of the total sample) and two characterized by “predominantly internalizing” of “predominantly externalizing” symptoms (19% and 6.5% of the total sample, respectively). Given the differences in the methodologies and that our sample was composed of adolescents that were once children help-seeking for mental health problems, we think that these results are in line with ours. These results highlight the importance of adolescence as a very sensitive period of life, in which the individual faces up to several changes in terms of biological development and new environmental requests. It is a challenging moment, which is in many cases characterized by a decrease in psychological

well-being. This impact on mental health is demonstrated to persist and affect youths' future development (Muratori et al., 2021).

Then, in the light of these previous results, thanks to the data collected in the ReMIND project wave 3 evaluation, we moved to a longitudinal perspective in the study of psychopathological traits. The preliminary results of the study presented in chapter 5 suggested the presence of specific internalizing manifestations trajectories from adolescence to adulthood. Individuals belonging to clusters characterized by stable high presence of anxious, depressive and somatic problems, which were approximately 23% of the total sample, presented higher emotional/behavioral dysregulation during preadolescence, with both higher internalizing and externalizing problems. The presence of clinical level of psychopathology during preadolescence is a strong predictor of its persistence through lifespan development. Moreover, the results evidence the presence of both homotypic and heterotypic continuities: both internalizing and externalizing traits during preadolescence predicted the belonging to the clusters characterized by persistence of high anxious, depressive and somatic symptoms.

This highlights the necessity of early identification and to follow over time children and preadolescence that manifest internalizing and externalizing problems. Further investigations are necessary to better depict these trajectories and the role of biological and environmental factors in shaping those courses. Our preliminary evidence suggested the importance for clinicians and researchers to account for both homotypic and heterotypic continuity in psychopathological traits when planning interventions of treatment and prevention.

My second aim was pursued thanks to the possibility of measuring the impact of environmental (i.e., SLEs, perinatal adversities, SES, parenting style) and genetic and epigenetic factors in influencing these psychopathological traits. Experimental studies presented in chapters 3 were expressly focused on the evaluation of the impact of parenting practice and parental psychopathological symptoms on internalizing and externalizing symptoms in children and on the evolution of genetic and neuroimaging variables on attention problems in children. They were both conducted on wave 2 evaluation of clinically referred subsample. We observed that positive parenting practices, in particular affirmation, have a relevant role in mediating the link between mother and child symptomatology presentation. This is particularly relevant when programming interventions and represents an implication to be taken into account in clinical and educational areas. Our second experimental study highlighted that the complex relationship between genetic makeup and

psychopathological traits might be mediated by neural structures, particularly important for identification and treatment of early-onset psychiatric disorders.

Moreover, we found that HIGh and LOW psychopathology clusters, identified at wave 2 (Experimental study reported in chapter 4), were characterized by differences in the exposure to prenatal adversities and methylation of DNA in candidate genes involved in psychopathology. The results of the analysis conducted with a decision tree classifier underlined the importance of both early environmental experience and epigenetics in causing the presence of psychopathological traits during adolescence. In fact, the presence of peculiar methylation patterns of IGF2, BDNF and OXTR in the HIGH subgroup of adolescents, part of a previously child-referred sample, is in line with previous results. The fact that this subgroup is also characterized by higher frequency of perinatal risk factors, suggested to us that our data confirm the presence of a strong association between adverse events and DNA methylation. As previously mentioned, preliminary results of the experimental study presented in chapter 5 underlined another important risk factor that must be taken into consideration: the stability of psychopathological traits. Our preliminary evidence suggested the importance for clinicians and researchers to account for both homotypic and heterotypic continuity in psychopathological traits when planning interventions of treatment and prevention.

5.5 Conclusions and future directions

The results of my work highlighted the importance of considering psychopathology as traits in order to have a wider vision of its complexity. In my opinion this allows researchers and clinicians to not lose sight of the specificity of the patients and of their problems to chase the right categorical diagnoses.

The results highlighted that internalizing and externalizing symptoms are stable through development, but also that they often are present simultaneously. It is extremely important to take into consideration both homotypic and heterotypic continuity in the study of developmental psychopathology.

Moreover, in the light of the results of this research work, it was clear that it is necessary to consider psychopathology as a very complex object of study. It is mandatory to consider psychopathological traits in a complex, multifactorial and developmental perspective in order to identify risk factors for the onset, the persistence and the worsening of psychopathological outcomes. This is the only way to put in place effective intervention programs.

There is great need for longitudinal studies which take into account risk and resilience factors of different nature and enable to better delineate the genetic, neuronal and environmental mechanisms contributing to developmental risk pathways: the results of these kink of studies would have important implications for effective prevention, identification and treatment of early-onset psychiatric disorders.

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Chapter 4 appendix

Methylation protocol

The methylation status of specific portions of the *BDNF*, *FKBP5*, *IGF2*, and *OXTR* genes was assessed by PCR amplification of bisulfite-treated DNA followed by next-generation sequencing (NGS). We analyzed *IGF2* differentially methylated region (DMR) (chr11:2169373-2169658, 5 CpGs); *BDNF* intron 1 (chr11:27723077-27723244, 11 CpGs); three regions of *OXTR*: promoter (chr3:8811488-8811837, 7 of 9 CpGs analyzed), intron 1 (chr3:8810654-8810919, 13 CpGs), and exon 3 (chr3:8809340-8809530, 15 CpGs); *FKBP5* intron 7 (chr6:35558405-35558550, 3 CpGs).

Methylation levels were determined in DNA from saliva using bisulfite modification followed by NGS. Saliva samples for epigenetic analysis were obtained either by placing a salivary swab in the child's mouth (for younger children) or by asking the child to spit into a tube (for older children). The saliva collection procedure was performed non-invasively. The buccal swabs were processed using the ORAcollect OC-175 kits (DNA Genotek, Ottawa, Canada) and stored at +4°C. Genomic DNA was extracted following manufacturer's protocols and quantified on a Qubit 2.0 fluorometer (Invitrogen). Bisulfite conversion was performed on 200 ng of genomic DNA using the EZ DNA methylation lightning kit (ZymoResearch, Inc, Irvine, CA, USA). Primers were designed using Bisulfite Primer Seeker. Specific tails were added to the primers in order to allow synthesis of SureSelect-style libraries of methylated fragments. Gene-specific PCR amplifications were performed on 20 ng of bisulfite-treated DNA using Taq Gold (Life Technologies, Inc.). Cycling consisted of 5 min pre-activation at 95°C, followed by 35 cycles of 94°C denaturation for 15 s, 58°C annealing for 20 s (56°C for FKBP_i7, 55°C for OXTR_E3), 72°C elongation for 1 min 30 s. All PCR products were tested on 2% agarose TAE gels, treated with Ilustra Exo Pro-STAR (GE Healthcare) to eliminate unincorporated primers, and quantified on a Bioanalyzer 2100 (Agilent).

Secondary PCR was conducted by amplifying pooled equimolar amounts of all gene-specific PCRs from each subject with Agilent Adapters1+2 using Herculase 1 (Agilent). Cycling consisted of 2 min pre-activation at 68°C, followed by 8 cycles of 98°C denaturation for 30 s, 58°C annealing for 30 s, 72°C elongation for 1 min. Products were again purified with Ilustra Exo Pro-STAR and 4 ml aliquots of each sample were amplified with a SureSelect Custom Amplicon Index Kit (Agilent) containing eight forward (i5) and twelve reverse (i7) index primers, allowing unique tagging of up to 96 samples. Cycling consisted of 2 min pre-activation at 98°C, followed by 14 cycles of 98°C denaturation for 30 s, 58°C annealing for 30 s, 72°C elongation for 1 min. Again all PCR products were checked on a 2% agarose TAE gel, purified with AMPure XP beads (Beckman Coulter), quantified on a Bioanalyzer 2100, then approximately equimolar aliquots of each product were pooled in the final library, containing six specific DNA fragments from up to 96 subjects. The library was sequenced on a NEXTSeq 500 (Illumina Inc., San Diego, CA, USA) using a v2 Reagent kit, 300 cycles PE.

Paired ends reads from each sample were independently aligned to all the reference sequences by a parallel striped Smith-Waterman algorithm. Only paired reads that aligned

coherently to the same reference sequence were retained. At each CpG site in each sequence, the 4 base frequencies were evaluated and reported along with the C→T percentage.

Table 1a - Distribution of methylation

Range, mean and standard deviation of individual CpG units within BDNF, FKBP5, IGF2, and OXTR regions

Methylation level	Min (%)	Max (%)	Mean (%)	SD (%)
BDNFCpg1	0.50	3.54	1.68	0.47
BDNFCpg2	0.00	3.37	0.41	0.27
BDNFCpg3	0.03	1.65	0.41	0.19
BDNFCpg4	0.05	1.20	0.30	0.16
BDNFCpg5	0.06	2.37	0.50	0.22
BDNFCpg6	0.08	1.88	0.46	0.19
BDNFCpg7	0.04	4.22	0.49	0.32
BDNFCpg8	0.06	3.88	0.78	0.32
BDNFCpg9	0.08	1.55	0.64	0.24
BDNFCpg10	0.13	2.41	0.83	0.29
BDNFCpg11	0.09	1.20	0.51	0.18
FKBP5Cpg1	52.32	82.94	72.35	5.12
FKBP5Cpg2	74.46	98.11	92.82	3.87
FKBP5Cpg3	70.39	97.21	90.05	4.80
IGF2Cpg1	12.27	61.46	49.27	5.40
IGF2Cpg2	15.75	46.28	38.37	4.66
IGF2Cpg3	26.15	53.86	39.95	4.65
IGF2Cpg4	21.74	56.50	34.06	5.06
IGF2Cpg5	0.00	0.25	0.08	0.05
OXTRE3Cpg1	0.11	24.93	7.94	3.23
OXTRE3Cpg2	0.00	22.68	6.50	2.94
OXTRE3Cpg3	0.11	19.25	4.68	2.38

OXTRE3Cpg4	0.00	20.84	4.52	2.63
OXTRE3Cpg5	0.06	9.84	1.99	1.31
OXTRE3Cpg6	0.12	26.99	8.39	3.85
OXTRE3Cpg7	0.00	21.53	5.90	3.04
OXTRE3Cpg8	0.09	13.58	3.04	1.91
OXTRE3Cpg9	0.12	20.26	5.32	2.93
OXTRE3Cpg10	0.33	20.02	5.80	3.04
OXTRE3Cpg11	0.24	23.89	5.75	3.42
OXTRE3Cpg12	0.03	15.91	3.07	2.13
OXTRE3Cpg13	0.06	23.02	4.96	3.29
OXTRE3Cpg14	0.18	15.33	2.98	2.04
OXTRE3Cpg15	0.32	17.75	4.13	2.42
OXTRI1Cpg1	0.06	4.80	1.73	0.77
OXTRI1Cpg2	0.06	6.71	2.75	1.02
OXTRI1Cpg3	1.95	16.12	7.26	2.07
OXTRI1Cpg4	0.84	17.28	2.84	1.43
OXTRI1Cpg5	23.02	53.09	36.12	4.91
OXTRI1Cpg6	15.10	57.52	38.50	5.58
OXTRI1Cpg7	46.11	74.84	62.09	5.07
OXTRI1Cpg8	28.89	57.48	43.52	4.90
OXTRI1Cpg9	9.03	40.46	23.38	4.88
OXTRI1Cpg10	0.07	15.68	8.27	2.44
OXTRI1Cpg11	0.17	20.24	10.68	3.00
OXTRI1Cpg12	0.07	25.62	12.08	3.60
OXTRI1Cpg13	6.10	25.23	13.22	3.36
OXTRPRCpg1	84.80	99.82	91.35	2.26
OXTRPRCpg2	61.12	87.95	78.55	4.38

OXTRPRCpg3	66.11	88.19	79.26	4.05
OXTRPRCpg4	33.89	80.86	64.30	5.34
OXTRPRCpg5	77.54	99.08	84.74	2.76
OXTRPRCpg6	28.20	67.18	46.98	5.04
OXTRPRCpg7	60.10	99.85	70.65	4.41

note: BDNF = Brain-derived neurotrophic factor; FKBP5 = FK506-binding protein 5 gene; IGF2 = Insulin-like growth factor-2; OXTRE3 = Oxytocin receptor exon 3; OXTRI1 = Oxytocin receptor intron 1; OXTRPR = Oxytocin receptor promoter

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