Tiny Clues, Monumental Impact: Navigating from microRNAs to Brain Development in the Landscape of Adolescent Depression Risk

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Abstract

The surge in the worldwide prevalence of major depressive disorder (MDD), exacerbated by the recent pandemic, among adolescents underscores their heightened vulnerability during this period of life - a phase marked by ongoing neuronal organization. This doctoral thesis addresses the pressing need to identify adolescents at risk for MDD by exploring molecular profiling of peripheral biosamples and early developmental trajectory differences driven by gene networks within the prefrontal cortex (PFC). Spanning five chapters, this research integrates multidisciplinary approaches to reveal markers implicated in psychopathology and identify measurable predictors of depression risk.

Chapter 1 serves as the introduction, delving into the burden of depression, the potential of microRNAs as disease biomarkers, and current advancements in the field. It identifies knowledge gaps, particularly in the study of early-onset disorder, and justifies the focus on vulnerability to MDD during the adolescent phase. It underscores the need for high-throughput analysis of circulating microRNA profiles in longitudinally followed cohorts of adolescents via minimally invasive biosampling. Additionally, it calls for nuanced investigations into candidate genes, building upon the established research involving miR-218 and its gene target DCC, which are known for their roles in PFC maturation during adolescence and their dysregulated expression in MDD.

Chapter 2 navigates the methodology of utilizing dried blood spots (DBS) as a minimally invasive source for profiling peripheral microRNA expression. The optimized protocol outlines procedural steps, including RNA extraction, quantification, and adapter use to mitigate sequencing biases. Standardizing microRNA readouts from DBS offers a cost-effective, reliable method for microRNA profiling, potentially aiding in the development of personalized medicine. Leveraging DBS for psychiatric studies enables streamlined disease prediction and monitoring within the framework of routine clinical care.

Chapter 3 moves to the core of identifying markers associated with molecular dysregulation in MDD. By analyzing microRNA profiles in DBS samples from adolescents with and without clinical depression, this study uncovers differentially expressed candidates tied to vulnerability to the condition. Gene ontology analysis delineates the targets of these

microRNAs and involvement in neurodevelopmental processes. Circulating microRNAs reflect the regulatory changes in the disorder, providing insight into the intricate interplay between neurodevelopment and microRNA dynamics during the onset of the psychiatric disorder.

Chapter 4 employs genetic variance profiling to characterize the DCC gene network's activity and function of in the PFC within community-sampled child cohorts. The study establishes a link between variations in this gene network and individual brain size differences by constructing DCC expression-based polygenic risk score (ePRS) rooted in PFC. The analysis reveals a novel association, with an elevated ePRS corresponding to larger total brain volume in children, offering a distinctive perspective from molecular to systems levels during early-age brain development.

In Chapter 5, I consolidate the findings obtained throughout my studies, offering a comprehensive overview. I conduct a thorough examination of the limitations inherent to each study, illuminating areas where further investigation is warranted. Additionally, I present proposals for future research directions, envisioning potential avenues to enhance our understanding of psychiatric pathology.

This thesis consolidates emerging risk detection strategies that aim to address the critical need to understand the dynamic biological mechanisms underlying vulnerability to mental illness. Together, these chapters depict the complex interplay between microRNAs, gene networks, and brain development. This multidisciplinary research fosters a thorough grasp of the molecular underpinnings of adolescent susceptibility to major depressive disorder, thereby laying the groundwork for early detection and intervention efforts through readily measurable markers.

Résumé

La hausse de la prévalence mondiale du trouble dépressif majeur (TDM), exacerbée par la récente pandémie, parmi les adolescents souligne leur vulnérabilité accrue au cours de cette période de la vie, qui est une phase marquée par une organisation neuronale en cours. Cette thèse de doctorat aborde le besoin pressant d'identifier les adolescents à risque de TDM en explorant le profilage moléculaire des bioéchantillions périphériques et les différences de trajectoire développementale précoce induites par les réseaux géniques dans le cortex préfrontal (CPF). S'étalant sur cinq chapitres, cette recherche intègre des approches multidisciplinaires pour révéler des marqueurs impliqués dans la psychopathologie et identifier des prédicteurs mesurables du risque de dépression.

Le chapitre 1 sert d'introduction, plongeant dans le fardeau de la dépression, le potentiel des microARN en tant que biomarqueurs de la maladie et les avancées actuelles dans le domaine. Il identifie des lacunes de connaissance, en particulier dans l'étude des troubles à début précoce, et justifie la focalisation sur la vulnérabilité au TDM pendant la phase adolescente. Il souligne la nécessité d'une analyse à haut débit des profils de microARN circulants dans des cohortes d'adolescents suivies via des prélèvements biologiques minimalement invasifs. De plus, il appelle à des investigations nuancées sur les gènes candidats, s'appuyant sur la recherche établie impliquant miR-218 et sa cible génique DCC, connus pour leurs rôles dans la maturation du CPF pendant l'adolescence et leur expression dérégulée dans le TDM.

Le chapitre 2 aborde la méthodologie de l'utilisation de taches de sang séché (TSS) comme une source minimalement invasive pour le profilage de l'expression périphérique des microARN. Le protocole optimisé décrit les étapes procédurales, y compris l'extraction de l'ARN, la quantification et l'utilisation d'adaptateurs pour atténuer les biais de séquençage. La normalisation des résultats de microARN à partir de TSS offre une méthode rentable et fiable pour le profilage des microARN, pouvant potentiellement contribuer au développement de la médecine personnalisée. L'utilisation de TSS pour les études psychiatriques permet une prédiction et un suivi précoces de la maladie dans le cadre des soins cliniques de routine.

Le chapitre 3 se concentre sur l'identification des marqueurs associés à la dérégulation moléculaire dans le TDM. En analysant les profils de microARN dans les échantillons de TSS d'adolescents avec et sans dépression clinique, cette étude découvre des microARN différentiellement exprimés liés à la vulnérabilité à la condition. L'analyse de l'ontologie génique délimite les cibles de ces microARN et leurs implications dans les processus neurodéveloppementaux. Les microARN circulants reflètent les changements régulatoires dans le trouble, fournissant un aperçu de l'interaction complexe entre le neurodéveloppement et la dynamique des microARN lors du début du trouble psychiatrique.

Le chapitre 4 utilise le profilage de la variance génétique pour caractériser l'activité du réseau génique DCC et la fonction du cortex préfrontal (CPF) au sein de cohortes d'enfants échantillonnées dans la communauté. L'étude établit un lien entre les variations dans ce réseau génique et les différences de taille cérébrale individuelle en construisant un pointage de risque polygénique basé sur l'expression de DCC (ePRS) enraciné dans le CPF. L'analyse révèle une nouvelle association, avec un ePRS élevé correspondant à un plus grand volume cérébral total chez les enfants, offrant une perspective distinctive des niveaux moléculaires aux niveaux systémiques lors du développement cérébral à un jeune âge.

Dans le chapitre 5, je consolide les résultats obtenus tout au long de mes études, offrant un aperçu complet. J'effectue un examen approfondi des limitations inhérentes à chaque étude, mettant en lumière les domaines où des investigations supplémentaires sont nécessaires. De plus, je présente des propositions pour les orientations futures de la recherche, envisageant des voies potentielles pour améliorer notre compréhension de la pathologie psychiatrique.

Cette thèse consolide des stratégies émergentes de détection des risques visant à répondre à la nécessité cruciale de comprendre les mécanismes biologiques dynamiques sous-jacents à la vulnérabilité aux troubles mentaux. Ensemble, ces chapitres dépeignent l'interaction complexe entre les microARN, les réseaux géniques et le développement cérébral. Cette recherche multidisciplinaire favorise une compréhension approfondie des fondements moléculaires de la susceptibilité adolescente au trouble dépressif majeur, jetant ainsi les bases

pour des efforts de détection précoce et d'intervention à travers des marqueurs facilement mesurables.

"So few grains of happiness
measured against all the dark
and still the scales balance"

The Weighing by Jane Hirshfield

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This dissertation is the culmination of a long and arduous journey. I would not have gotten here without the good hearted, kind people. You truly tip the scale.

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who have given me the optimal genetic and environmental foundations to build upon.

Babulya,
Aleshenka and
my cousins
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The most extraordinary, precious and marvelous woman, who spoils and showers me with love. To you I owe everything. You continuously inspire and surprise me with your drive, wisdom, positivity, grace, and divine brilliance.
Thank you.

Preface to the thesis

This thesis is presented in the manuscript-based format according to the Doctoral Thesis guidelines, as described in the Thesis Preparation Guidelines by the McGill University Department of Graduate and Postdoctoral Studies. The studies constitute three of the chapters and were performed under the supervision of Dr. Cecilia Flores.

The introductory Chapter I is composed of background literature complemented by figures and a table. I also incorporate the published review "*MicroRNA regulation of prefrontal cortex development and psychiatric risk in adolescence*" published in Seminars in Cell and Developmental Biology.

Chapter II "*Preparation and processing of dried blood spots for microRNA sequencing*" has been published in the protocol format in Biology Methods and Protocols.

Chapter III "*Peripheral microRNA signatures in adolescent depression*" is a manuscript currently in preparation for publication.

Chapter IV "*DCC gene network in the prefrontal cortex is associated with total intracranial volume in community-based samples of children*" was published in Journal of Psychiatry and Neuroscience.

Additional segments that provide conceptual links between manuscripts are provided after chapters III and IV as connecting statements. Chapter V provides a summary and discussion of the findings in Chapters II to IV, including important limitations, future directions, and concluding remarks.

Contribution to original knowledge

This thesis comprises three original manuscripts, which I will describe here in chronological order rather than following the sequence of the thesis. My first publication, "*DCC* gene network in the prefrontal cortex is associated with total intracranial volume in community-based samples of children," originated from my rotations in the labs of Dr. Flores and Dr. Silveira. The functional roles of the *DCC* gene are primarily known in embryonic development and as I had joined Dr. Flores' lab, the studies at that time have just showed that *DCC* expression is dysregulated in postmortem brains of individuals with depression who died by suicide. However, the role of this gene in neurodevelopmental processes during childhood and adolescence, whether in healthy or diseased trajectories, was previously unknown. Drawing from the unique approach of Dr. Silveira's lab, we constructed a prefrontal cortex based *DCC* co-expression gene network score that integrated genotype data of kids from a community-based cohort study. The neuroimaging data available for this cohort was used to assess the implication of the network in the global organization of the brain. This study provides evidence of the continuity of DCC in development of the human brain and functional outcomes.

In mouse models for the study of depressive behavior, I contributed to a project exploring epigenetic regulators of Dcc, finding that monitoring miR-218 from adolescence to adulthood distinguishes mice prone to stress-induced depressive behaviors. This sparked the interest in collaborating with clinical researchers to investigate circulating microRNAs in adolescent cohorts. Concurrently, we were invited to submit two review articles, one in Seminars in Cell and Developmental Biology, and another as a Hot Topics mini review in Neuropsychopharmacology. In these reviews, I illustrate the existing gap in the literature, emphasize the necessity for a developmental approach in biomarker studies within psychiatric research, and take the opportunity to propose the specific line of research that I begin to address in this thesis.

We began with a pilot study assessing feasibility of measuring microRNAs in available samples, which were saliva and dried-blood spots. The samples were generously provided by Dr. Ian Gotlib and Dr. Tiffany Ho, lead scientists of two adolescent cohorts. For processing of RNA sequencing data and data analysis, I had the fortunate opportunity to join forces with and

learn from the bioinformatician Dr. Nicholas O'Toole in the laboratory of Dr. Michael Meaney. We identified that the expression profiles of microRNAs were distinct between saliva and those in blood. Deciding to complement the existing literature on adult populations with psychiatric diagnoses with blood samples (including its derivatives, such as plasma and serum), and considering the sample size requirements as estimated by my power analysis, we proceeded to profile microRNAs in dried-blood spots.

Although microRNAs have been profiled in dried-blood spots before, there were no studies in the field of psychiatry when we embarked on this journey. My goal was to approach the untargeted profiling of circulating microRNAs, to guide further assessment of candidate markers. This journey posed some challenges, mainly that the RNA quality in our tested dried blood spots did not pass the standard RNA sequencing quality control measures. Indeed ribosomal RNA, which is used to level the sample input, is not preserved in dried-blood spots, unlike small RNA. With guidance by Dr. Corina Nagy and the practices within the field of small RNA, specifically those associated with extracellular vesicles, we adapted and optimized the methodological approaches for the dried-blood samples. I worked closely with Pascal Ibrahim, a graduate student co-supervised by Dr. Gustavo Turecki, to acquire the fundamentals of library preparation for subsequent small RNA sequencing. Beyond addressing my project objectives, we published the methodological approach in Biology Methods and Protocols "*Preparation and Processing of Dried Blood Spots for microRNA Sequencing*" to promote the widespread use of the method and advocate for open data standards.

Following that, I applied the methodological knowledge to the entire adolescent cohort with clinically-based behavioral measures. In characterizing differentially expressed microRNAs in a single drop of blood this study brings the molecular biology field closer to clinical psychiatry. This study "Peripheral microRNA signatures in adolescent depression" unveils previously unexplored molecular connections associated with depression, elucidating the intricate relationships between specific microRNAs and the biological processes governed by the genes they regulate. It is surely the first of many to come, as dried-blood spot biosampling enables seamless integration into the clinical setting and is cost efficient. The insights gained

from identifying microRNA markers can then be integrated to enhance our capacity for predicting, preventing, and treating depression in adolescents.

The rationale for the nonchronological order of my thesis chapters stems from my overarching research strategy, which progresses from unbiased molecular profiling towards the construction of brain-based networks utilizing the identified gene targets. This approach is instrumental in dissecting the neurodevelopmental mechanisms at the core of depression from the systemic changes apparent in blood samples. For instance, the next phase of my research involves combining microRNA profiling with genotype data from the same cohort to infer expression-based gene networks in the brains of the adolescents. These networks will serve as a means to infer brain pathways relevant to depression and unravel the intricate mechanisms at the core of psychiatric pathology.

Peer Reviewed Publications resulting from graduate work

Alice Morgunova, Nicholas O'Toole, Fatima Abboud, Saché M. Coury, Gary Gang Chen, Gustavo Turecki, Patricia Pelufo Silveira, Michael J. Meaney, Anthony Gifuni, Ian H. Gotlib, Corina Nagy, Tiffany C. Ho, Cecilia Flores. "Peripheral microRNA signatures in adolescent depression, insights from dried blood spots" *in preparation*.

Dariusz Żurawek, **Alice Morgunova**, Laura M. Fiori, Jennie Yang, Claudia Belliveau, Pascal Ibrahim, Sidney H. Kennedy, Raymond W. Lam, Roumen Milev, Susan Rotzinger, Claudio Soares, V. Taylor, Rudolf Uher, Jane A. Foster, Benicio N. Frey, Cecilia Flores, Corina Nagy, Gustavo Turecki. "miR-151a-5p cargo in neuron-derived extracellular vesicles mediates antidepressant response" *in preparation*.

Alice Morgunova, Pascal Ibrahim, Gary Gang Chen, Saché M. Coury, Gustavo Turecki, Michael J. Meaney, Anthony Gifuni, Ian H. Gotlib, Corina Nagy, Tiffany C. Ho, Cecilia Flores (2023). "Preparation and processing of dried blood spots for microRNA sequencing" in Biology Methods and Protocols.

Alice Morgunova and Cecilia Flores (2021). "MicroRNAs as promising peripheral sensors of prefrontal cortex developmental trajectory and psychiatric risk." Part of *Hot Topics* series in Neuropsychopharmacology. DOI:10.1038/s41386-021-01113-3

Alice Morgunova and Cecilia Flores (2021). "MicroRNA regulation of prefrontal cortex development and psychiatric risk in adolescence" *Review* article for the special issue on "Cortical development: from embryo to adolescence" in Seminars in Cell and Developmental Biology. Academic Press;S1084-9521(21)00084-7. DOI: 10.1016/j.semcdb.2021.04.011

Alice Morgunova, Irina Pokhvisneva, Sonja Entringer, Pathik Wadhwa, John Gilmore, Martin Styner, Claudia Buss, Roberto Britto Sassi, Geoffrey B.C. Hall, Kieran J. O'Donnell, Michael J. Meaney, Patricia P. Silveira, Cecilia A. Flores (2020). "DCC gene network in the prefrontal cortex is associated with total intracranial volume in community-based samples of children." Journal of Psychiatry and Neuroscience;46(1):200081. DOI: 10.1503/jpn.200081.

Torres-Berrío Angélica, **Alice Morgunova**, Michel Giroux, Santiago Cuesta, Eric J. Nestler, and Cecilia Flores. (2020). miR-218 in adolescence predicts and mediates vulnerability to stress. Biological Psychiatry. DOI:https://doi.org/10.1016/j.biopsych.2020.10.015

Santiago Cuesta, Dominique Nouel, Lauren M Reynolds, **Alice Morgunova**, Angélica Torres-Berrío, Amanda White, Giovanni Hernandez, Helen M Cooper, Cecilia Flores (2020). "Dopamine axon targeting in the nucleus accumbens in adolescence requires Netrin-1" Frontiers in Cell and Developmental Biology. DOI: 10.3389/fcell.2020.00487

Author Contributions

Chapter I: *Introduction*

The thesis introduction composition was written by Alice Morgunova under the supervision of Dr. Cecilia Flores. Some sections were adapted from the two reviews written by Alice Morgunova and Cecilia Flores.

Chapter II: "*Preparation and processing of dried blood spots for microRNA sequencing*"

Conceptualization and design of the study: Alice Morgunova, Anthony Gifuni, Ian H. Gotlib, Corina Nagy, Tiffany C. Ho, Cecilia Flores

Research execution: Alice Morgunova, Pascal Ibrahim, Gary Gang Chen, Saché M. Coury, Tiffany C. Ho.

Data analysis: Alice Morgunova, Gary Gang Chen, Corina Nagy, Cecilia Flores

Resources: Gustavo Turecki, Michael J. Meaney, Ian H. Gotlib, Corina Nagy, Tiffany C. Ho, Cecilia Flores

Writing of the manuscript: Alice Morgunova and Cecilia Flores wrote the manuscript with valuable review and editing from Pascal Ibrahim, Gary Gang Chen, Anthony Gifuni, Ian H. Gotlib, Corina Nagy, and Tiffany C. Ho.

Chapter III: "*Peripheral microRNA signatures in adolescent depression*"

Conceptualization and design of the study: Alice Morgunova, Anthony Gifuni, Ian H. Gotlib, Michael J. Meaney, Patricia Pelufo Silveira, Corina Nagy, Tiffany C. Ho, Cecilia Flores

Research execution: Alice Morgunova, Gary Gang Chen, Saché M. Coury, Tiffany C. Ho.

Data analysis: Alice Morgunova, Nicholas O'Toole, Fatima Abboud, Corina Nagy, Tiffany C. Ho, Cecilia Flores

Resources: Gustavo Turecki, Michael J. Meaney, Ian H. Gotlib, Corina Nagy, Tiffany C. Ho, Cecilia Flores

Writing of the manuscript (in preparation): Alice Morgunova and Cecilia Flores.

Chapter IV: "*DCC gene network in the prefrontal cortex is associated with total intracranial volume in community-based samples of children*"

Design of the study: Alice Morgunova, Patricia Pelufo Silveira, Cecilia Flores, Pathik Wadhwa, Kieran J. O'Donnell, Michael J. Meaney

Research execution: Alice Morgunova, Patricia Pelufo Silveira, Sonja Entringer, Pathik Wadhwa, John Gilmore, Martin Styner, Claudia Buss, Roberto Britto Sassi, Geoffrey B.C. Hall, Kieran J. O'Donnell, Michael J. Meaney

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Connecting Statements and Chapter V Discussion:

Alice Morgunova wrote the connecting sections and discussion under the supervision of Dr. Cecilia Flores.

Abbreviations

ACC	Anterior Cingulate Cortex
DE	Differential Expression
DCC	*Deleted in colorectal cancer*
DCC	*Human gene*
Dcc	*Mouse gene*
DCC	Protein
ePRS	Expression-based Polygenic Risk Score
GWAS	Genome-Wide Association Study
MDD	Major Depressive Disorder
MicroRNA/ miRNA	Microribonucleic acids
mPFC	Medial Prefrontal Cortex
mRNA	Messenger ribonucleic acids
PFC	Prefrontal Cortex
SNP	Single Nucleotide Polymorphism

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***All manuscript related Figures and Tables can be found in the according Results sections when first referred to.

Chapter I: Introduction

1.1 Adolescence: The great transformation

Adolescence is far from being *just a phase* – it is the time of objective developmental transformations with monumental ramifications. This period is characterized by physiological changes like puberty, as well as quests for novel experiences, evolving social dynamics, and cognitive growth (Spear, 2000). The more apparent observable changes from childhood that mark the beginning of adolescence are the physical changes associated with puberty (Sisk and Foster 2004; Breehl and Caban, 2022). This may include growth spurts and development of secondary sexual characteristics. Beneath these changes are active signals, such as hormones originating from the nervous system and circulating throughout the body (Blakemore et al., 2010). There is considerable variability among individuals reaching puberty, and definitions of adolescence may differ based on historical and cultural perspectives. While some aspects, like reproductive maturation gauged by the Tanner scale, can be objectively assessed, marking biological milestones such as cortical development necessitates extensive neuroimaging techniques (e.g., Pines et al., 2020). Neurodevelopment is a dynamic, nonlinear process unfolding over time (Larsen et al., 2023). As it spans ages around 10 to 18, individuals in this age group may be referred to as youth, teenagers, or young adults. Indeed, some debate has been raised about terminology and the age range, pushing the upper boundary to mid-twenties (Sawyer et al., 2018). Adolescence is particularly notable for transformations in the brain that encompass both physiological and functional developments.

1.1.1 Ongoing maturation of the brain and cognitive development in adolescence

The adolescent brain undergoes maturation along architectonic gradients, from primary cortices responsible for sensory and motor functions and gradually progressing to higher-order, transmodal association cortices (Gogtay et al., 2004; Paus, 2005; Tottenham, 2020; Dong et al., 2021; Sydnor et al., 2021). While the volume of cortical grey matter peaks in early adolescence, the development of connections driven by the white matter continues up until the third decade of life (Giedd et al., 2015; Paquola et al., 2019).

The structural maturation and mechanisms underlying it, carry functional ramifications that form the basis for cognitive and emotional development (Blakemore, 2008; Arain et al., 2013; Luna et al., 2015; Vijayakumar et al., 2018). For example, the prefrontal cortex (PFC), which is one of the last brain regions to reach maturation, plays a pivotal role in self-related processing (Rutter & Rutter, 1993; Casey et al., 2008). Adolescents experience significant gains in cognitive flexibility and transition from egocentric to more nuanced, multidimensional thought processes, such as abstract thinking and problem-solving (Rubia et al., 2006; Pfeifer and Berkman 2018; Pfeifer and Allen, 2021). This development enables strategic thinking and deeper introspection. As adolescents navigate diverse environments and form close relationships (Blakemore, 2008), they engage in self-exploration and identity formation, which are crucial for social functioning and integration in adulthood (Pfeifer and Berkman, 2018). The refined organization of the brain and shifts in cognitive functions operate in a complex synergy, shaping the holistic development of the individual in conjunction with environmental influences.

1.2 Depression's hidden depths in youth

The malleable window of adolescence, particularly that of developing brain circuitry, is sensitive to external perturbations, such as stress, which can negatively affect the ongoing neurobiological processes, placing individuals at heightened risk for mental illness (Henje Blom et al., 2016; Shaw et al., 2020; Pizzagalli and Roberts, 2022). A prominent psychiatric disorder facing this age group is the major depressive disorder (MDD; Otte et al., 2016; Daly, 2022). Experiencing depression during these formative years is associated with functional impairment in academic and social settings, deliberate self-harm, and can escalate the risk of more severe depression and premature mortality in adulthood (Birmaher et al., 2002; Dunn and Goodyer, 2006; Hawton et al., 2012; Archer et al., 2018).

There are two main classifications for depression that are used in clinical practice around the world, the Diagnostic and Statistical Manual of Mental Disorders, DSM-5 (American Psychiatric Association, 2014) published by the American Psychiatric Association, and the International Classification of Diseases (ICD-11, 2019) reference published by the World Health

Organization. They offer slightly different categorizations of depressive disorders and both are criticized for poor distinction between child (ages 5-11), adolescent (also referred to as young person 12-18) and adult depression (Thapar et al., 2012; Henje Blom et al., 2016; Bernaras et al., 2019; Herrman et al., 2022).

Based on the DSM-5 guide, there are 9 possible symptoms (Figure 1) indicative of the impairment. The operational disease definitions and the categorical approach are some of its debated features in both the clinic and research (Bernaras et al., 2019; Mücke-Heim et al., 2022; Herrman et al., 2022). In clinical assessments aimed at determining vulnerability or diagnosis, practitioners are tasked with considering a myriad of factors: “patient’s circumstances, preferences, values, maturity, developmental level, clinical profile, comorbidities, neurodevelopmental disorders, communication needs and capacity” (Luxton and Kyriakopoulos, 2022; Herrman et al., 2022). Compounding this challenge, young people may be reticent to share their inner experiences (Babajide et al., 2020; Herrman et al., 2022). This multifaceted subjectivity likely contributes to the inconsistency and variability commonly observed across cohort studies, posing critical challenges for research and clinical practice alike (Fried and Nesse, 2015; Buch and Liston, 2021).

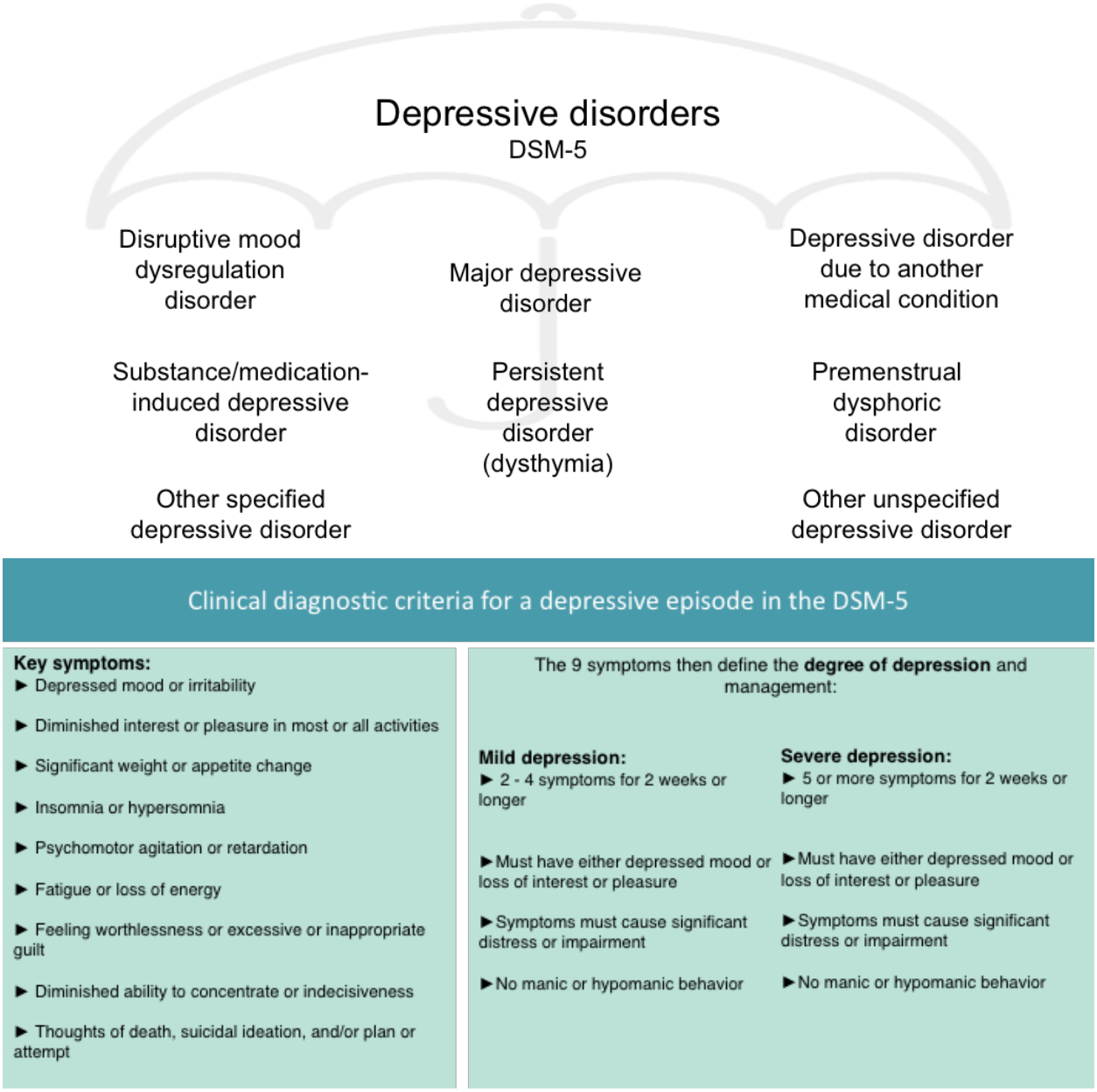

Figure 1. Top panel: Depressive disorders is an umbrella term encompassing several conditions. Bottom panel: Diagnostic criteria used in the clinic and research. Adapted from American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders (DSM), version 5, 2013.

In addition to the classification, there are other challenges facing adolescent mental health care. Long wait lists for specialized mental health care are not uncommon (Kowalewski et al., 2011; Edbrooke-Childs and Deighton, 2020); as a result, it is often recommended that mild depression without comorbidity is managed by primary care (Lawton and Moghraby, 2016; Babajide et al., 2020; Luxton and Kyriakopoulos, 2022). Structural barriers, such as prolonged wait times for clinical appointments, are linked to subsequent reduced patient compliance (Gallucci et al., 2005; Reardon et al., 2017). However, there are additional service gaps that young people experience. It is estimated that 12% of adolescents diagnosed with depression will make at least one suicide attempt (Cash et al., 2009; Serra et al., 2022). Alarmingly, this is likely a significant underestimate because the suicide-related diagnostic codes used by emergency departments fail to identify a third of children who present with self-injurious thoughts and behaviors, and more than half are missed when relying solely on the suicide-related chief complaints (Edgcomb et al., 2023). Moreover, the first-line treatment for depression, selective serotonin reuptake inhibitors (SSRI), triples the risk of suicide attempts when prescribed to individuals under 25 years of age (Sarginson et a., 2017; Lagerberg et al., 2023). In low and middle-income countries where mental health resources are severely limited, these challenges are further magnified (Wainberg et al., 2017; Zajkowska, 2021). Adolescence is a demographic group that is both understudied and underserved in specialized mental health care services.

The highly heterogeneous symptom presentation under the major depressive disorder umbrella poses challenges for both individual treatment outcomes and MDD research studies. There is a clear need for the current nosology framework represented by the DSM-V and ICD-11 to be complemented by biological and molecular measures (Comes et al 2018). Biological and genetic systems are integral frameworks upon which resilience or vulnerability to psychiatric illness is interwoven, with the timing of adverse exposures serving as a critical factor (Jirtle and Skinner, 2007; Boersma et al., 2014). The intersecting pathways of adolescent neurodevelopment and mental health risks make it imperative to examine the neurobiological basis of developmental trajectories that could tilt the balance toward either vulnerability or resilience (Henje Blom et al., 2016).

1.2.1 Overview of multifaceted factors in MDD

Risk factors for depression span multiple domains, including demographics, cognition, and interpersonal factors (Herrman et al., 2022). Indeed, these risk factors serve as distinct differences between individuals who experience the onset of MDD in adolescence and those in adulthood (Jaffee et al., 2002; Rice, 2010). One recurring observation is the higher prevalence of depression in females than in males that emerges during adolescence. Although the hormonal sex differences play a role, they alone do not fully account for the complexity of the disease etiology (Soares et al., 2008; Kessler and Bromet, 2013; Thapar et al., 2012; Galvo et al., 2014; Kuehner et al., 2017; Labala et al., 2018; Mena and Benoit, 2019).

The rise in major depressive disorder (MDD) during adolescence aligns with the biologically initiated events of puberty, underscoring the inherent connection between the developmental and biological aspects of MDD. Adolescent depression may arise from certain configurations of three developmental factors: (i) normative changes that occur during adolescence, such as brain maturation, shifts in interactions with the environment, and hormonal surges; (ii) sensitive periods when external influences have pronounced effects on developing biological systems, and (iii) maturational shifts that act as catalysts for the pathognomonic trajectory (Andersen & Teicher, 2008).

The complexity of MDD involves multiple systems and, over centuries, has been examined through various, at times transient, approaches (Kendler, 2019; Malik et al., 2021; Herrman et al., 2022). While covering the vast research on depression, implicated systems and associated risk factors is beyond the scope of this thesis, key features are summarized in Table 1. This serves to acknowledge the intricate puzzle in the realm of MDD biomarker research, as well as to caution against the shortcomings of current practices that overlook the biological facets intrinsically linked to the disease.

Table 1. Selected systems and factors involved in depression risk.

Implicated systems	**Highlighted features**	**References**
Structural	Network wide: frontostriatal, frontolimbic and corticocortical	Price et al., 2012; Henje Blom et al., 2015; Miller et al., 2015; Boccia et al., 2016; Miles et al., 2021; Pizzagalli and Roberts, 2022
	Regions: prefrontal cortex, anterior cingulate, amygdala, striatum	Forbes and Dahl., 2012; O'Callaghan and Stringaris, 2019; Bittar and Labonté, 2021
	Neural basis: dendrite and myelination	Pizzagalli and Roberts, 2022; Ho et al., 2021; Qiao et al., 2016
Neurochemical	Monoaminergic systems (e.g., norepinephrine, dopamine, serotonin),	Jiang et al., 2022; Hirschfeld et al., 2000; Belmaker and Agam, 2008; Hamon et al., 2013; Moncrieff et al., 2022
	Neurotransmitters (e.g., glutamate)	Khoodoruth et al., 2022; Duman et al., 2019; Jun et al., 2014
Neuroendocrine	Hypothalamic-Pituitary Axis (HPA), cortisol levels	Harkness and Wynne-Edwards, 2011; Zorn et al., 2017; Stetler and Miller, 2011; Lopez-Duran et al., 2015; Ulrike and Dirk, 2013; Nikkheslat et al., 2020; Zajkowska, 2021
	Hormonal imbalances (gonadal, oestradiol, testosterone)	Naninck et al., 2011; Bangasser and Valentino, 2014; Angold et al., 2006
Genetic	Heritability	Sullivan et al., 2000; Kendler et al., 2018; Zhang et al., 2023;
	GWAS studies	Zhang et al., 2019; Li et al., 2021; Howard et al., 2019; Buch and Liston, 2021
Environmental	Stress, isolation, socioeconomic status, early life stress and/or trauma	Andersen and Teicher, 2008; Zajkowska et al., 2021; LeMoult et al., 2020; Rao and Chen, 2022
Epigenetic	DNA methylome, histone modifications, chromatin remodeling, non-coding RNAs (e.g., microRNAs)	Barker et al., 2018; Misra et al., 2019; Paoli et al., 2022; Wu et al., 2022; Bagot et al., 2022; Torres-Berrío et al., 2022
Inflammatory	Upregulated cytokine levels (e.g., TNF-α, IL-6)	Latham et al., 2022; Colasanto et al., 2020
	Gut-brain axis	Calarge et al., 2019; Simkin et al., 2019; Freimer et al., 2020
	Brain barrier permeability	Wu et al., 2023; Serna-Rodríguez et al., 2022
Physiological correlates	Heart rate, blood pressure, cardiovascular and metabolic diseases	Baumeister-Lingens et al., 2023; Vazquez et al., 2016; Goldstein et al., 2015; Walker et al., 2015.

Susceptibility to disease risk factors is not uniform across individuals, and the unique combination of any of these factors for each individual can result in a phenotype that is broadly defined by the current nosology (Herrman et al., 2022). MDD is a systemic condition with changes at multiple levels (from molecules to systems) and thus a multi-layered approach encompassing genetic and cognitive perspectives is imperative for a more comprehensive understanding of MDD (Kendler et al., 2019; Zonca, 2021; Marx et al., 2023).

1.3 MicroRNAs, the maestros

Risk variants identified in genome wide association studies (GWAS), including those for depression, have yielded a curious observation that there are many intergenic hits that impact phenotypes, as opposed to exclusively in the protein encoding exons (Edwards et al., 2013; Ciuculete et al., 2020). The non-coding sections of the genome make up the vast majority and include highly important regulatory sequences. Within these regions, non-coding RNAs can be transcribed from both strands of the DNA and divided into two broad categories according to size: (i) long noncoding RNAs (lncRNAs; >200 bp); and (ii) small RNAs (<200 bp), such as microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), and piwi-interacting RNAs (piRNAs) (Mattick and Makunin, 2006). Of the non-coding RNA class, miRNAs are the most commonly studied as they offer a convenient path for basic and clinical research in terms of accessibility and functionality. Analogous to a film score that adds nuance and emotional depth, microRNAs serve to modulate and fine-tune gene expression, thereby achieving a balanced cellular environment. Their abundance and evolutionally conservation across species (Gebert and MacRae, 2019) is a telltale sign of their critical involvement in biological processes, and indeed miRNAs have been noted to be involved in the development and differentiation of the nervous system, synaptic plasticity, maintenance and survival of neurons (Sempere et al 2004; Graff et al., 2011; Liu et al., 2018).

The unfolding of gene expression, such as during the typological hierarchy of brain development, underscores the importance of the regimented transcriptome timing (onset, rate and duration) (Somel et al., 2009; Krol et al., 2010; Haeussler et al., 2017). Non-coding RNAs possess an epigenetic capacity to promptly modify gene expression, essential for brain function

and responsive to environmental circumstances (Graff et al., 2011). MicroRNAs constitute one of the fastest and most dynamic mechanisms for regulating gene transcription and protein translation in response to stress (Lopizzo, 2019). Beyond target regulation, miRNAs reduce stochastic gene expression noise (Schmiedel et al 2015; Siciliano et al., 2013) and regulate themselves (Hill and Tran, 2022). Of additional relevance to the multifactorial MDD, microRNAs are responsive to the effects of endogenous factors such as hormones and cytokines (Gulyaeva and Kushlinskiy, 2016).

The molecular mechanisms and timelines that underpin resilience or susceptibility to dysfunction in psychiatric disorders remain poorly understood. MicroRNAs serve as the maestros of neurodevelopmental orchestration, akin to the role of conductors in a complex musical symphony. Just as a conductor's nuanced direction can make or break the performance, guiding it towards harmony or dissonance, microRNAs make critical adjustments at pivotal moments. Their fine-tuned modulation can set the stage for either a trajectory towards well-being or a precarious path leading to mental health challenges such as depression. In the following review, I delve into the literature to uncover how microRNAs may play a role in shaping maturation of the brain circuitry during adolescence and their link to vulnerability to depression.

1.4 MicroRNA Regulation of Prefrontal Cortex Development and Psychiatric Risk in Adolescence

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Abstract

In this review, we examine the role of microRNAs in the development of the prefrontal cortex (PFC) in adolescence and in individual differences in vulnerability to mental illness. We describe results from clinical and preclinical research indicating that adolescence coincides with drastic changes in local microRNA expression, including microRNAs that control gene networks involved in PFC and cognitive refinement. We highlight that altered levels of microRNAs in the PFC are associated with psychopathologies of adolescent onset, notably depression and schizophrenia. We show that microRNAs can be measured non-invasively in peripheral samples and could serve as longitudinal physiological readouts of brain expression and psychiatric risk in youth.

Introduction

Adolescence is a developmental period marked by sexual maturation, novel-experience seeking, and transition in cognitive skills. This malleable window is sensitive to perturbations including drugs of abuse and stress, which can render individuals at risk for mental illness. Enhanced psychiatric vulnerability in adolescence has been attributed to substantial changes occurring in the maturing prefrontal cortex (PFC) during this time. Dysfunction of the PFC is linked to changes in personality, emotional response, memory, attention, and social behavior (Harlow, 1868; Choroschko, 1923; Feuchtwanger, 1923; Brickner, 1934; Ackerly, 1935; Uchida et al., 2010; Fuster, 2015), and is observed in psychopathologies that emerge in adolescence (Gao et al., 2012; Goldstein and Volkow, 2011; Tan et al., 2009; Rice et al., 2019). The molecular mechanisms and the timeline underlying susceptibility or resilience to PFC dysfunction remain elusive. MicroRNAs are essential coordinators of developmental programing, and mediators between environmental factors and changes in gene expression. In this review we propose that microRNA-mediated shaping of PFC circuitry maturation in adolescence may be an important determinant of lifetime mental health.

MicroRNAs: functional complexity

Microribonucleic acids, or microRNAs, are small RNA molecules that serve as key regulators of large-scale gene expression. MicroRNAs are non-coding RNA 19-25 nucleotides long and are single-stranded. They can match and bind seed regions of several different messenger RNAs (mRNAs). This property allows a single microRNA to control and coordinate activity of entire gene networks and cellular pathways (Rajman and Schratt, 2017). MicroRNA biogenesis is a multi-step process. Briefly, the more common canonical biogenesis starts with the transcription of primary microRNAs, which are double-stranded long hairpin-like structures with a polyA tail. The polyA tail is then cleaved by the microprocessor complex (Drosha/DGCR8), generating a double-stranded precursor microRNA, which is exported from the nucleus to the cytoplasm by the Exportin-5 protein. Once in the cytoplasm, Dicer endoribonuclease cleaves the precursor microRNA, to produce a single-stranded and functional mature microRNA, either the forward 5’ (5p) or the reverse 3’ direction (3p) of the original strand. The mature microRNA is then loaded

into an Argonaute family protein (AGO 1-4 in humans) to form a microRNA-induced silencing complex (miRISC) that interacts with the 3'-untranslated region (UTR) of the mRNA target (for detailed reviews see Ha and Kim, 2014; O'Brien et al., 2018).

The binding of microRNAs to perfectly complementary mRNA sequences (2-7 nucleotides) of target genes usually induces transcript degradation and/or prevention of mRNA translation (Ha and Kim, 2014). However, increased mRNA translation upon microRNA binding to mRNAs targets has also been described (Vasudevan et al., 2007; Rocchi et al., 2019). Mature functional microRNAs can be found in the cell's cytoplasm and in the nucleus, and their distribution can change in response to environmental factors (Li et al., 2013; Turunen et al., 2019). MicroRNAs localized to the cytoplasm and those in the nucleus seem to be involved in different processes. Nuclear microRNAs can trigger gene transcription by binding and activating enhancer regions (Xiao et al., 2017) or by directly binding to promoter regions (Matsui et al., 2013). The variety of mechanisms involved in microRNA control over gene expression indicates the complexity of the system and that much remains to be uncovered.

Contribution of microRNAs to early cortical development

The majority of microRNAs are evolutionary conserved and play analogous biological functions across species, including in neurodevelopment. In this review we focus on the role of microRNAs in the development of the cerebral cortex, the most superficial sheet of the mammalian brain, which is involved in higher-order cognitive function and sensory processing. MicroRNAs are proving to be essential regulators of cortical development (Volvert et al., 2012), playing critical roles in the production of progenitor cells (Desai and McConnell, 2000; Briam et al., 2013; Chen et al., 2014; Abdullah et al., 2016) and in the survival, differentiation and spatiotemporal organization of cortical neurons (Tonelli et al., 2008; Schwamborn et al., 2019; Cremisi, 2013; Barca-Mayo and Tonelli, 2014). A microRNA network appears to have evolved to specifically shape the developmental fate of corticospinal and callosal projection neurons, which are at the root of mammalian (more specifically eutherian) brain anatomy and function (Diaz et al., 2020). Obstruction of microRNA function or of enzymes involved in microRNA synthesis (e.g. Dicer, DGCR8) induces dramatic disruption of cortical development, from cell viability and

premature progenitor differentiation during corticogenesis to cortical malformations (Barca-Mayo and Tonelli, 2014; Marinaro et al., 2017; Wang et al., 2007). MicroRNA control over cortical development extends to adolescence (Torres-Berrio et al., 2020), when the prefrontal portion of the cortex (PFC) continues to undergo substantial maturation (Gogtay et al., 2004; Luna, 2009; Sturman and Moghaddam, 2011; Ordaz et al., 2013; Larsen and Luna, 2018; Kalsbeek et al., 1988; Naneix et al., 2012; Willing et al., 2017; Hoops and Flores, 2017), remaining particularly vulnerable to environmental factors.

Adolescence is a critical period for PFC development, microRNA expression, and psychiatric vulnerability

The PFC is often characterized as the "executive" center where information is processed and integrated to carry out complex functions, including planning, decision-making and goal-directed behavior. One third of the cerebral cortex in primates is the anterior region of the frontal lobe (Fuster, 1996), although in rodents the PFC takes smaller percentage of the total cortical area. As reviewed elsewhere, differences in PFC cytoarchitecture between primates and rodents are important limitations to the translation of findings across species (Gao et al., 2012; Carlen, 2017; Laubach et al., 2018). However, both anatomically and functionally, the PFC is believed to be homologous between primates and rodents, with some disagreement remaining as to the specific correspondences between PFC subregions between primates and rodents (Laubach et al., 2018).

A common feature of the PFC in rodents and primates is that it continues to sustain significant structural and functional maturation across the adolescent period and into early adulthood (Gogtay et al., 2004; Luna, 2009; Sturman and Moghaddam, 2011; Ordaz et al., 2013; Larsen and Luna, 2018; Hoops and Flores, 2017). This protracted development is accompanied by corresponding transitions in behaviors and cognitive function, including the gradual stabilization of emotional reactivity, novelty seeking, cognitive control, and decision-making (Sturman and Moghaddam, 2011; Spear, 2010). During adolescence, the PFC undergoes gray matter volume reduction, white matter content increase, and refinement in circuitry

cytoarchitecture, including axonal myelinization, dendritic morphology and synaptic density (Sowell et al., 2003; Gogtay et al., 2004; Larsen and Luna, 2018). The *pari passu* unfolding of structural and behavioral correlates indicates that proper PFC development is critical for the maturation of cognition and behavior. Adolescence is in fact a sensitive developmental time that sets the stage for lifelong mental health (see Marin, 2016).

Here we review the evidence on how maturation of the PFC and emergence of psychiatric disorders may be mediated by microRNAs. Nevertheless, changes in microRNA expression in the cortex at any point of the developmental continuum can affect the function of gene networks controlling ongoing homeostatic processes (Somel et al., 2010). Emerging evidence from rodent studies supports the notion that microRNAs are key epigenetic mediators of early environmental programing on neurocircuitry, cognitive development and function throughout the lifespan (Torres-Berrio et al., 2020; Allen and Dwivedi, 2020; Cattaneo et al., 2020), even lasting across generations (Rodgers et al., 2013; Zucchi et al., 2013).

Human postmortem brain studies have shown that microRNA expression in the PFC is highly influenced by age with some microRNAs being differentially expressed between infancy, early and late childhood, and adolescence (Ziats and Rennert, 2014). Remarkably, the adolescent period coincides with a shift in the pattern of global microRNA expression. A study analyzing average expression of microRNAs in the PFC from neonates to older adults found that precisely in adolescence the pattern of microRNA expression splits into two diverging directions: while some microRNAs begin to be upregulated, others are downregulated, with the new pattern of expression maintained for the rest of life (Beveridge et al., 2014). To probe possible biological implications of the age-associated microRNA expression split, the authors performed gene target, pathway and function analyses. They found that gene targets of the “split” microRNAs are involved in nervous system development and function, cell-to-cell signaling and interactions: e.g. synaptic transmission and axon and neurite morphology, and have been associated with schizophrenia, bipolar and other mood disorders. This study also revealed that the expression of genes involved in microRNA biogenesis, notably *EXPORTIN-5* and *DICER*, also switches from teenagehood onward (Beveridge et al., 2014). These findings suggest that abnormalities in the

adolescent pattern of microRNA expression trajectories in the PFC could lead to predisposition to mental illness.

Dysfunction of the PFC has been strongly implicated in the etiology of psychiatric disorders of adolescent onset, including schizophrenia, major depressive disorder (MDD) and substance use disorder (see: Gao et al., 2012; O'Donnell, 2011; Goldstein and Volkow, 2011; Myers-Schulz and Koenigs, 2012). An increasing number of studies, including those by our group, have identified alterations in microRNA expression in postmortem PFC samples of psychiatric patients. A summary of results from studies in schizophrenia, bipolar disorder, depression, and substance use disorder, are presented in Table 1. Some microRNAs have been found to be dysregulated in multiple disorders, suggesting that they may play a role in core neurodevelopmental processes. MiR-29, for example, is involved in cortical maturation (Kole et al., 2011), neuronal differentiation (Zhang et al., 2017), neuron survival (Roshan et al., 2014), and synapse formation (Lippi et al., 2011), and appears to be altered in both schizophrenia and bipolar disorder. In fact, a recent study in rodents links miR-29 to neurobehavioral deficits through the regulation of postnatal cortical maturation (Swahari et al., 2021). Studies are needed to assess the role and timing of specific microRNAs in the pathogenesis of these disorders.

Role of miR-218 in psychiatric disorders of PFC dysfunction and adolescent onset

Major depression is one of the most widespread psychiatric disorders, ranked by the World Health Organization as the leading cause of disability worldwide (Ferrari et al., 2013), and as many as 40% of patients diagnosed with MDD do not respond to common antidepressant therapies (Rush et al., 2006; Zhou et al., 2014; Cuijpers et al., 2020). The incidence of depression is high between the ages 12 – 25 (Wittchen et al., 1998; Lewinsohn et al., 1993; Beesdo et al., 2009; Keyes et al., 2019) and an alarming 25% of adolescents meet the criteria for depression (Saluja et al., 2004; Avenevoli et al., 2008; Merikangas et al., 2009; Lee et al., 2014). Experiencing a depressive episode in adolescence increases the risk of depression and of higher severity in adulthood up to 3-fold (Pine et al., 1998; Weissman et al., 1999; Kessler et al., 2005).

In our studies investigating microRNAs involved in PFC maturation and MDD vulnerability, we identified the microRNA miR-218-5p (whose matured sequence originates from the 5' strand

of its miR-218-1 or miR-218-2 stem loop precursors), as a repressor of the DCC gene. DCC is a receptor for the guidance cue Netrin-1 (Torres-Berrio et al., 2017) and is intimately involved in the formation of neuronal networks during early neurodevelopment. Notably, DCC-mediated Netrin-1 signaling controls the protracted maturation of the PFC circuitry specifically in adolescence (Manitt et al., 2013; Reynolds et al., 2018; Hoops et al., 2018). Using postmortem brain samples, we showed that *DCC* expression in the PFC of adult individuals who were diagnosed with MDD and died by suicide is significantly upregulated compared to control individuals who died by sudden death (Torres-Berrio et al., 20017). We replicated this finding in two independent cohorts (Torres-Berrio et al., 2017; Manitt et al., 2013). To probe the role of miR-218-5p (henceforth referred to as miR-218) in the *DCC* changes observed in MDD, we assessed its expression in the same postmortem PFC samples and found that miR-218 is *downregulated* in MDD by approximately 50% compared to controls. Reduced levels of miR-218 correlate with elevated expression of *DCC* in the PFC of individuals with depression, suggesting a causal link between miR-218 and *DCC* alterations in MDD pathogenesis (Torres-Berrio et al., 2017).

A rapidly increasing number of studies show that changes in *DCC* are linked to vulnerability to psychopathologies of adolescent onset and involving PFC dysfunction, most prominently MDD (reviews from (Vosberg et al., 2019; Torres-Berrio et al., 2020). Furthermore, we have shown that variation in the *DCC* gene co-expression network within the PFC is associated with total brain volume across childhood, highlighting the role of this system in broad postnatal neurodevelopment (Morgunova et al., 2021). Whether genetic variants (single nucleotide polymorphisms) within *DCC* (see: Grant et al., 2012) affect the binding of microRNAs that modulate them, or whether changes in expression of the *DCC* network are linked to altered microRNA function, remains to be shown.

Postmortem studies cannot reveal whether alterations in microRNA expression in MDD mediate atypical development of the PFC and/or play a causal role in disorder symptomatology. MicroRNAs are highly conserved, and miR-218-5p is homologous between humans, macaques, and rodents (UCSC genome browser; Kuhn et al., 2013), offering a link between preclinical and translational studies. The social defeat stress paradigm is a well-established model used in

rodents to study stress-induced behavioral abnormalities that resemble depression-like traits (Golden et al., 2011; Berton et al., 2006). Using this model, we assessed the effects of degrading miR-218 directly in the PFC of adult male rodents via anti-sense oligonucleotides ("antagomirs"). Consistent with the reduced expression of miR-218 in the PFC of MDD patients, we found that downregulating miR-218 in the adult mouse PFC *increases* DCC expression and elicits vulnerability to stress-induced depression-like behavioral abnormalities (Torres-Berrio et al., 2020). Furthermore, in mice that show resilience to stress, intranasal administration of miR-218 antagomir induces a similar vulnerability. Upregulating miR-218 in the PFC, via viral-mediated gene transfer, instead *reduces* DCC expression and protects against stress-induced depression-like behavioral traits, pointing at miR-218 as a potential therapeutic target. Altered miR-218 levels in the PFC have also been reported in adult stress-exposed rats (either through the chronic stress paradigm or corticosterone injections; Dwivedi et al., 2015), and in adult mice exposed to chronic unpredictable mild stress (Ma et al., 2016).

Given that DCC receptors control the adolescent maturation of the PFC (Manitt et al., 2013; Reynolds et al., 2018; Hoops et al., 2018), we investigated miR-218 expression in the mouse PFC across postnatal life. Consistent with the adolescent shift in global microRNA expression observed in the PFC in humans (Ziats et al., 2014; Beveridge et al., 2014), miR-218 levels in mice increase from early adolescence to adulthood. Postnatal PFC miR-218 and *Dcc* expression in mice correlate negatively (Torres-Berrio et al., 2020). Disrupting this developmental pattern has enduring behavioral consequences: downregulation of miR-218 in the PFC of adolescent mice via antagomir microinfusion, induces resilience to detrimental effects of chronic social defeat stress in adulthood. This finding is opposite to the effects seen following miR-218 downregulation in the adult matured PFC, yet in line with the idea that changes in the adolescent pattern of microRNA expression in the PFC are associated with vulnerability to developing psychiatric traits. Interestingly, results from a preliminary experiment show that intranasal administration of miR-218 antagomir in adolescent male mice leads to reduced anxiety-like behavior in adulthood, without affecting motor abilities (Amin et al., 2015; Hoye et al., 2017; Cerro-Herreros et al., 2018), indicating that targeting microRNA expression in adolescence may have enduring preventative and/or treatment benefits.

The opposite effects that adolescent versus adult antagomir-218 microinfusions in the prefrontal cortex have on stress vulnerability are intriguing (Torres-Berrio et al., 2020; Torres-Berrio et al., 2017; Torres-Berrio et al., 2021) [33,79,90], particularly within the context of miR-218 regulation of *Dcc* expression across postnatal life (Torres-Berrio et al., 2021). The Netrin-1/DCC guidance cue pathway organizes neural connectivity in the developing and matured brain via age-specific molecular and cellular processes. In adolescence, DCC-mediated Netrin-1 signaling is involved in axonal targeting and growth, whereas in the adult brain this signaling pathway controls the refinement of already established circuitries, by modifying neuronal structure, including dendritic spine morphology (reviews: Vosberg et al., 2019; Torres-Berrio et al., 2020). The role of miR-218 in shaping psychiatric vulnerability or resilience is likely to be dictated by the function of the Netrin-1/DCC pathway at that particular developmental period. Downregulating miR-218 in the adolescent PFC, when its levels are significantly lower than in adulthood, would prolong high expression of *Dcc* and likely extend the postnatal window of axonal targeting and growth and synapse formation. In contrast, reducing miR-218 in the adult PFC, would bring *Dcc* expression to levels observed in the immature brain, eliciting aberrant changes in the organization of synaptic circuitry. RNA sequencing based strategies assessing alterations in PFC gene expression under antagomir-218 treatment in adolescence versus adulthood would aid in elucidating gene networks and pathways involved in the distinct behavioral outcomes.

microRNAs may coordinate the organization of synaptic circuits in the developing adolescent PFC

The maturation of the PFC requires tight regulation of gene expression to drive proper neuronal connectivity and plasticity (Hoops and Flores, 2017; Caballero et al., 2016). In cultured cortical neurons, microRNAs have been shown to modulate synaptogenesis (Kos et al., 2016) and axon extension (Kos et al., 2016; Zhang et al., 2013; Li et al., 2014; Kos et al., 2017). In rodent studies with a focus on early postnatal life, microRNAs have been found to regulate cortical pyramidal neuron dendritic structure (Christensen et al., 2010) and spine morphology (Siegel et al., 2009), which are key determinants of intercellular communication and circuitry organization. The influence of microRNAs over PFC development in adolescence likely involves coordinating expression of gene networks that control the establishment of synaptic connections, including

those influencing dendritic spine morphology and plasticity. Consistent with this idea, postnatal deficiency in microRNA production in pyramidal neurons, due to conditional downregulation or deletion of the DGCR8 microprocessor, results in altered dendritic morphology and synaptic transmission in the PFC (Hsu et al., 2012; Schofield et al., 2011). MiR-218 is highly expressed in dendritic spine compartments of PFC pyramidal neurons (Siegel et al., 2009), and changes in its expression are associated with modification of spine morphogenesis in these cells (Torres-Berrio et al., 2020). Changes in somatodendritic properties of PFC pyramidal neurons are well documented in psychiatric disorders of adolescent onset (Lewis et al., 2005; Kang et al., 2012). Abnormal neuronal size (Rajkowska et al., 2001), dendritic outgrowth (Black et al., 2004), reduced basal dendrite (Glantz et al., 2000) and spine density (Broadbelt et al., 2002) have all been reported in schizophrenia. In MDD, dendritic spine morphology, density of PFC pyramidal neurons, and loss of synapses in PFC circuitry have also been consistently documented (Kang et al., 2012; Qiao et al., 2016). Altered microRNA expression in the PFC in adolescence may disrupt ongoing synaptic pruning and the balance of excitatory and inhibitory signaling, inducing psychiatric vulnerability (Roshan et al., 2014; Lewis et al., 2005).

Circulating microRNAs as biomarkers of psychiatric risk in adolescence

The neurobiological processes underlying the elevated risk for psychiatric disorders in adolescence has not been representatively studied and there is a pressing need to identify biological factors that would aid in early identification and prevention. Depressive symptoms and rates in adolescents have been on a rapid rise with the ongoing global viral pandemic (Esterwood and Saeed, 2020; de Figueiredo et al., 2020; Fegert et al., 2019; Nwachukwu et al., 2020; Ravens-Sieberer et al., 2021). The abrupt change in psychosocial interaction and elevated stress stemming from home confinement already appears to be disproportionately exacerbating depressive symptoms in youth (Esterwood and Saeed, 2020; Fegert et al., 2019; Nwachukwu et al., 2020). Unfortunately, the number of studies in psychiatry aimed at discovering biological markers of risk in adolescence is scant. Using keywords “biomarker” and “psychiatry” in the Scopus® search engine yields a track record from the year 1989 with ~ 500 articles added per year in the last two decades (Figure 1A). Adding the keyword “adolescence” to the search shows

a track record from the year 2000 with only ~80 articles published annually in the last 6 years (Figure 1B). More studies aimed at discovering biomarkers to detect psychiatric vulnerability in adolescence are urgently needed.

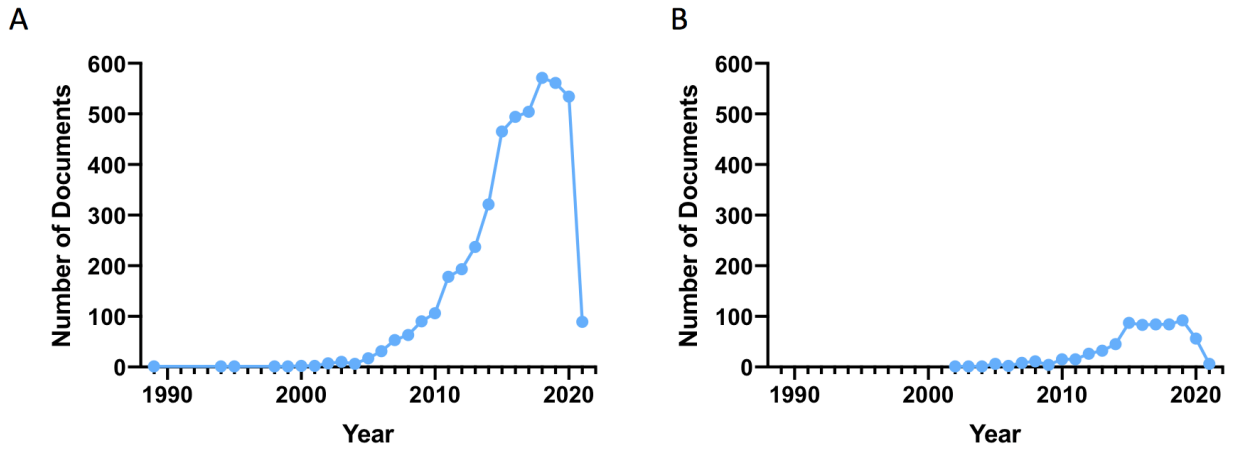

Figure 1. Number of articles published in the topic of biomarkers for psychiatric risk according to searches using Scopus ® Elsevier B.V. (A) Using keywords "biomarker" and "psychiatry" yields a track record, as of February 2021, from the year 1989 with ~ 500 articles added per year in the last two decades. (B) Using the keywords "biomarker", "psychiatry" and "adolescence" to the search shows a track record from the year 2000 with only ~80 articles published annually in the last 6 years.

MicroRNAs are emerging as promising diagnostic and therapeutic tools in human disease (Atri et al., 2019). They are very stable, do not degrade due to heat (Jung et al., 2010) or after prolonged storage (Nelson et al., 2006; Mitchell et a., 2008), and are abundant and readily detectable in a variety of peripheral fluids, such as blood, saliva, and urine (Layne et al., 2019; Fujimoto et al., 2019). MicroRNAs are secreted into peripheral fluids and transported out of cells via exosomes, microvesicles, or by binding to proteins, achieving post-transcriptional regulation of gene expression at far away targets. Parallel changes in microRNA expression have been observed between brain and peripheral samples (Torres-Berrio et al., 2021; Torres-Berrio et al., 2020; Issler et al., 2014; An et al., 2019; Jeng et al., 2011; Wang et al., 2018). In our miR-218 studies in mice, we have found that the dynamic pattern of miR-218 expression in the postnatal PFC is also observed in blood and that stress-induced reduction of miR-218 levels in the PFC is also detected in blood samples (Torres-Berrio et al., 2021). In humans, miR-218 levels in blood

also appear to be downregulated in MDD, as shown in a study in aging individuals with MDD and cognitive impairment (Mendes-Silva et al., 2017). Remarkably, direct upregulation and downregulation of miR-218 in the PFC of adult mice, including specifically within pyramidal neurons, lead to corresponding changes in peripheral blood (Torres-Berrio et al., 2020). MicroRNAs in peripheral fluids may serve as readout of brain expression and function, and be used for early prediction of risk severity for mental illness (Roy et al., 2020).

To address whether peripheral microRNAs in adolescence could serve as biomarkers of psychiatric vulnerability, we collected blood samples from adolescent male mice that were subjected to chronic social defeat stress in adulthood. Indeed, we found that circulating miR-218 in adolescence predict vulnerability to stress-induced depression-like behavioral abnormalities in adulthood. In comparison to control and resilient groups, adult mice that showed susceptibility to stress had elevated blood levels of miR-218 in adolescence (Torres-Berrio et al., 2021). Previous studies assessing the role of microRNAs as early biomarkers of psychiatric risk in humans have provided an exciting direction for the field. However these studies have focused on associating participant-reported adverse childhood events with later outcomes or diagnosis (Allen and Dwivedi, 2020), or comparing a child cohort with a separate adult group (Jawaid et al., 2020), rather than longitudinally following up on adolescent cohorts. We propose that future longitudinal studies be designed to identify microRNA profiles in peripheral samples from adolescent boys and girls to determine whether they can serve as identification markers of risk and guide preventative and intervention measures specifically during this age (Figure 2). Analysis of global miRNA expression through unbiased small RNA sequencing of peripheral sampling is a promising strategy we are currently using to investigate changes associated with the development of maladaptive behaviors in longitudinal adolescent cohorts. These studies may identify microRNAs not previously linked to psychiatric traits as well as provide more information regarding the potential role of microRNAs differentially expressed in psychiatric disorders (e.g. Table 1) in adolescent neurodevelopment. Finding associations between circulating microRNAs and changes in cognitive function and behavior during adolescence will also shed light into how specific microRNAs may shape developmental trajectories in the human brain.

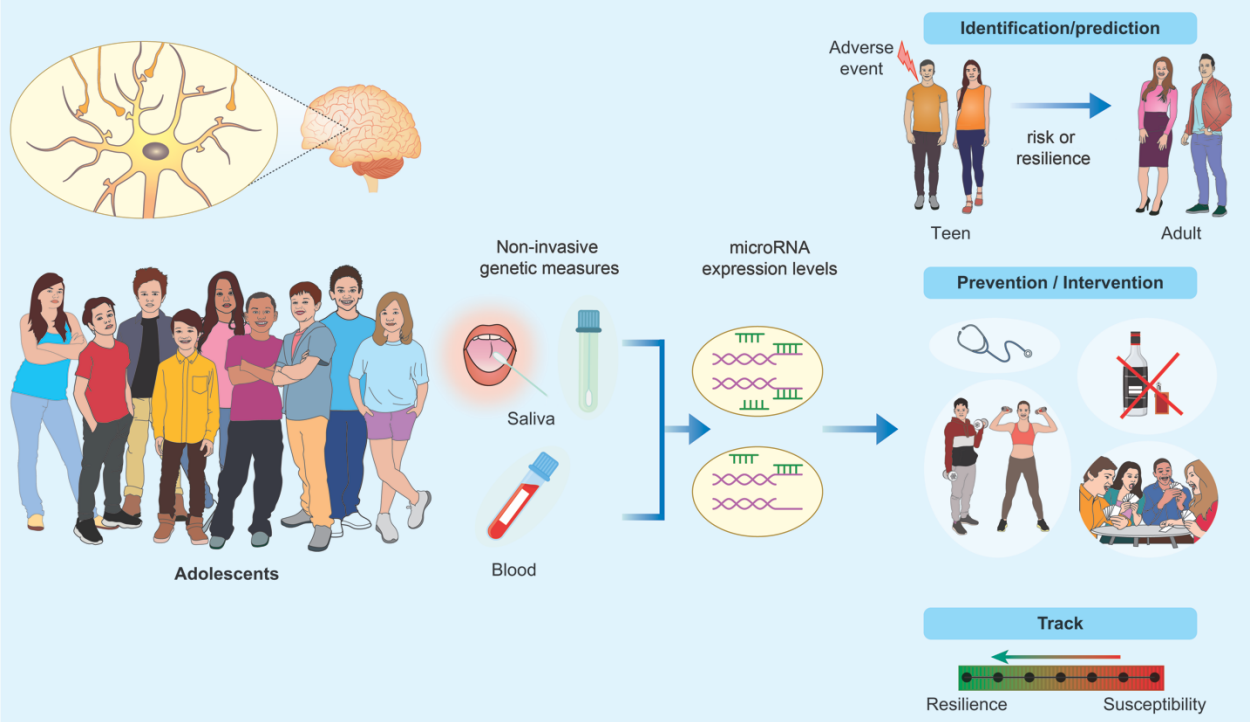

Figure 2. Peripheral microRNAs in adolescence as indicators of neurodevelopmental state and psychiatric risk. Assessment of microRNA profiles in peripheral samples from adolescent boys and girls in longitudinal studies could (i) help identifying markers of risk and resilience, (ii) guide prevention and intervention programs specifically for this age, and (ii) allow monitoring of disease severity.

The transition from adolescence to adulthood does not have a clearly defined differentiating boundary (Arain et al., 2013; Schneider et al., 2013; Somerville, 2016). Given our studies in rodents showing that circulating miR-218 levels in adolescence and in adulthood predict opposite outcomes regarding stress susceptibility, diagnosis prediction using peripheral microRNAs in late adolescence may be limited and would need to be considered with caution. Assessing microRNA biomarkers in combination with clinical observations and markers of developmental stages, including the Tanner pubertal staging and hypothalamic-pituitary-adrenal axis responses (Halligan et al., 2007), is an important next step.

Conclusions and future directions

This review shows that microRNA control over gene networks that organize PFC circuitry during adolescence may be a key mechanism in the development of vulnerability or resilience to mental illness. MicroRNAs measured in saliva or blood in adolescence may serve as indicators of

PFC maturational stage and function and be used to predict behavioral outcomes to stress later in life. In biofluids, microRNAs are enriched in exosomes (Gallo et al., 2012) and the approach of isolating circulating brain-specific exosomes (Kanninen et al., 2016; Tavakolizadeh et al., 20118; Saeedi et al., 2019) during adolescence could serve as a more objective measure of brain function and maturational state, enhancing the specificity and sensitivity of microRNAs as diagnostic tools. In this review we focused on microRNA expression in the PFC. However, microRNAs in non-cortical regions are also likely to contribute to PFC development and to the etiology of MDD, schizophrenia and substance use disorders. Indeed, miR-218 levels have been shown to be also elevated in the postmortem lateral amygdala of individuals with neuroticism and anxiety (Jurkiewicz et al., 2020). In rodents, the disruption of PFC dopamine development by exposure to recreational-like doses of stimulant drugs of abuse in adolescence requires miR-218 upregulation in the ventral tegmental area (Cuesta et al., 2018). Manipulations of brain-wide microRNA expression via systemic administration of antagomir will aid in prevention, and the discovery of therapeutic treatments (Ari et al., 2019).

The incidence rate and onset of PFC-related psychiatric disorders that emerge during adolescence are sex specific (Zahn-Waxler et al., 2008; Shaw et al., 2020). This poses an important limitation to the results derived from studies assessing the role of microRNAs on PFC development and adolescent risk, which have largely been conducted exclusively in males. As the prevalence of mental illness in adolescent boys and girls is on an unprecedented rise, there is a pressing need for basic and clinical research in both males and females. In our own unpublished studies we are finding sexual dimorphisms in the pattern of microRNA expression in the postnatal PFC and in our translational studies we are prioritizing cohorts with representative male and female ratios.

How experiences in adolescence impact microRNA systems in the developing brain needs to be assessed in more detail. Particularly, whether and how adaptive and coping mechanisms, including biological, psychological, or social factors, result in resilience despite adversity-related risk (Southwick et al., 2014). Individuals with the highest risk for mental illness are often the ones who benefit the most from early positive interventions (Boyce, 2019). A few recent studies have begun to address the involvement of microRNAs in this regard (Issler et al., 2014; Dias et al., 2014;

Chen et al., 2015; Higuchi et al., 2016; Lopizzo et al., 2019; Yang et al., 2020), but the developmental adolescent perspective in this line of research is missing. Efforts to understand microRNA function in PFC development prior to the closing of the formative adolescent window may ultimately help improving mental health outcomes for youth.

Table 1. Evidence showing alterations in microRNA expression in postmortem prefrontal cortex samples of psychiatric patients. The methods for measuring microRNA expression vary across studies, most likely explaining the variability of findings.

microRNAs	Cortex region	Human condition	Reference
miR-26b, miR-30b, miR-29b, miR-195, miR-92, miR-30a-5p, miR-30d, miR-20b, miR-29c, miR-29a, miR-212, miR-106b, miR-7, miR-24, miR-30e, miR-9-3p	PFC BA-9	Schizophrenia vs. non-psychiatric control subjects	Perkins et al., 2007 (149)
miR-346	BA46		Zhu et al., 2009 (150)
miR-34a, miR-132, miR-212, miR-544, miR-7, miR-154,	Dorsolateral PFC BA-46	Schizophrenia vs. control individuals	Kim et al., 2010 (151)
miR-504, miR-454, miR-29a, miR-520c-3p, miR-140-3p, miR-145, miR-767-5p, miR-22, miR-145, miR-874, miR-133b, miR-154, miR-32, miR-573, miR-889		Bipolar vs. control individuals	
let-7d, miR-128a, miR-16, miR-181a,b, miR-20a, miR-219, miR-27a, miR-29c, miR-7	Dorsolateral PFC BA-9	Schizophrenia vs. control individuals	Beveridge et al., 2010 (152)
miR-553, miR-369-3p, miR-18a, miR-339-5p, miR-1, miR-7, miR-196a, miR-301a, miR-144, let-7g, miR-153, let-7f, miR-203, miR-34c-5p, miR-101, miR-376c, miR-665, miR-152, miR-194, miR-423-5p, miR-515-3p, miR-374b, miR-140, miR-519b-3p, miR-586, miR_135b, miR-92a, miR-15b, miR-580, miR-146a, miR-454-3p, miR-380, miR-652, miR-802, miR-196b	PFC	Alcohol user vs. control	Lewohl et al., 2011 (153)
miR-328, miR-17-5p, miR-134, miR-652, miR-382, and miR-107	Dorsolateral PFC BA-46	Schizophrenia/schizoaffective disorder vs. control individuals	Santarelli et al., 2011 (154)

miR-330, miR-33, miR-193b, miR-545, miR-138, miR-151, miR-210, miR-324-3p, miR-22, miR-425, miR-181a, miR-106b, miR-193a, miR-192, miR-301, miR-27b, miR-148b, miR-338, miR-639, miR-15a, miR-186, miR-99a, miR-190, miR-339	BA-9	Schizophrenia vs. control individuals AND Bipolar vs. control individuals	Moreau et al., 2011 (155)
miR-132	Dorsolateral BA-46	Schizophrenia vs. control individuals	Miller et al., 2012 (156)
miR-383, miR-32, miR-490-5p, miR-165b, miR-513-5p, miR-876-3p, miR-449b, miR-297, miR-188-5p, miR-187		Bipolar vs. control individuals	
miR-142-5p, miR-137, miR-489, miR-148b, miR-101, miR-324-5p, miR-301a, miR-146a, miR-335, miR-494, miR-20b, miR-376a, miR-190, miR-155, miR-660, miR-130a, miR-27a, miR-497, miR-10a, miR-20a, miR-142-3p	PFC BA-9	Depressed suicide vs. control individuals	Smalheiser et al., 2012 (157)
miR-185	Anterior PFC BA-10	Depression vs. control individuals	Maussion et al., 2012 (158)
hsa-miR-375, hsa-miR-3065-5p, hsa-miR-488-star, hsa-miR-299-3p, hsa-miR-377, hsa-mir-516a-2, hsa-miR-767-5p, hsa-miR-493, hsa-miR-379, hsa-miR-105, hsa-miR-29b, hsa-miR-149	Frontal BA-9	Alcohol user vs. control	Manzardo et al., 2013 (159)
miR-31, miR-33, miR-96, miR-28, miR-30e-5p, miR-199a, miR-501, miR-504, miR-15b, miR-29c, miR-455, miR-380-3p, miR-323, miR-527, miR-93, miR-32, miR-20b, miR-516-5p, miR-92, miR-30a-3p, miR-497 etc.	PFC BA-9	Differential expression for schizophrenia, bipolar and control groups	Banigan et al., 2013 (160)
miR-17-5p, miR-331-5p, miR-16-5p, miR-187-3p, miR-106b-	PFC BA-10	Schizophrenia vs. control individuals	

5p, miR-485-5p, miR-129-2-3p, miR-454-3p, miR-185-5p, miR-429-3p, miR-511, miR-18a-5p, miR-590-5p, miR-106a-5p, miR-145-5p, miR-642a-5p, miR-625-5p, miR-508-3p, miR-219-2-3p			Smalheiser et al., 2014 (161)
miR-17-5p, miR-145-5p, miR-579, miR-106b-5p, miR-485-5p, miR-370, miR-500a-5p, miR-34a-5p, miR-29c-3p		Bipolar vs. control individuals	
miR-508-3p, miR-152-3p		Depression vs. control individuals	
miR-34c-5p, miR-139-5p, miR-195, miR-320c	Ventrolateral PFC BA-44	Depression vs. control individuals	Lopez et al., 2014 (162)
miR-1202	Ventrolateral PFC BA-44	Depression vs. control individuals	Lopez et al., 2014 (163)
miR-218	Ventrolateral PFC BA-44	Depression vs. control individuals	Torres-Berrio et al., 2017 (79)
miR-124	Dorsolateral PFC BA-46	Depression vs. control individuals	Roy et al., 2017 (164)
miR-146a-5p, miR-146b-5p, miR-24-3p, miR-425-3p	Ventrolateral PFC BA-44	Depression vs. control individuals	Lopez et al., 2017 (165)
miR-19a-13p	Dorsolateral BA-10	Depression vs. control individuals	Wang et al., 2018 (166)
miR-3162, miR-936	BA-46	Schizophrenia vs. control individuals	Hu et al., 2019 (167)
miR-30e	Dorsolateral PFC BA-9	Depression vs. control individuals	Gorinski et al., 2019 (168)

1.5 The imperative for minimally invasive markers in biometric psychiatry

The apparent knowledge lacuna concerning the diagnosis and management of depression across diverse life stages would benefit from integration of biological metrics, which could provide a more nuanced and dynamic understanding of mental health conditions. The inclusion of peripheral biological signatures would complement traditional diagnostic approaches, including self-reporting symptoms and clinical observation. This integration promises more robust diagnosis, effective risk stratification, and enhanced long-term disease monitoring.

A biomarker is an objectively measurable characteristic that serves as an indicator of either normal or pathogenic biological processes or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working, 2001; FDA, 2016). Biomarkers can have a singular or multiple purposes, including predicting patient outcomes, confirming the presence or absence of a disease or its subtype, monitoring disease status over time, and assessing safety or toxicity risks associated with treatment.

Biomarkers obtained via neuroimaging techniques have been proposed for depression (Lai et al., 2019; Li et al., 2022). Tracking every individual's brain structural and functional trajectories would entail high costs and require specialized resources, making them less accessible on a wide scale or in countries with limited healthcare infrastructure. The direct examination of human brain tissue, largely unfeasible except in postmortem studies, could offer unparalleled molecular and cellular insights into depressive disorders (Rajkowska, 2003; Krishnan and Nestler, 2010). However, these studies are limited by their focus on end-stage pathologies.

Serological biosamples, such as blood serum or plasma, provide a more practical alternative, which already constitute the most common biological samples used in the clinical settings (Chen et al., 2023). They are relatively easy to collect with a venipuncture draw and offer access to a broad array of potential biomarkers, including hormones, cytokines, and other circulating molecules. Although not brain-specific, serological samples can nonetheless offer a valuable proxy for identifying factors correlated with psychiatric conditions. Circulating blood serves as a rich source of information that can be transmitted throughout the body. Neuronal transcriptional and epigenetic changes in disease have been observed to be reflected in the

blood (Menezes et al., 2019). Notably, miRNAs have shown promise as crucial signaling molecules in circulation, which may serve as viable biomarkers for psychiatric conditions (Rao et al., 2013; Pirritano et al., 2018). In Figure 2, I compliment the review in the previous section by providing the basics of canonical miRNA biogenesis and the different carriers of microRNAs in the circulating blood (figure adapted from Cui et al., 2019; Gerbert and MacRae, 2019).

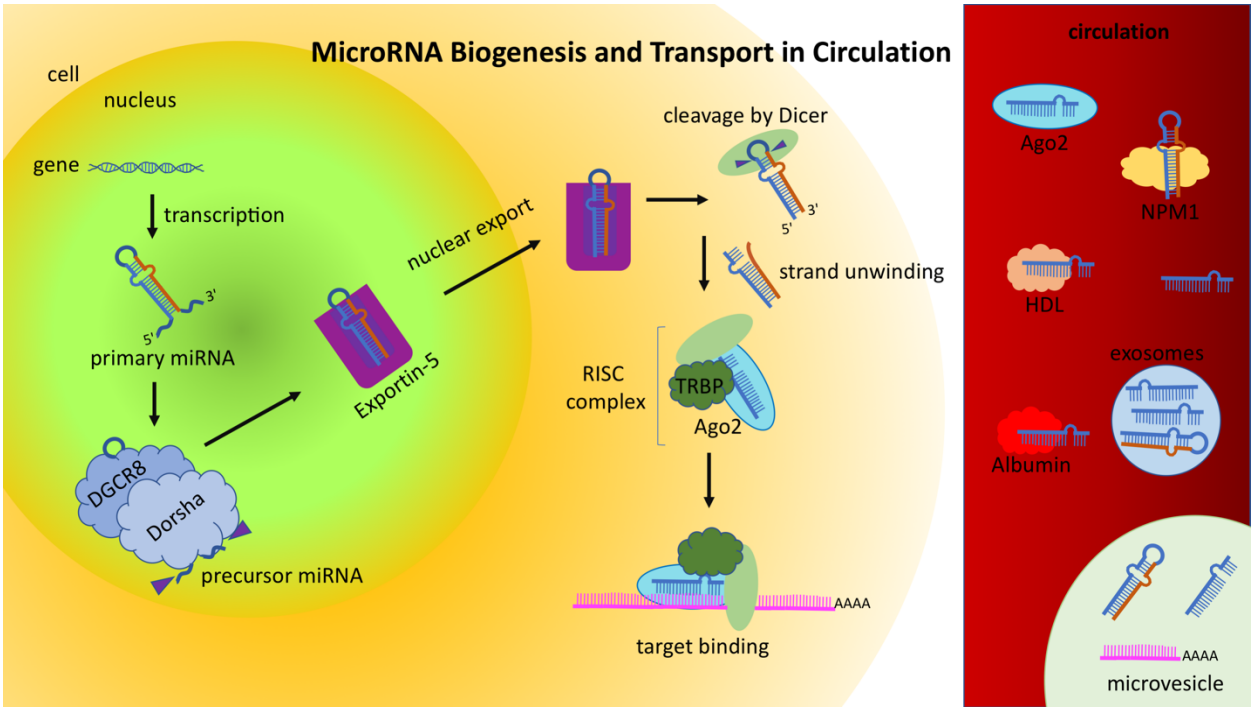

Figure 2. Simplified schematic of the canonical microRNA biogenesis, and carriers that stabilize and protect microRNAs in circulation. Abbreviations: DGCR8 (DiGeorge Syndrome Critical Region 8), TRBP (Transactivation Response Element RNA-Binding Protein), RISC (RNA-Induced Silencing Complex) complex, Agonaute 2 (Ago2), Nucleophosmin 1 (NPM1), High-density Lipoproteins (HDL). Adapted from: Cui et al., 2019; Gebert and MacRae, 2019.

An alternative to traditional venipuncture for blood collection is the use of dried blood spot sampling (DBS; not to be confused with the abbreviation for deep brain stimulation!). DBS are in fact routinely used in the medical field for newborn screening and metabolic disease assessments (Lim et al., 2018). With a prick to the finger, blood is collected on a filter paper card which can then be punched with a regular hole puncher for subsequent processing. The fibrous matrix of the card allows for rapid desiccation, thereby halting enzymatic activity, and provides a chemically inert material that preserves the biological state and ensures an even distribution of the analyte (Malsagova et al., 2020). DBS sampling is especially beneficial for large-scale epidemiological and longitudinal studies as it is cost-effective and minimizes participant discomfort, thereby encouraging continued participation over time. The need for

minimally invasive methods is particularly pressing when studying vulnerable populations such as children and adolescents.

Addressing the critical need for reliable biomarkers in psychiatric disorders, particularly depression in adolescents, is a pressing healthcare priority. Despite this urgent need, research efforts specifically focusing on molecular biomarkers for MDD in this vulnerable age group remain remarkably sparse (as shown in section 1.4, Figure 1; Zonca, 2021). Beyond the measurement of risk and neurobiological alterations, the attributes of a paragon biomarker include simple utilization by both patients and clinical staff, along with resource and cost efficiency (Ritsner et al., 2009; Thapar, 2012; Rao et al., 2013; Holland, 2016). The advent of next-generation sequencing (NGS) technologies, which enable high-throughput analysis of millions of molecular transcripts (Goodwin et al., 2016; Hu et al., 2021), can accelerate the discovery and validation of such biomarkers.

Rational and aims

In summary, adolescence is a critical period that presents both a challenge and an opportunity for mental health research and care. Today, adolescents are diagnosed by self-reported symptoms and clinical interviews. The subjectiveness and symptom heterogeneity propagates into missed patients, misdiagnosis and mistreatment. Major obstacles to clinical implementation stem from the underexplored biological mechanisms underlying the major depressive disorder, as well as the logistical complexities that such biological assessments could impose on an already strained public health system. The first step toward addressing these challenges involves conducting targeted studies that prioritize specific stages of the disease, such as the emergence of vulnerability in adolescence, while using minimally invasive yet easily implementable tools.

The formative stage of adolescence represents a critical period for foundational maturation that has lifelong implications for functioning. There is a compelling rationale for focusing on adolescents at risk for MDD by exploring the molecular mechanisms responsible for brain dysfunction, as such mechanisms can provide clues about the neurodevelopmental origins of the disorder and its trajectory. This idea stems from previous research that identified DCC, an axon guidance receptor, to orchestrate PFC's structural maturation in adolescence, where its expression is modulated by the small non-coding RNA miR-218, and altered expression levels of both are observed in adults with MDD who died by suicide. With this neurodevelopmental framework already demonstrated in late-stage disease, my interest lies in pursuing translational studies focused on the early episodes of depression in adolescence. MicroRNAs present a promising avenue for biomarker discovery, given their ability to be measured in peripheral blood as a proxy for brain expression and their regulatory influence over neurodevelopmental processes.

Based on the points of interests and gaps in the literature discussed above, the specific aims for my research are:

Aim I – to set up a high-throughput sequencing workflow for examining microRNA profiles in minimally invasive samples from human participants. Specifically, I explore the utility of dried blood spots, a microsampling technique that necessitates only a single dried drop of blood. An

untargeted approach with next-generation sequencing technology offers distinct advantages, specifically the ability to provide an unbiased snapshot of the global microRNA transcriptional landscape while achieving single-variant resolution.

Aim II – to evaluate circulating markers that are differentially expressed (DE) between adolescents diagnosed with depression and healthy peers, based on the proposed approach in aim I. Sequencing of microRNAs in peripheral fluids is an invaluable tool for understanding the epigenetic mechanisms affecting physiological functions under disease conditions. By employing bioinformatic tools and datasets, I compute the gene targets that are modulated by the top DE microRNAs and evaluate related biological functions based on ontology analysis.

Aim III – to begin understanding how genes, such as those influenced by miRNA modulation, may play a direct role in the brain and in shaping neurodevelopmental trajectories by investigating these genes within a specific biological context. A good starting point is the candidate gene DCC, as it has already been shown to be dysregulated in depression, yet its role in ongoing development remains an open question. Thus, I begin investigating DCC-associated gene network in the prefrontal cortex by computing an expression-based polygenic score. For this analysis I use genotype data from a community-sampled cohort and available neuroimaging data to assess associations with morphological outcomes.

Through an integrative research methodology, this work aims to offer a more nuanced understanding of the complex pathology of depression, viewed through the lens of neurodevelopment, in human datasets. The proposed approaches are minimally invasive for participants. Ultimately, this work can pave the way for early detection strategies for at-risk youth.

CHAPTER II

Preparation and Processing of Dried Blood Spots for MicroRNA Sequencing

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Abstract

Dried blood spots (DBS) are biological samples commonly collected from newborns and in geographic areas distanced from laboratory settings for the purposes of disease testing and identification. MicroRNAs – small non-coding RNAs that regulate gene activity at the post-transcriptional level – are emerging as critical markers and mediators of disease, including cancer, infectious diseases and mental disorders. This protocol describes optimized procedural steps for utilizing DBS as a reliable source of biological material for obtaining peripheral microRNA expression profiles. We outline key practices, such as the method of DBS rehydration that maximizes RNA extraction yield, and the use of degenerate oligonucleotide adapters to mitigate ligase-dependent biases that are associated with small RNA sequencing. The standardization of microRNA readout from DBS offers numerous benefits: cost-effectiveness in sample collection and processing, enhanced reliability and consistency of microRNA profiling, and minimal invasiveness that facilitates repeated testing and retention of participants. The use of DBS-based microRNA sequencing is a promising method to investigate disease mechanisms and to advance personalized medicine.

Introduction

The Strengths of Using Dried Blood Spot Samples for Disease Identification

Reliable measurement of molecules is a cornerstone of biological methods aimed at identifying candidate biomarkers with clinical applicability. Blood samples serve as a primary biospecimen in identifying molecular biomarkers of disease (Perry et al., 2019). The protocol presented here addresses the need for a streamlined, cost-effective, and minimally burdensome approach for collecting, processing and measuring molecular markers in serological samples.

Dried Blood Spots (DBS) are routinely used in newborn screening (e.g., heel prick in infants) and in adults (e.g., finger prick for diabetes monitoring) to identify genetic and metabolic diseases (for more information see Little Feet Make Big Footprints in Health by CDC; Walsh, 2022). This globally accepted practice has high analytical sensitivity and specificity – that is, correctly detecting individuals with a disease and verifying negative result in healthy persons (Dollard et al., 2021). Although DBS are collected for a singular purpose where sampled blood is used up for immediate analysis, in some instances, residual samples are preserved for secondary use (Rothwell et al., 2019). The application of DBS-based samples in biomolecular marker studies spans diverse fields, from monitoring environmental health and infectious diseases (McClendon-Weary et al., 2020; Tuaillon et al., 2020) to cancer detection and therapeutic evaluation (Nguyen-Dumont et al., 2015; Kahraman et al., 2017; Heider et al., 2020). The minimally invasive nature of DBS sampling facilitates frequent sample collection across a wide range of demographic groups and conditions, offering substantial advantages in disease risk assessment, diagnostics, and monitoring (McClendon-Weary et al., 2020; Sauer et al., 2022).

DBS collection presents a viable alternative to venipuncture. It requires minimal training, does not necessitate a laboratory setting or infrastructure, and is well-suited for individuals sensitive to pain. Molecular profiles obtained via DBS are highly correlated with those obtained from whole blood or plasma samples (Resau et al., 2018; Ponnusamy et al., 2016), indicating a broad representation of biological activity. Moreover, this method enhances participant retention and promotes efficient disease monitoring in longitudinal assessments. The analytical

reliability of archived DBS samples for up to 20 years (Gauffin et al., 2009; Therrell et al., 2012; Grauholm et al., 2015; Carpentieri et al., 2021) provides an invaluable opportunity to serve as a snapshot of the past for epidemiological and longitudinal biomarker studies.

Other biosampling techniques, such as liquid whole blood or plasma, demand professional handling in the pre-analytical stage (Pritchard et al., 2012; Ponnusamy et al., 2016; Haberberger et al., 2018), along with a stabilizing agent (e.g., preservation tubes as PAXgene and EDTA) for long-term storage, prompt centrifugation (Page et al., 2013), and significantly larger freezer and transport accommodations than are required for paper cards (Diener et al., 2019). Therefore, the collection of DBS specimens can also prevent potential pre-analytical errors. In Canada and the U.S., DBS is classified as a non-regulated and exempt material, allowing easy shipping in an envelope (CDC Bloodspot Transportation Guidelines), and providing access to residual samples from clinical testing for research purposes. It is advisable, however, for researchers to consult the regional and country-specific regulations. Special attention does need to be paid to the size of DBS punches, shipping time and exposure to high temperature or humidity (Crimmins et al., 2020; Borrajo et al., 2021). Although DBS specimens do not require freezing, opting for temperatures below -20°C maximizes the reliability of gene expression measurements (Wei et al., 2014).

DBS can provide a valuable insight into both intra- and extra-cellular components of blood (Ponnusamy et al., 2016). The matrix of the filter paper binds a variety of biomolecules including cells, protein, antibodies, antigens, DNA, RNA, and various other forms of nucleic acids (Zhuang et al., 2022). As such, DBS is a useful tool for researchers studying complex conditions that affect systemic functions, intracellular markers, microenvironments, or candidate molecules that have organ- and/or cell-specific expression.

MicroRNAs as Regulators of Gene Expression in Response to Environmental Factors

Changes in gene expression can be used as an indicator of the ongoing interplay between environmental factors and biological function at a given time. MicroRNAs (miRNAs) have garnered considerable attention for their role as molecular markers of pathology risk (Cui et al., 2019; Condrat et al., 2020; Morgunova and Flores, 2021; Morgunova and Flores, 2022) and as

potential therapeutic agents (Si et al., 2019; Yi et al., 2020) due to their ability to regulate gene expression on a broad scale. The functional implications of miRNAs range from fine-tuning local intracellular gene activity to modulating entire networks of signaling pathways.

MiRNAs, released from their cell of origin in response to specific signals, are abundant in body tissues and biofluids, and serve as signaling molecules across different cells and organs (Keller et al., 2022). Their expression levels can be assessed in peripheral fluids, such as whole blood and saliva, and can be used as a proxy for their expression levels in specific tissues, including the brain (Sempere et al., 2004; Stoicea et al., 2016; Torres-Berrio et al., 2020). MiRNAs are present in cell-free body fluids (e.g., cerebrospinal fluid or plasma), shielded by RNA-binding proteins or encapsulated within extracellular vesicles (Mori et al., 2019). MiRNAs are highly stable and readily detectable in peripheral fluids, making them a valuable asset for potential use as therapeutic agents, with the capacity to reach the brain (Diener et al., 2019; Layne et al., 2019; Lee et al., 2019).

MiRNAs' robust resistance to degradation under conditions that would break down most RNAs and widespread influence over the post-transcriptional landscape makes them ideal candidates for biomarker studies across developmental, aging, and disease-related pathways (Cortez et al., 2011; O'Brien et al., 2018). Researchers have begun to appreciate the stability of the miRNome, as studies have found miRNA levels in DBS samples on par with those in liquid blood (Kahraman et al., 2017; Reust et al., 2018). As miRNA processing and degradation are tightly orchestrated (Köberle et al., 2013; Coenen-Stass et al., 2019; Wang and Liu, 2022), temporal snapshots of circulating miRNAs can reveal dynamic shifts in cellular processes, thus serving as a chronicle of biological events or interventions. The robust measurement and recovery of miRNAs in DBS samples paves the way for the study of various topics, including fetal programming of metabolic diseases (Rodil-Garcia et al., 2017) and individual fitness scores (Krammer et al, 2022).

Harnessing the Power and Advancements of Small RNA Next Generation Sequencing

DBS biospecimens have been analyzed with Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR; Matsubara et al., 1992), microarray (Haak et al., 2009),

genotyping (Hollegaard et al., 2009A), genome-wide analysis (Hollegaard et al., 2009B; Ding et al., 2023), and next generation sequencing (NGS; Bybjerg-Grauholm et al., 2017). Methods such as RT-qPCR require controls, have limited throughput, and can be sensitive to user practices and reaction efficiency. In contrast, NGS offers a species-independent method, eliminating reliance on endogenous controls, enabling re-analysis with updated human genome templates, and revealing miRNA variants. NGS surpasses microarray technology by demonstrating superior intra-sample and inter-lab gene expression replicability (Hoen et al., 2008), and overcoming several limitations, such as reliance on pre-designed mature miRNAs detection probes, background noise (cross-hybridization), lack of isoform or anti-sense transcript identification, and limited sensitivity for low-abundance transcripts (Chen et al., 2009; Buermans et al., 2010; Zhao et al., 2014).

To prepare samples for sequencing and create a sequencing-ready library from short miRNA molecules, adaptors are ligated to the ends of the miRNAs. These adaptors serve the dual purpose of adding primer-binding sites for reverse transcription and enabling subsequent amplification steps. The protocol we outline here addresses a common limitation of most miRNA sequencing studies: the sequence bias from preferential adapter binding affinity at the 5’ and 3’ ends of the input sequences (Linsen et al., 2009; Hafner et al., 2011; Raabe et al., 2014; Wright et al., 2019). This bias can be mitigated by introducing modified forms of RNA ligases (T4 RNA ligase), random bases on adapter ends and optimizing polyethylene glycol (PEG) concentrations (Dard-Dascot et al., 2018; Giraldez et al., 2018; Wright et al., 2019; Androvic et al., 2022). While various approaches are being explored, employing random bases (also known as degenerate bases) at the ligation boundary has emerged as an effective strategy to overcome ligation-based biases (Baran-Gale et al., 2015; Fuchs et al., 2015; Belair et al., 2019; Benesova et al., 2021; Gómez-Martín et al., 2023). MiRNAs have been observed to preferentially and more efficiently ligate to sequences that enable structural interactions (Jayaprakash et al., 2011; Sorefan et al., 2012; Zhuang et al., 2012; Fuchs et al., 2015). The incorporation of random bases accommodates natural sequence variation in miRNAs, reducing the likelihood of self-ligation and facilitating the capture of miRNA diversity. For in-house library preparation, we took the approach of Extracellular RNA Communication Consortium initiative

(Das et al., 2019) and the Galas et al. protocol (Galas Lab 4N RNA library preparation protocol A - Version 1.0 - Pacific Northwest Research Institute, Seattle University, United States; Giraldez et al., 2018), documented to have high reproducibility across labs, to capture sequence diversity, and to consistently deliver reliable results - even in situations of low RNA input, including in extracellular-vesicle derived miRNA samples (Saeedi et al., 2021).

Preserving RNA quality (e.g., messenger RNA) in DBS is a shared challenge with postmortem tissue studies, given potential varying degradation rates among samples (Koppelkamm et al., 2011) and volume diminishment in unstable temperature conditions. However, the association between RNA integrity number (RIN) values, indicating the degradation of full-length RNA, and small RNAs is virtually nonexistent (Jung et al., 2010). Our protocol confronts the issue of low RNA input by adopting a modified approach that quantifies RNA with linear fluorescence detection (Table 1) for successful library preparation and NGS analysis of miRNAs.

	Nanodrop		RiboGreen
Sample ID	**ng/ul**	**260/280**	**pg**
T43	-1.71	0.71	0.77
E164	-1.65	0.75	0.40
G1	1.7	2.23	0.95
G2	29.2	1.46	2.577864
G3	14.1	1.45	1.498758
G4	11.3	1.45	0.415353
S1	0.9	2.12	0.00072
S2	2.3	2.04	1.21
T1	37.9	1.45	4.690695
T2	0.9	10.32	2.36
T3	17.4	1.47	2.10065
T4	10.2	1.5	1.050205
M1	2.4	3.1	0.82
M2	16.8	1.37	1.118038
M3	1.6	4.55	0.71
M4	11.5	1.51	0.864861
M5	8.5	1.49	0.14068

Table 1. The specifications for Nanodrop indicate a range of detection between 2ng/µl up to 12,000ng/µl, which is not a suitable quantification method for DBS RNA. Fluorescence-based quantification methods, such as the modified method described in this protocol with Quant-iT RiboGreen kit are significantly more sensitive.

Method	Sample ID	pg/uL
RLT lysis buffer	G	0.95
	S	0.001
	M	0.82
QIAzol/ Phenol based	G	23.13
	T	16.43
	M	12.28
RLT buffer + β-mercaptoethanol	S	1.21
	T	2.36
	M	0.71
Tris–EDTA Buffer	G	16.58
	T	15.40
	M	10.64
Overnight with QIAzol	G	7.97
	T	6.83
	M	5.64

Table 2. We tested five modification of RNA extraction protocols to select the procedure that yields the highest concentration of total RNA. Incubating the DBS with QIAzol, followed by sonification and addition of chloroform, as per the miRNeasy protocol, shows consistently high concentrations.

By refining and amalgamating different protocols, here we describe a highly selective sequencing approach for miRNA detection, incorporating advancements in DBS RNA extraction and library preparation techniques suitable for low-input miRNA samples. The extraction (Table 2) and library construction procedures we outline minimize noise, maximize efficient use of sequencing resources (Figures 1 and 2), and select for small non-coding RNAs - predominantly composed of miRNAs (Figure 3). Given the dramatic decrease in NGS costs (https://www.illumina.com/science/technology/next-generation-sequencing/beginners/ngs-cost.html), our approach allows for the assessment of detailed global miRNome profiling at an affordable cost. In addition, freely accessible and user-friendly institutional/web services for small noncoding RNA sequencing analysis are now available to aid in standardizing data processing and analysis, including miRMaster2 (Fehlmann et al., 2021); sRNAPipe via Galaxy (Pogorelcnik et al., 2018), Genboree (Rozowsky et a., 2019) and miRDeep2 (Friedländer et al.,

2012). The analysis in the protocol we detail below was guided by the exceRpt pipeline on Genboree server, designed to identify different types of small RNAs (http://genboree.org/java-bin/workbench.jsp).

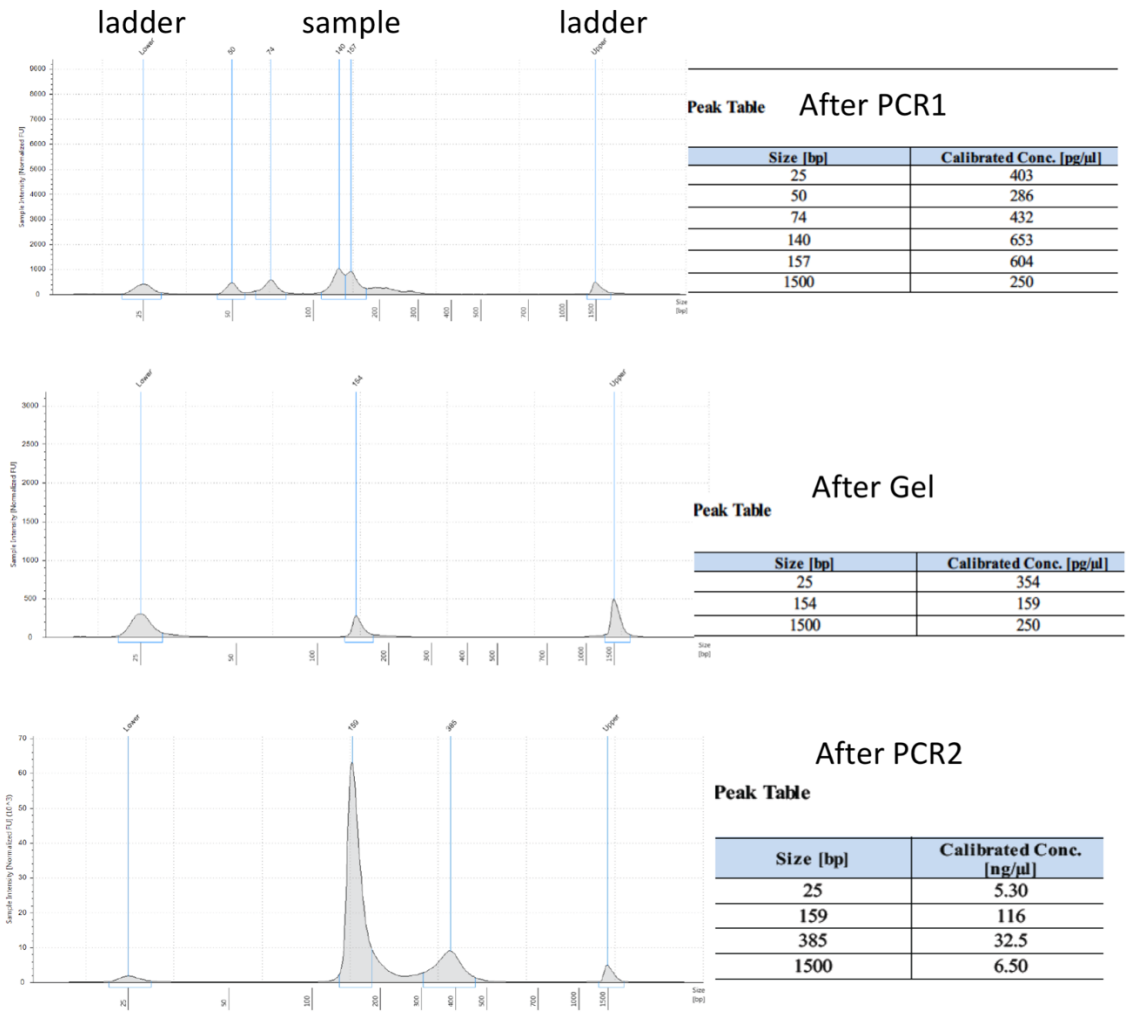

Figure 1: Example concentration peaks of a single DBS sample measured with TapeStation across the library preparation steps: after PCR#1, after gel purification with BluePippin, and after PCR#2. The final library amplification step (note the change from picograms to nanograms) plays a crucial role in enhancing the representation of small RNA molecules, ensuring their adequate abundance for downstream sequencing analysis.

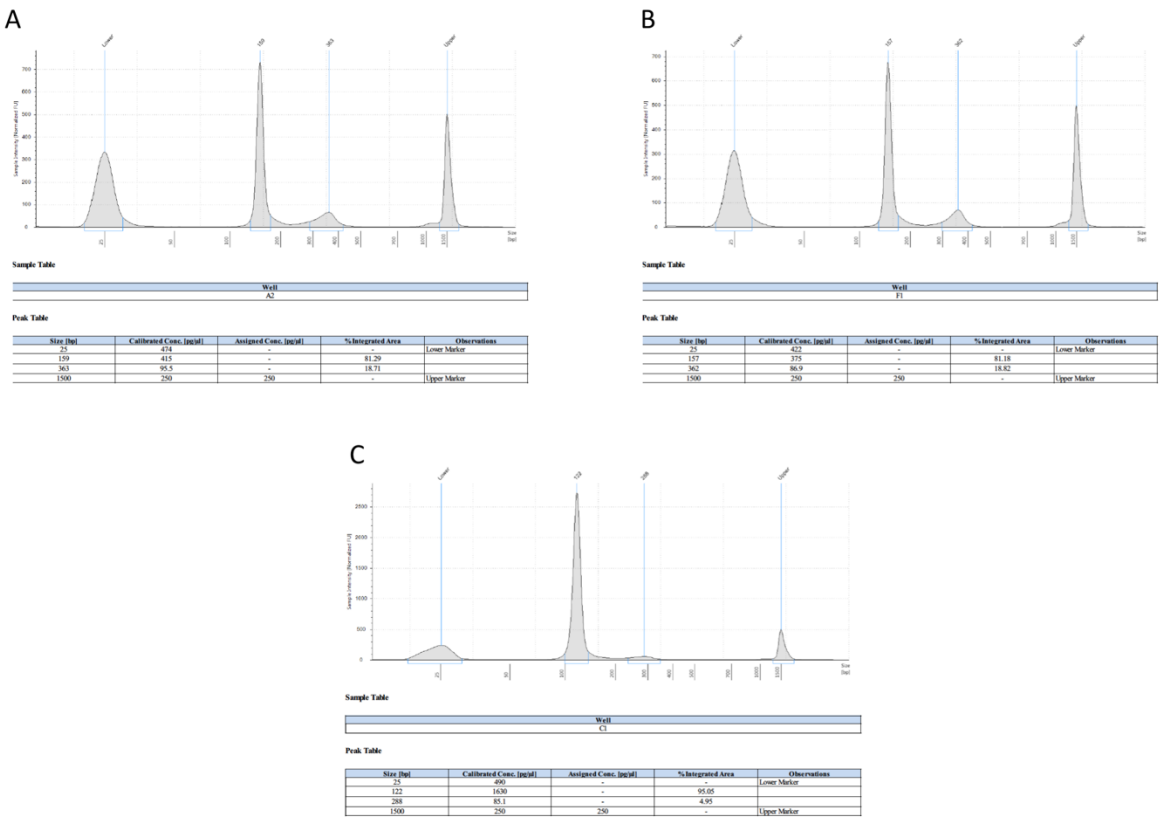

Figure 2: Comparison of AMPure beads purification versus reconditioning PCR to eliminate the PCR "bubble" products, of a library diluted to 2ng/ul. A) The sample (size 159bp) concentration 415pg/µl and bubble product (size 363bp) at 95.5pg/µl after a single and B) a second round of purification with AMPure beads, with final sample (size 157bp) concentration of 375pg/µl and bubble product (362bp) 86.9pg/µl. The beads purification slightly reduced the size of the PCR bubble at a cost of losing sample concentration, and a repeated purification did not improve the sample to bubble ratio. An alternative method is to expose the library to a single cycle of PCR#2 (C) which substantially reduces the large PCR bubble product (size 288bp 85.1 pg/µl) without losing the sample (size 122bp 1.63 ng/µl).

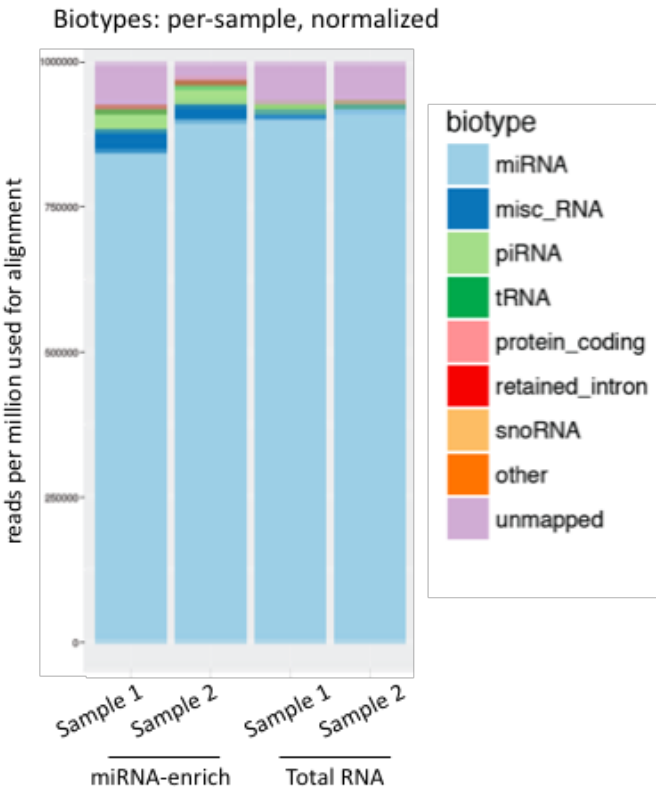

Figure 3: Reads by biotype reveal substantially greater proportion of miRNAs across all samples (either enriched for small RNA during extraction versus total RNA). Figure obtained from ExceRpt pipeline results.

Our method of sequencing microRNA in dried blood spots provides a straightforward yet robust approach for comprehensive molecular profiling, suitable for hypothesis-driven and exploratory research alike. DBS biosampling circumvents technical challenges in sample processing and storage, enabling the capture of representative miRNA profiles in blood that facilitates comprehensive (e.g., repeated assessments within an individual) and large-scale (e.g., population-level) testing. Since only a single punch of each DBS sample is needed for the miRNA sequencing, the rest of the paper card can be preserved for additional analysis, such as genomic, proteomic, or metabolic profiling, in the same subject.

Specific Considerations for Implementing DBS MiRNA Sequencing Protocol

The procedure is structured into the following sections: (i) sample collection and storage; (ii) sample preparation and RNA extraction; (iii) RNA quantification; (iv) library preparation; and (v) RNA sequencing and analysis.

The critical elements of the first section involve collecting blood without smearing, maintaining storage at -20°C or lower, and ensuring controlled humidity conditions. The blood volume procured from finger-pricks relates to lancet penetration depth, with recommended length not exceeding 2.4 millimeters (mm) for individuals 8 years of age and older (for detailed guidelines, refer to WHO Guidelines on Drawing Blood). In the literature, there is variability in the DBS punched disk diameter (ranging from 3 to 9 mm) and the number of spots used in downstream analysis. Based on adequate spot sizes observed in adolescent and young adult participants (Ho et al., 2021), we opted for a single punch of a 6 mm diameter.

We conducted a comparative analysis of several RNA extraction protocol modifications, based on different approaches described in the literature, and using three DBS samples. As shown in Table 2, we tested: (1) addition of RLT buffer (proprietary name for a lysis buffer in the RNeasy kit) during DBS incubation and agitation (McDade et al., 2016); (2) an adaptation of miRNeasy kit with QIAzol added prior to DBS incubation and agitation, followed by sonication, and subsequent addition of chloroform (Salamin et al., 2019); (3) incubation with RLT with β-mercaptoethanol (Wu et al., 2019); (4) incubation with Tris–EDTA (TE) buffer (Rodil-Garcia et al., 2017); (5) and overnight incubation with QIAzol (Kahraman et al., 2017). The highest concentrations were observed consistently with the QIAzol agitation and sonication approach, which we adopted in this protocol.

Our protocol approach relies on purifying the miRNA enriched fraction (< 200 nucleotides) to remove genomic DNA and large RNAs (this fraction can also be retained). MiRNA enriched samples compared to total RNA fractions within the same subjects show higher diversity in profiled reads (Table 3). Although the sorting of small RNAs is desired, it poses a challenge for the RIN, which relies on ribosomal RNA; thus, making conventional measures such as Nanodrop unsuitable for accurately estimating the input material (Table 1). We used an adaptation of the Quant-iT RiboGreen RNA Assay kit, allowing us to estimate RNA on the picogram scale (Table 2). As an alternative to the RiboGreen assay, it is possible to assess RNA quantity, quality, and the presence of small RNAs with Bioanalyzer (Agilent Technologies), although we should note that the accuracy of the Bioanalyzer for measuring low quantities can be limited, and intercalating dyes, such as RiboGreen, may enhance the reliability and consistency of the results.

Our recommended small RNA and complementary DNA (cDNA) library preparation mitigates the potential biases related to ligase binding and sequence-specific predilection. The cost of reagents is lower compared to commercial options. The limited number of Illumina indices possible in one pool of samples per lane can be overcome with a custom design of the barcodes.

	miR-enriched extraction		Total RNA extraction	
miRNA ID	**Sample 1**	**Sample 2**	**Sample 1**	**Sample 2**
miR-451a	24.34%	10.98%	52.61%	44.46%
miR-486-5p	13.19%	27.47%	3.99%	8.54%
miR-92a-3p	8.62%	12.46%	3.25%	4.17%
let-7a-5p	5.37%	4.15%	3.44%	3.41%
hsa-miR-16-5p	3.28%	2.48%	3.01%	3.40%

Table 3: Biased representation of a single miRNA in total RNA extraction. Shown top five miRNAs, with each value indicating percent of expression out of all mapped miRNA reads. miRNA enriched extraction shows higher diversity of reads profiled.

This protocol accommodates low sample input preferences, obviates the need for high quality RIN values or internal controls, and incorporates a library preparation that substantially minimizes binding biases. To our knowledge, some limitations remain, including potential preferential PCR amplification of shorter fragments in samples with low RNA input, and the under-amplification of microRNAs possessing complex secondary structures.

Reagents and equipment

Materials

Sample collection and storage

- Mini contact-activated lancets (BD 366594 Microtainer, BD Biosciences)
- 3 mm filter paper cards (Whatman #903, GE Healthcare)
- Double-lock Ziplock bags
- Desiccant packs
- Humidity indicator card - 30, 40, 50% (e.g., ULINE S-1547)
- Alcohol pads (70%)
- Band-aids and gauze

Sample preparation

- Single hole punching pliers with a 6 mm diameter punch head
- Particle-free and gentle wiping material – e.g., Kimwipes (e.g., Fisher scientific Cat. No. 06-666)
- RNAase free, labeled Eppendorf tubes 1.5 – 2 mL (e.g., Invitrogen Fisher scientific Cat. No. AM12425)
- Holder for Eppendorf tubes
- Forceps
- RNaseZap (e.g., Fisher scientific Cat. No. AM9780), diethylpyrocarbonate-treated water (DEPC H_2O) for cleaning
- PCR tubes 0.2 mL 8-tube strips (Eppendorf Cat. no. 0030124286)
- Dry ice

Sample RNA quantification

- Microplate with optical transparency, such as the Greiner Bio-One FLUOTRAC 96-well non-treated microplates (Fisher scientific Cat. No. 07000721 or equivalent)
- Pipet-Lite Multi Pipette L8-200XLS+ (Rainin, cat. no. 17013805)

Absolute quantification of libraries

- PCR tubes 0.2 mL 8-tube strips (Eppendorf Cat. no. 0030124286)
- 96-well PCR plate e.g., (Eppendorf, cat. no. 30129504)
- Adhesive PCR Plate Seal e.g., Bio-Rad, cat. no. MSB1001

Reagents

RNA extraction

- miRNeasy Micro kit (Qiagen Cat. No. 217084)
- Ethanol 100% (Sigma, cat. no. 459836-500ML)

- RNase-free chloroform

Sample RNA quantification

- Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen Cat. No. R11490)

Library preparation

Oligonucleotide sequences (custom order from Integrated DNA Technologies (IDT))

- 5' adapter (desalted)– 5' rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrCr(N:25252525)r(N)r(N)r(N)
- 3' adapter (HPLC purification)– 5' /5rApp/(N:25252525)(N)(N)(N)TGGAATTCTCGGGTGCCAAGG/3ddC/
- RT primer (desalted)– 5' GCCTTGGCACCCGAGAATTCCA
- RP1 PCR primer (HPLC Purification)– same as Illumina RP1 PCR primer
- Indexed Illumina PCR primers, e.g., RPI1 - RPI48 (HPLC Purification)
- Universal PCR primer F (desalted)– 5' AATGATACGGCGACCACCGAG
- Universal PCR primer R (desalted)– 5' CAAGCAGAAGACGGCATACGA

Reagents for 3' Ligation

- T4 RNA ligase 2 truncated KQ (NEB M0373)
- 10X T4 RNA ligase reaction buffer (included with T4 RNA ligase)
- 50% PEG 8000 (included with T4 RNA ligase)
- RNAseOut RNAse inhibitor (Invitrogen 10777-019)
- Strip tubes (Axygen PCR-0208-CP-C or equivalent)

Reagents for adapter depletion

- E. coli single-stranded DNA binding protein (SSB) (Promega M3011)
- 5' deadenylase (NEB M0331)
- RecJf (NEB M0264)

Reagents for 5' Ligation

- 10 mM ATP (NEB P0756)
- T4 RNA ligase 1 (NEB M0204)

Reagents for Reverse Transcription

- Strip tubes (Axygen PCR-0208-CP-C or equivalent)
- Superscript III (Invitrogen 18080-044)
- 5x First strand buffer (included with Superscript III)
- 0.1 M Dithiothreitol (DTT) (included with Superscript III)
- 25 mM Deoxynucleotide triphosphates (dNTP) mix (Thermo Scientific R1121)
- RNAseOut RNAse inhibitor (Invitrogen 10777-019)

Reagents for PCR Amplification #1

- NEBNext Ultra II Q5 PCR master mix (NEB M0544) or other high fidelity PCR master mix
- DNA Clean and Concentrator - 5 columns (Zymo D4004)

Reagents for Gel Purification

- 3% agarose cassettes for BluePippin system (Sage Science BDF3010)

Reagents for PCR Amplification #2

- KAPA 2X real-time PCR master mix (KAPA KK2701) or other high fidelity PCR master mix
- DNA Clean and Concentrator 5 columns (Zymo D4004)

Reagents for Library Validation

- TapeStation High Sensitivity D1000 Sample Buffer (Agilent, cat. no. 5067-5603)
- TapeStation High Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584)

Absolute quantification of libraries

- KAPA SYBR FAST qPCR Master Mix (Roche KK4600)
- PhiX Control v3 (Illumina FC-110-3001)
- Illumina P5 primer (IDT custom: 250 nmole DNA Oligo 5' AATGATACGGCGACCACCGA)
- Illumina P7 primer (IDT custom: 250 nmole DNA Oligo 5' CAAGCAGAAGACGGCATACGA)

Equipment

Sample storage

- Freezer (-20°C short term, -80°C long term)

RNA extraction

- Sonicator
- Agitator with regulated temperature setting
- Centrifuge

Sample RNA quantification

- A fluorescence plate reader, e.g. Tecan Spark

Library preparation

- Vacuum concentrator, e.g., Savant SpeedVac Concentrator (Thermo Fisher Scientific)
- Thermocycler
- TapeStation system 2200 (Agilent) or equivalent equipment
- BluePippin System (BLU0001; required for BluePippin gel cassettes for the automatic size selection step)
- qPCR machine, e.g. QuantStudio™ 6 Flex System

Absolute quantification of libraries

- qPCR machine, e.g. QuantStudio™ 6 Flex System

Procedure

Sample collection and storage

1. Request that the participant warm their non-dominant hand under comfortably hot water for 2 minutes.

2. Label the collection paper card (Whatman).

3. Dry participant's hands with paper towel and immediately clean the middle/ring finger of the non-dominant hand with isopropyl alcohol wipes. Allow the finger to air dry for 30 seconds.

4. Use the lancet (BD Microtainer) to prick the side of the selected finger, avoiding the fingerpad.

5. Gently apply pressure to the sides of the finger to increase blood flow and formation of a blood droplet. If necessary, ask participant to stand and relax their arm while you massage their hand to further stimulate blood flow.

6. Capture the initial blood drop with a gauze pad and dispose of it in a biohazard bag.

7. Position the participant's hand above the paper card, if necessary, applying gentle pressure, to produce another drop of blood. Touch the blood droplet with the filter paper, absorbing the blood without the paper contacting the skin.

IMPORTANT: Refrain from smearing the blood onto the collection paper. Avoid placing additional blood on a previously spotted area, even if the initial spot appears small. Overlaying blood drops can concentrate the sample and compromise the uniform diffusion properties of the paper.

8. Saturate each circle indicated on the paper card with a drop of blood (1 drop per circle).

9. If necessary, repeat for all circles using the same finger prick.

10. Apply a bandage to the pricked finger.

11. Record the sample collection time and any additional notes on the data collection form.

12. Store the labeled card in a temporary storage box made of non-absorbent material. This box will protect the cards against direct sunlight and heat. Allow the card to dry fully at room temperature under low humidity conditions for a minimum of three hours, or leave it overnight, maintaining consistent drying times across all samples. Do not stack

the cards during storage.

13. Once dry, the card should be placed in a resealable plastic bag with a desiccant sachet. Store the bag at a consistent temperature of -20°C or colder.

Sample preparation (spot punching)

14. Note that the size of punched circle should be consistent across samples, thus it is crucial to use the same hole-puncher for all specimens. We suggest punchers that produce a 6 mm diameter spot and one spot per tube for downstream processing. It is essential to clean the punching pliers between each sample with ethanol and to allow them to dry thoroughly.

15. Maintain a clean working environment by disinfecting the counter with RNAaseZap (Thermo Fisher) + DEPC H_2O. If the samples were stored at freezing temperatures, place a few Ziplock bags containing samples (~2 to begin with, progress to 4, max 6 at a time) atop dry ice – CAUTION: use proper ventilation and take care to avoid freeze burns.

16. Sterilize the forceps that will handle the paper cards and hole-puncher tool with ethanol, ensuring they are **fully dry** before use. Label the tubes with the sample ID on both the top and the side.

17. When picking up the samples, use caution that no dry-ice precipitation forms on the Ziplock bag, so it doesn't get inside or onto the card. If necessary, wipe the Ziplock bag with a Kimwipe before opening. Grab the sample card by the edge with forceps. Place the puncher over the DBS, while holding the Eppendorf tube (Invitrogen) with another hand – CAUTION to punch the center of the DBS – 1 punch per sample per 2mL tube, not the edge.

18. After the punching process, place the card back with care, slowly sealing the Ziplock bag while gently pressing out any excess of air – to avoid the possibility of condensation from temperature fluctuations. Immediately transfer the bag to a container with dry ice. Close the tube and place on dry ice.

19. Clean the hole-puncher and the forceps (if used) with ethanol pads and wipe them dry with Kimwipes (Fisher Scientific) after each sample.

20. Repeat this process for the remaining samples.

RNA extraction

Note: Due to inconsistency in the literature in regard to RNA extraction procedure, we tested several modifications, including McDade et al., 2016; Wu et al., 2019; Salamin et al., 2019;

Kahraman et al., 2017 (Table 2). The optimized protocol that yields the highest RNA concentration and microRNA proportion (Table 2; Figure 3) is detailed below.

21. Place the 2 mL tube with DBS on dry-ice until the addition of 1 mL of QIAzol lysis reagent (included in miRNeasy kit; Qiagen).

22. Agitate the tubes at 450 rpm for 15 min at 37°C.

23. Place the tubes in Sonicator at room temperature (15 - 25°C) for 15 min.

24. Agitate the tubes once again, 450 rpm for 15 min at 37°C.

25. Add 250 µL of chloroform and vortex the tube prior to incubation at room temperature for 5 min.

26. Centrifuge the tube at 12,000 × g for 15 min.

27. Remove the tube carefully from the centrifuge, ensuring the integrity of the different phases within the tube is maintained. Pipette only the upper aqueous phase and transfer to a new 2mL tube. If carryover occurs, repeat from *step 26*, centrifuging for at least 5 minutes.

28. Add 100% 800 µL of ethanol and mix.

Purify the homogenate for miRNA-enriched fractions, following Appendix A of the manufacturer's miRNeasy handbook (Qiagen):

29. Pipet the sample into RNeasy MinElute spin column within a 2 mL collection tube. Gently close the tube, centrifuge at 12,000 × g for 30 seconds at room temperature. Pipet the flow-through with the microRNA fraction into a new 2 mL tube. Discard the RNeasy MinElute spin column.

30. Add 500 µL of 100% ethanol (x 0.65 volumes) to the flow-through and vortex to mix thoroughly.

31. Pipet 700 µL of the sample into a new RNeasy MinElute spin column placed in a 2 mL collection tube. Centrifuge for 30 sec at ≥8000 x g at room temperature. Discard the flow-through. Repeat until entire volume of the sample has been processed.

32. Add 700 µL of Buffer RWT to the spin column, close the lid, and centrifuge for 30 sec at ≥8000 x g at room temperature. Discard the flow-through.

33. Add 500 µL of Buffer RPE to the spin column, close the lid, and centrifuge for 30 sec at ≥8000 x g at room temperature. Discard the flow-through.

34. Add 500 µL of 80% ethanol to the spin column, close the lid, and centrifuge for 2 min at ≥8000 x g at room temperature. Place the spin column into a new 2 mL collection tube, discarding the one with the flow-through to avoid ethanol carryover.

35. Centrifuge the spin column in the collection tube with open lid for 5 min at ≥8000 x g at room temperature, positioning the caps into an empty preceding space of the rotor to minimize the risk of caps being torn off.

36. Elute the extracted RNA with 20 µL of RNase-free water into a 1.5 mL collection tube, centrifuging for 1 min at ≥8000 x g at room temperature.

    If not proceeding to sample quantification immediately, secure the labeled tube and keep in -80°C for long term storage.

Sample quantification

Note: Nanodrop can be used for RNA quantification on these samples, however the 260/280 ratios are likely to be inadequate or abnormal due to enrichment of shorter RNA fragments and low concentrations in the majority of samples (Table 1). To this end, we modified the RiboGreen protocol (Invitrogen) which identifies RNA on a scale ranging from 1 ng to 100 pg.

37. Prepare 2 µg/mL RNA Standard and 200-fold dilution of RiboGreen (10 µL per Standard) with the final concentrations being 1 ng/µL, 500 pg/µL, 250 pg/µL, 100 pg/µL and blank (1xTE buffer only), with total volumes of 20 µL per well, as such:

1x TE (uL)	**2 ug/mL RNA Standard**	**200-Fold Dilution RiboGreen**	**Final RNA concentration ng/µL**
0	10	10	1 ng/µL
5	5	10	500 pg/µL
7.5	2.5	10	250 pg/µL
9	1	10	100 pg/µL
10	0	10	Blank

38. Mix 1 µL of each stock RNA (sample) with 9 µL TE buffer in separate tubes.

39. To a microplate with optical transparency, pipette the RNA Standard ladder in duplicates (10 µL per well) and the diluted samples, keeping track of the sample-well

placement.

40. Top up the sample wells with the 10 µL of the RiboGreen dilution, gently pipette the solution up and down, and incubate the plate at room temp for 5 minutes covered with foil.

41. Using a fluorescence plate reader machine (e.g.,Tecan Spark), select the appropriate choice for the plate setting, with shaking for 5 sec, fluorescence intensity: excitation 480, emission 520.

42. Perform the calculations of the concentrations. The average blank value will be subtracted from all the averaged RNA standard values and the samples, and these sample values multiplied x 10 to return ng/µL concentrations.

Library preparation and pooling

Note: We adopted the Galas protocol "Library Preparation for small RNA sequencing using 4N adapters" to prepare the samples for sequencing, incorporating minor modifications:

43. Bring the samples to equal concentrations as per calculations from *step 42*.

44. Halve the stated concentrations of the adapters, RT primer, dNTP mix, and Universal primer cocktail compared to the indicated concentration (due to low RNA input and sufficient efficiency). Illumina forward primer and RPI1 through 48 reverse index primers can be used at 10 mM concentrations.

45. Add 3 µL of 50% PEG 8000 (included as such with T4 RNA ligase; NEB) to each strip tube per sample, slowly pipetting the volume of the viscous solution, which can be dehydrated in a vacuum centrifuge/concentrator (set for 37°C, 1 - 2 hours) to create a highly concentrated pellet.

46. For 3' ligation, in each tube with the PEG pellet, mix 7 µL of stock RNA with 1 µL adenylated 3' adapter.

    Heat the tube to 70°C for 2 minutes in a thermocycler, then snap cool on ice.

    To each tube of denatured RNA and adapter add:

    1 µL of 10x T4 RNA ligase reaction buffer
    1 µL RNAseOut RNAse inhibitor
    1 µL T4 RNA ligase 2 truncated KQ

Incubate at 25°C in a thermocycler for 2 hours.

Add 1 µg E. coli SSB (diluted in 1X ligase buffer; Promega) and incubate at 25°C for 10 minutes.

Add 1 µL 5' deadenylase (NEB) and incubate at 30°C for 1 hour.

Add 1 µL of RecJf (NEB) and incubate at 37°C for 1 hour.

47. In separate tubes, add 1 µL of 5' adapter (25 µM) per ligation and denature at 70°C for 2 minutes, then snap cool on ice.

    Per tube of denatured 5' adapter, add:

    1 µL 10 mM ATP (NEB).

    1 µL T4 RNA ligase 1 (NEB).

    Add the 3 µL mix (5' adapter, ATP and ligase) to the completed 3' ligation from *step 46*. The total volume will make up 18 µL, pipette up and down to mix.

    Incubate in a thermocycler at 25°C for 1 hour.

48. To begin the reverse transcription, to a new strip tube per sample add 1 µL of RT primer (5 µM) and 6 µL of the ligated RNA, from *step 47*. Leftover RNA to be stored at -70°C or below.

    Incubate at 70°C for 2 minutes, then snap cool on ice.

    Create a master mix of:

    1 µL Superscript III (Invitrogen)
    2 µL 5x First strand buffer (included with Superscript III)
    0.5 µL 12.5 mM dNTP mix (included with Superscript III)
    1 µL DTT (included with Superscript III)
    1 µL RNAseOut (Invitrogen)
    5.5 µL Total

    Add the 5.5 µL of the master mix to each tube of the 7 µL denatured RNA and primer.

    Incubate at 55°C for 1 hour, then at 70°C for 15 minutes.

49. For the first PCR amplification, create a master mix of:

25 µL PCR master mix (NEBNext Ultra II Q5 or equivalent)
2 µL Illumina RP1
8 µL RNAse free water
35 µL Total

Add to the tubes with cDNA from *step 48*.

Add 2 µL Illumina index primer (RPI 1 – 48, up to 48 samples to be indexed and potentially pooled; important to keep track of index-sample combination)

Amplify for 10 cycles:

98°C	30 sec	1X
98°C	10 sec	10X
60°C	30 sec	
65°C	35 sec	
65°C	2 min	1X

50. Purify and concentrate the PCR product with DNA Clean and Concentrator columns (Zymo) to elute to the final volume of 11 µL. As per the Zymo DNA Clean & Concentrator – 5 kit instructions, up to 5 µg of total DNA per column into as little as 6 µL can be processed by performing the steps below:

    If using a new kit, ensure to add the indicated amount of 100% ethanol to the DNA Wash Buffer (e.g., for 25 mL DNA Wash Buffer, add 96 mL 100% ethanol).

    a. In a 1.5 mL microcentrifuge tube, add 5 times the volume of DNA Binding Buffer to each volume of DNA sample. Based on the rounded volume of the PCR product from *step 49* add 250 µL. Mix briefly by vortexing.

    b. Transfer the mixture to a provided Zymo-Spin Column in a Collection Tube.

    c. Centrifuge for 30 seconds. Discard the flow-through.

    d. Add 200 µL DNA Wash Buffer to the column, then centrifuge for 30 seconds. Repeat this wash step.

    e. Add 11 µL DNA Elution Buffer (10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA) or water (pH > 6) directly to the column matrix (not the walls of the column) and incubate at room temperature for one minute. Transfer the column to a 1.5 mL microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.

51. Quantify the PCR product with TapeStation (Agilent) HighSensitivity D1000 tapes (detection range 35 – 1000 bp) with 2 µL of the DNA sample and 2 µL of the HS D1000 Sample Buffer. Cap firmly and vortex thoroughly for at least 1 minute. Spin down prior to loading the tube strip into the TapeStation machine.

52. Using BluePippin (Sage Science) for the single gel purification step, with a target of 138bp size (Figure 1), using the entire volume of the samples collected from *step 50* (~ 9 µL) bring the volume up to 30 µL with 1xTE as per the manufacturer's protocol, then add 10 µL (room temp) of the supplied marker/internal standard mix.

    At the end of the run collect the samples from the elution well.

53. Purify and concentrate the eluted sample as in *step 50*.

54. Quantify the purified samples with the TapeStation system.

55. For the second PCR amplification, create the following master mix:

    25 µL KAPA 2X real-time master mix (KAPA)
    2.5 µL Universal primer cocktail (10 µM of each, forward and reverse primers)
    27.5 µL Total

    Bring up the cDNA volume to 22.5 µL with nuclease-free water, and add master mix.

56. Run for 6 cycles, which creates sufficient amplification and minimizes "bubble" product from being created (see Troubleshooting section for more details; Figure 2)

98°C	45 sec.	1X
98°C	15 sec	6X
60°C	30 sec.	
72°C	20 sec.	
72°C	2 min.	1X

57. Purify and concentrate the PCR product with DNA Clean and Concentrator columns, as in *step 50*. Elute to 20 µL.

58. Quantify the PCR product with the TapeStation HighSensitivity D1000 tapes (quantitative range 10 pg/µL – 1 ng) with 2 µL of sample or with 1 µL of sample D100 ScreenTape (quantitative range 0.1 ng/µL – 50 ng/µL).

    Singular peaks with size (bp) ranging from 140 – 160 are expected and the second gel purification is not needed (Figure 2).

To achieve precise absolute quantification of libraries and to ensure an accurate pooling of samples, we recommend performing a qPCR test. The pooling process should take into consideration variations in samples, including balancing factors such as sex, age, and condition in each group. Here, we utilized PhiX as the control template/DNA Standard. PhiX, an adapter-ligated library, is commonly used as a control in Illumina sequencing runs. It is particularly useful for ensuring the quality of sequences in runs involving libraries with low diversity.

59. Dilute libraries with either TE buffer (Tris & EDTA) or distilled water, based on molarity estimated with TapeStation (e.g., range between 1 – 10 ng or 1 – 10 nM), so the diluted concentrations fall within the dynamic range of the standard curve (detailed example below).

60. Prepare a set of fresh serial dilutions of PhiX (10nM; Illumina) in a 0.2 mL eight-tube strip, following the example provided, with a range of 0.1 – 0.0015625 nM:

PhiX	**Concentration**
1	0.1 nM
2	0.05 nM
3	0.025 nM
4	0.0125 nM
5	0.00625 nM
6	0.003125 nM
7	0.0015625 nM
Negative control	0 nM

61. Prepare sufficient reaction mix for the required number of reactions with a total volume of 10 µL, assayed in triplicate:

    5 µL KAPA-2X SYBR FAST Universal qPCR Master Mix (2X)

    4 µL of each library
    OR 4 µL PhiX dilution

    1 µL Primer premix (Illumina P5&P7 at 10 µM each)

62. Seal the plate using adhesive seal and centrifuge the plate to 250 x g for 1 minute

63. Run qPCR, selecting absolute quantification option and the following parameters:

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	95°C	5 min	1X
Denaturation	95°C	10 sec	40X

Annealing/Extension	60°C	35 sec	
Melt curve analysis	65-95°C		

64. Standard curve is generated based on average Cq (quantification cycle) scores of the PhiX dilutions against their known concentrations, removing replicate outliers e.g., Cq replicates > 0.5 from other, or based on statistical outlier detection. This curve enables estimation of sample concentrations based on their Cq values, allowing determination of the corresponding sample concentration by comparing its Cq value to the standard curve.

Sequencing and analysis

Post-preparation, the samples can be submitted to a service company equipped with appropriate sequencing machines (e.g., Illumina NovaSeq 6000 system). Refer to their specific sample volume and concentration submission requirements, and account for some volume to be used for quality control. Our suggested coverage per sample is 20M reads.

For analysis, we recommend the exceRpt pipeline (Figure 3; Supplementary Figures), which can be downloaded directly (depending on memory capacity) or accessed via Genboree portal. Maintaining a record of analysis package versions is crucial because the existing Genboree packages, such as exceRpt and DESeq2, may not be the latest versions available. Consequently, variations may arise when replicating the code using different versions. The use of the 4N adapters needs to be specified in the analysis pipeline or manually removed via cutadapt package. We highly recommend that the maximum allowed mismatched bases in the aligned portion of the read should be set to zero (the default is 1), as miRNAs are short and allowing for less stringent alignment can change the results.

Troubleshooting

- Library preparation final PCR results in double peak: In cases in which the sample concentrations are high to begin with (>100ng post PCR#2), the samples are likely to show a "bubble" product, approximately double the band of the peak concentration (Figure 2). Although this product will not interfere with sequencing, an efficient way to reduce or remove it is to run an additional PCR cycle on all libraries to eliminate it. All samples would need to undergo an additional single PCR cycle:

98°C	45 sec.	1X
98°C	15 sec	1X
60°C	30 sec.	
72°C	20 sec.	
72°C	2 min.	1X

- Few to no sequences mapped to the genome: Using an Illumina MiSeq machine with a 50bp protocol, the automated exceRpt trimming of the sequences was sufficient. However, sequencing with NovaSeq6000 S1100bp leads to the issue of inadequate trimming and few sequences mapped to the genome. To this end, manually removing the 4N adapters and adjusting the sequence length (via command such as: --cut -50 -a NNNNTGGAATTCTCGGGTGCCAAGG -o) prior to file submission to a pipeline, as specified in the previous section, will successfully prepare the reads for mapping to genome.

Time taken

The following section provides estimated time frames for the different phases of the protocol, that may vary depending on the number of participants recruited and the equipment used:

(i) Sample collection - collecting blood generally requires less than 30 minutes per participant, with drying times for the cards ranging from 3 hours to overnight.

(ii) Sample preparation and RNA extraction - ideally conducted in batches of 12-16 (depending on the centrifuge model and available space), DBS punching and extraction should be executed sequentially to minimize the freeze-thaw cycles. Around 32 samples can be comfortably processed within 8 hours, if run manually/without equipment that automates and speeds up the process.

(iii) RNA quantification - a single plate, which has 96 wells for about 86 samples would take approximately 3 hours.

(iv) Library preparation – The stages up to reverse transcription, including preparation, 3’ ligation, and 5’ ligation are expected to take 6 to 8 hours, accommodating around 30 samples. Reverse transcription, PCR amplification and PCR product purification can comfortably be done within 8 hours for the same number of samples. Gel purification speed is limited due to the number of samples or libraries that can be processed at a time using the BluePippin cassette, and expected time from sample preparation to collection per plate is about 2 hours. A TapeStation measurement should take about an hour. PCR amplification #2 and PCR product purification can comfortably be done within 4 hours for about 30 samples.

(v) Library quantification – setting up the dilutions and preparing reagents and samples may take approximately 2 hours. The qPCR run and subsequent analysis each take less than an hour.

Anticipated results

- Based on the absence of contamination indicated by the humidity index, placed inside the Ziplock bags with the DBS sample cards and in the container holding all Ziplock bags, it is anticipated that transport, storage, and use of the DBS cards for the punching procedure will not contribute to variability in the outcomes.
- Low RNA concentrations are to be expected for microRNA enriched fractions, as described (Table 1 and 2). The commercial kits for constructing a library specify recommended RNA volume, ranging from 1 ng to 200 ng. A study on DBS derived miRNA sequencing describes 500 picogram input to produce sufficient library (Pirritano et al., 2018). The protocol described here has been tested on 50 picograms (with the smallest RNA concentration from a single DBS being 3.5 pg/uL) and produces superior mapping efficiency – MiSeq at the depth of 38M for 4 samples pool, 84-90% of reads mapped to human genome (Supplementary Figures).
- Library quality shows high percent of reads used for alignment and reads mapped to genome, with minimal failed quality or contamination (Supplementary Figures).
- MicroRNA reads compose the highest concentrations compared to other types of RNA (Figure 3).
- Drawing from our substantial experience in analyzing DBS samples from pediatric and adolescent cohorts, including individuals with psychiatric conditions and healthy counterparts, we expect that the fold changes in differentially expressed microRNAs will be relatively modest compared to what is typically observed in gene expression studies.

Limitations

Certain limitations associated with the DBS-sourced miRNA sequencing approach should be recognized, particularly those relating to pre-analytical factors. Although research indicates that technical variability is minor compared to biological factors, it is critical to ensure proper drying of the blood on the card, and to maintain controlled humidity throughout the process using desiccant packets and humidity index cards (Patnaik et al., 2010). These precautions help mitigate potential variations that may arise from factors such as the number of freeze-thaw cycles, multispotted versus a single punch, or the location within the blood spot (Atneosen-Åsegg et al., 2021).

Individual variations in hematocrit can contribute to variability in the proportion of red blood cells in DBS samples (Velghe et al., 2019; Daousani et al., 2019; Ackermans et al., 2021; Carpentieri et al., 2021). Diluting the samples to the lowest RNA concentration after extraction helps account for and mitigate hematocrit-related variations in RNA yield. Comparing liquid blood (and its fractions such as plasma) and DBS sample miRNA expression should be done with caution, as hemolysis can introduce bias to miRNA levels (McDonald et al., 2011; Kirschner et al., 2011, 2013; Mayr et al., 2017; Nemcekova et al., 2021) and to analytes overrepresented in erythrocytes.

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Connecting statement chapter II to chapter III

The methodology manuscript for Chapter II was formulated as I was troubleshooting and optimizing the protocol as part of a pilot study, which was important prior to embarking on the next chapter with precious samples from a full clinical cohort. It was critical to establish that the protocol we use captures all microRNAs from the small input in a way that minimizes any potential biases associated with the preparation of the transcripts for sequencing.

With the establishment of the protocol, I proceeded with taking punches from the cards with dried blood samples from the cohort called Teen Inflammation Glutamate Emotion Research, which we refer to as TIGER. This cohort is part of a longitudinal study that has been following up on adolescents (recruited at 13 – 18 years of age) over 2 time points, 9 months apart, with the third follow up being delayed due to the global viral pandemic. This novel study was designed to examine the effects of peripheral inflammation on neurodevelopmental outcomes by measuring cortical glutamate in individuals diagnosed with depression. The primary measures include clinical interviews, self-reports, magnetic resonance imaging (MRI), cognitive and blood-based measures.

The dried-blood samples were collected with the intention to measure inflammatory cytokines. My goal was to use the remaining dried blood spots to measure microRNAs to identify top differentially expressed targets and associated pathways potentially underlying the vulnerability to early-onset depression. This work builds the foundation for complimenting psychiatric research with easily accessible biological samples and highlighting targets that can serve as potential markers of vulnerability.

I have taken the lead in this multi-institutional collaboration with the samples coming from Dr. Tiffany Chen Ho at University of California Los Angeles and Dr. Ian Gotlib at Stanford, acquired training in library preparation from Dr. Corina Nagy and Dr. Gustavo Turecki, and in bioinformatics and clinical statistics from the laboratories of Dr. Patricia Silveira and Dr. Michael Meaney at McGill. It certainly takes a village to drive the scientific advancement.

The impact of this unique research is multifold: this study is one of a handful looking at circulating miRNAs in adolescents with a psychiatric diagnosis; it is a proof of principle that DBS can be leveraged for microRNA profiling; and in addition to my immediate results presented in

the following chapter, the sequencing dataset will provide a timeless source for subsequent studies, including reanalysis, such as with updates to the reference genome.

CHAPTER III

Peripheral microRNA signatures in adolescent depression

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Abstract

Identifying reliable molecular indicators for psychiatric risk can greatly contribute to implementing early intervention and prevention strategies. MicroRNAs are emerging as powerful epigenetic regulators of neurodevelopmental processes and are implicated in mental health disorders, including those with adolescent onset such as depression. In this study, we examine microRNA profiles from peripheral blood samples of adolescent males and females, juxtaposing those with clinical depression to a control group. Leveraging dried blood spot samples in a longitudinal study on adolescent depression (N=62), we processed and sequenced these samples using a small RNA protocol tailored for microRNA identification. We identified nine differentially expressed (DE) microRNAs (p adj<0.05), all of which are upregulated in adolescents with depression (N=34) compared to controls (N=28). The expression levels of two DE microRNAs, miR-942-5p and miR-3613-5p, are also associated with future depression severity. Gene ontology analyses of predicted DE microRNA targets link these microRNAs to neurodevelopmental and cognitive processes, and indicate their involvement in depression. Thus, miRNA profiles derived from DBS in adolescents may serve as markers for developmental pathways that shape lifelong psychiatric risk.

Introduction

Major depressive disorder (MDD) is a pervasive and debilitating condition, consistently ranked among the leading causes of disability globally. Symptoms frequently emerge in adolescence and persist into adulthood (Kessler et al., 2007; Shore et al., 2018). The transition from adolescence to adulthood is a period of substantial psychosocial and cognitive growth accompanied by ongoing neuromaturational changes in brain regions (Larsen and Luna, 2018; Reynold and Flores, 2021) consistently found to exhibit structural and functional alterations in individuals with depression (Paus et al., 2018; Howard et al., 2019; Eckstrand et al., 2022; Pizzagalli and Roberts, 2022). Adolescent onset of MDD is associated with graver long-term outcomes, including chronic and severe forms of depression, as well as a poorer response to treatment (Mullen, 2018). Recent years have been marked with a dramatic surge in youth mental health related visits to emergency rooms (Bommersbach et al 2023) and have surpassed, already before the pandemic, physical conditions as the most prevalent form of impairment and limitation among young people (Green et al 2019).

Despite these alarming trends and clinical observations, the heterogenous characteristic of the disorder has made it challenging to identify early molecular markers of risk. Numerous population-based studies aimed to identify genetic factors contributing to MDD, have found only low to moderate heritability (6 - 40%) and few, if any, single nucleotide variants passing the genome-wide significance threshold (Sullivan et al 2000; Hasin et al., 2005; Kendler et al., 2014; Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium, 2013; Hyde et al., 2016; Wray et al 2018; Arnau-Soler et al 2019; Howard et al 2019; Harder et al 2022). Few studies involving human participants have investigated the genetic and molecular basis of adolescent depression. This represents a major research gap because there are evident disparities between MDD cases with adolescent versus adult onset, likely due to the neurodevelopmental components (Kwong et al., 2019; Rice et al., 2019; Zhao et a., 2020; Thapar and Riglin, 2020). Subphenotyping based on age of onset has emerged as a promising approach for reducing phenotypic heterogeneity and is revealing age-specific genetic architectures that could partially contribute to the disorder (Shi et al., 2011; Ferentinos et al.,

2015; Power et al., 2017; Wray et al 2018; Schwabe et al 2019; Jami et al., 2022). This evidence underscores the importance of defining molecular targets focusing on adolescent-onset MDD.

There is a general need for biological markers (biomarkers) as objective metrics of physiological disruption, linking molecular profiling with clinical presentation for diagnostic precision and treatment monitoring (Goetz and Schork, 2018; Conrat et al., 2020). In psychiatry, clinical observations based on individual's emotional and cognitive symptoms, their duration, and the impact on daily functioning have been established as the gold standard diagnostic approach. Incorporating biological markers would add to our understanding of the mechanisms underlying clinical depression and serve as objective indicators for early prediction efforts.

The non-coding region of the genome, which constitutes its majority (Mattick & Makunin, 2006), is pivotal in biomarker research, particularly through small molecules like microRNAs (miRNAs), which are potent regulatory elements of the post-transcriptional landscape and are notably stable and readily accessible in peripheral biosamples (Mori et al., 2019). By modulating gene and protein expression, miRNAs regulate core physiological functions across diverse cell populations. MiRNAs are known to play a role in neurodevelopment (Ma et al., 2019; Thomas and Zakharenko, 2021) and are increasingly being characterized in the context of depression (Morgunova and Flores, 2020; Zurawek and Turecki, 2021). Since they are secreted into the circulating blood, miRNAs enable cell-to-cell communication by controlling gene transcription and expression even in distant organs (Kosaka et al., 2010).

Building on this understanding of miRNAs as pivotal regulators and potential biomarkers, we have investigated their specific role in depression using a candidate-based approach. In two independent cohorts, we demonstrated downregulation of miR-218 expression in the prefrontal cortex (PFC) of adult individuals diagnosed with depression who died by suicide (Torres-Berrio et al., 2017). Using rodent models, we found that adult mice exposed to social defeat stress and exhibiting depressive-like behavioral susceptibility also have decreased levels of miR-218 in both the PFC and peripheral blood (Torres-Berrio et al., 2017; Torres-Berrio et al., 2019). Probing into miR-218 levels in the prefrontal cortex of adult mice correspondingly altered its levels in peripheral blood and either induced or protected against depressive-like susceptibility (Torres-Berrio et al., 2019). Notably, circulating miR-218 levels in *adolescent* mice

mirrored its brain expression and served as predictors of the severity of stress-induced depressive-like behavioral abnormalities later in life (Torres-Berrio et al., 2020).

Seeing the strength of peripheral miRNAs for risk prediction in adolescence, in this study, we conducted global profiling of circulating miRNAs in adolescents diagnosed with depression and psychiatrically healthy controls. We used Dried Blood Spots (DBS), a 'micro-sampling technique' consisting of just a few drops of blood and is well suited for multi-omics investigations on a population-wide scale (McClendon-Weary et al., 2020; Zhuang et al 2022). Importantly, DBS has demonstrated reliable and stable capture of circulating miRNAs (Kahraman et al., 2017; Diener et al., 2019; Atneosen-Åsegg et al., 2019). To generate high-throughput expression data, we employed small RNA sequencing pipeline that best captures miRNA profiles in an unbiased and objective manner (Morgunova et al., 2023). We focused on the most differentially expressed miRNAs based on diagnosis, investigated association between miRNA levels and self-reported symptoms from follow-up visits, and using a bioinformatics approach explored potential gene targets and their associated biological pathways.

Methods

Participants and study design

Participants (N=62) were selected from two longitudinal neuroimaging studies, based on availability of dried-blood spot samples and on demographic matching (similar to Ho et al., 2021). The cohort Teen Inflammation Glutamate Emotion Research (TIGER; NIH grant: K01MH117442; Walker et al., 2020) recruited participants ages between 13 and 18 years, meeting diagnostic criteria for clinical depression, and age- and sex-matched healthy controls. The Early Life Stress (ELS) cohort (NIH grant: R37MH101495) included participants based on age and pubertal development and not on the basis of psychiatric history, although psychiatric history or severity of early life stress were extensively assessed. Both studies were approved by the Institutional Review Boards at Stanford University and the University of California, San Francisco. All participants and their parents gave written assent and informed consent, respectively, in accordance with the Declaration of Helsinki, and were financially compensated for their participation.

To be included in the present study, participants were classified as having MDD (TIGER N=34) or healthy controls with no history of any psychiatric disorder (TIGER: 6, ELS: 22). Diagnostic criteria for a depressive disorder was determined per DSM-IV guidelines, assessed by psychiatric interview using the Kiddie Schedule for Affective Disorders and Schizophrenia, Present and Lifetime (K-SADS-PL). In the case of a subthreshold on the K-SADS-PL, a total t-score > 55 on the Children's Depression Rating Scale-Revised was also considered (for cohort details, see Walker et al., 2020; Ho et al., 2021). Each diagnosis was reviewed for consistency and reliability by a clinically-trained research team. Severity of depression (self-reported) was measured using the Reynolds Adolescent Depression Scale (RADS-2; Reynolds, 2002) with t-scores available for 29 participants for two sequential time points (for one sequential time point 9 months later).

Sample processing

Sample collection, RNA extraction and library preparation were performed using the protocol we described in detail in Morgunova et al. (2023).

Dried-blood spot collection: Blood spots were collected using mini contact-activated lancets (BD Biosciences) from participant's non-dominant hand on filter paper cards (GE Healthcare). The cards were dried overnight at room temperature and subsequently transferred to Ziplock bags with a desiccant and stored at -20°C. Remaining parts of the paper card were shipped insulated with dry ice to Douglas Research Institute, Montreal, where punches were taken for subsequent processing.

Small RNA Extraction: A 6mm diameter punch of the dried blood was placed into a 2mL tube (Eppendorf). The samples underwent lysis, agitation and sonication, followed by RNA extraction using the miRNeasy kit (Qiagen) with the small RNA fraction enrichment. On average 10ng of RNA was collected from each sample of the 62 samples, as quantified by the RiboGreen fluorescent assay.

Library generation and Next Generation Sequencing: To mitigate the small RNA input that a single punch of dried-blood spot poses, small RNA libraries were prepared with a protocol that effectively captures miRNAs and minimizes the selection biases of RNA sequences during

ligation with the use of 'degenerate' bases on ends of the Illumina TruSeq small RNA adapters (Galas Lab 4N RNA library prep protocol Version 1.0; Pacific Northwest Research Institute).

Sequencing: Libraries were sequenced at the Genome Quebec (Montreal, Canada) on NovaSeq6000 S1 (Illumina; 100 nucleotide single-end reads, covering on average 24M reads per sample).

Bioinformatics analysis

Sequencing reads were trimmed with cutadapt to remove technical sequences. In following standardization of RNA sequencing data analysis, we submitted trimmed reads to the ERCC's exceRpt small RNA-seq pipeline (https://github.gersteinlab.org/exceRpt) on an institutional server (Genboree; Rozowsky et a., 2019; exceRpt v 4.6.2), which aligned the reads to the human genome, with a zero-mismatch setting, and quantified miRNAs into counts. MiRNAs with zero reads across all subjects were removed with the rest analyzed for differential expression analysis with DESeq2 in R (package v 1.34.0). MicroRNAs that were differentially expressed between control and MDD above the false discovery threshold (FDR; padj<0.05), are referred to as 'top DE miRNAs' from here on.

MiRNA pathway analysis and tissue expression probing

Tissue based query of top DE miRNAs was done with Tissue Atlas Database (Ludwid et al., 2016) and the DIANA Tools miRNA Tissue Expression Database (DIANA-miTED; Kavakiotis et al., 2021; with the following settings: TCGA and SRA data collections, Log2RPM, all diseases, all health status). Expression values for top DE miRNAs were extracted specifically for regions within the brain available in the TissueAtlas database and are depicted in a heatmap.

Further inquiry into enrichment of top DE miRNAs by cell type was based on the Diana miRPath v4.0 (Tastsoglou et al., 2023) web-based computational tool with the following specifications: TarBase v8.0, direct targets, Homo sapiens, miRbase-v22.1 annotation, classic analysis with FDR correction and the Molecular Signatures Database (MSigDB).

We also used DIANA-miTED database to assess the expression profile overlap between the top 20 miRNAs expressed in the DBS samples to plasma, blood (liquid), white blood cell, and serum from healthy subjects.

Logistic regression on top DE miRNAs

Logistic regression analysis was used to assess the potential diagnostic and classification potency of the miRNAs, with categorical diagnosis as the outcome and miRNA expression levels as explanatory variables, using all data samples. We compared models selected using both stepwise and backwards selection to identify the optimal predictive model. For stepwise selection, the Akaike information criteria (AIC) was used when comparing models. The final selected model was subsequently used for Receiver Operating Characteristic (ROC) analysis.

Expression of top DE miRNAs and Future Self-Reported Depression Scores

To assess a potential association between top DE miRNAs and future self-reported depression scores, we conducted individual linear regression analyses using the Reynolds Adolescent Depression Scale (RADS) t-scores. We also used linear regression analyses to assess the association between top DE miRNAs and Multidimensional Anxiety Scale for Children (MASC) scores. Cook's distance was employed as an estimate to identify potential outliers, with a cutoff value of above 1.

Gene target prediction

Gene target prediction analysis of the top DE miRNAs was done using an integrative database of human miRNA target predictors, mirDIP (version 5.3.0.1, Database version 5.2.3.1), which aggregates target predictors from multiple algorithms (Tokar et al., 2018; Shirdel et al., 2011). We considered genes that are in the top 1% score class. Genes that are predicted targets of *two or more* top DE miRNAs were annotated with GO analysis using enrichGO R library as well as using enrichment analysis with the MetaCore™ database (Clarivate Analytics). We retained only terms that were adjusted for multiple comparisons using the FDR correction.

Statistical analysis

For demographics, R package gtsummary (v 1.7.2) was used to create the table and to test for group differences. The correlation matrix was performed with R packages Hmisc (v 5.1-0) and corrplot (v 0.92). Postcounts obtained with DESeq2, were extracted (dds data) and transformed using the regularized logarithm for visualization. Statistical analysis and plots were done using GraphPad Prism v9.5 and R packages.

Results

Study sample characteristics

A summary of the participant demographic characteristics between healthy controls (N=28) and adolescents diagnosed with depression (N=34) is shown in Table 1. There are no statistically significant differences in most characteristics except for a minor age difference (p=0.045) between the control and MDD participants. The age distribution of the two groups is depicted in Supplementary Figure 1A. Participants were predominately of female sex, with five participants reported identifying as non-binary.

Mean ± SD, n (%)	**Control** (n = 28)	**MDD** (n = 34)	**p-value**
Age	16.3 ± 1.3	17.0 ± 1.3	0.043
Sex			0.611
Female	18 (64.3)	25 (73.5)	
Male	10 (35.7)	9 (26.5)	
Gender			0.086
Female	18 (64.3)	21 (61.8)	
Male	10 (35.7)	8 (23.5)	
Non-binary	0 (0)	5 (14.7)	
Ethnicity			1[b]
Hispanic/Latino	6 (21.4)	7 (20.6)	
Non-Hispanic/Latino	22 (78.6)	27 (79.4)	
Parent Level of Education			0.568
High school graduate or equivalent [a]	0 (0)	1 (3.0)	
Some college. No degree.	2 (7.1)	5 (15.1)	
Associate's degree [b]	3 (10.7)	2 (6.1)	
Bachelor's degree [c]	10 (35.7)	9 (27.3)	
Master's degree [d]	11 (39.3)	10 (30.3)	
Doctoral or Professional degree [e]	2 (7.1)	6 (18.2)	
Not Disclosed	0	1	

Table 1. Cohort demographics table. a e.g., GED; b e.g.; AA, AS; c e.g., BA, BS; d e.g., MA, MS, MED; e e.g., MD, DDS, DVM, PhD, EdD

Pre-sequencing processing and sequenced data mapping metrics

Following miRNAs sequencing from DBS, the samples were divided into two library pools and correspondingly, into two sequencing lanes to ensure deep coverage. The random selection of samples for each library pool ensured an equitable representation of both MDD and healthy controls groups (see Supplementary Figure 2A). Prior to sequencing, a quality control assessment revealed a singular peak in the library fragment length distribution around 150 base pairs, which corresponded to individual small RNA sequences augmented by adaptors and indices to mark sample origin (Supplementary Figure 2B).

The number of mapped reads to the human genome was on average 13M reads per sample, with ~79% mapping to miRNA sense and ~11% to precursor sense sequences. The distribution of mappings by biotype demonstrates distinct miRNA selectivity (Figure 1). Normalized mappings for each sample, extracted from exceRpt diagnostic plots by three batches, are shown in Supplementary Figure 3 A and B. Principal component analysis of miRNA transcripts does not reveal apparent clustering by age, diagnosis, sex or sequencing pool batch (Supplementary Figure 4). A total of 1332 mapped microRNA reads with nonzero total read count were processed with DESeq2 for differential analysis between MDD and control groups, resulting in comprehensive and unbiased miRNA profiling.

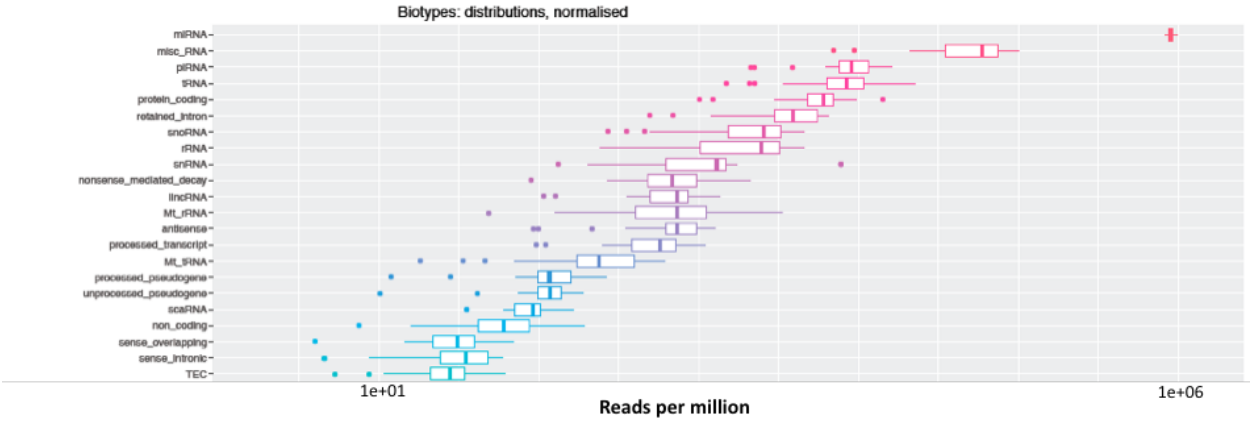


Figure 1. The vast majority of mapped reads are dominated by microRNAs. Showing representational image of mapping summary by exceRpt pipeline, for one third of the samples combined, as per random batch processing..

Distinct miRNA profiles in adolescents with MDD versus healthy controls

Following the differential expression analysis between control and MDD, miRNAs were sorted according to their p-value and FDR, with the full DESeq2 analysis output provided in Supplementary Table 1. The volcano plot encompassing all miRNAs (p<0.05) differentially expressed (DE) between MDD and control groups is depicted in Figure 2. Interestingly, the number of upregulated miRNAs in MDD surpasses those that are downregulated (>60% of miRNAs with p<0.05) and the top DE miRNAs (padj<0.05) are all upregulated in the MDD group. Table 2 lists the top DE miRNAs and showcases the detailed fold change and adjusted p-values. Three out of the 9 DE miRNAs are precursor sequences, potentially giving rise to mature forms through cleavage at either the 3' or 5' end (for process description see review by Gebert and MacRae, 2019). The correlation matrix showing primary variables and covariates is presented in Figure 3 and highlights significant associations primarily among top DE miRNAs.

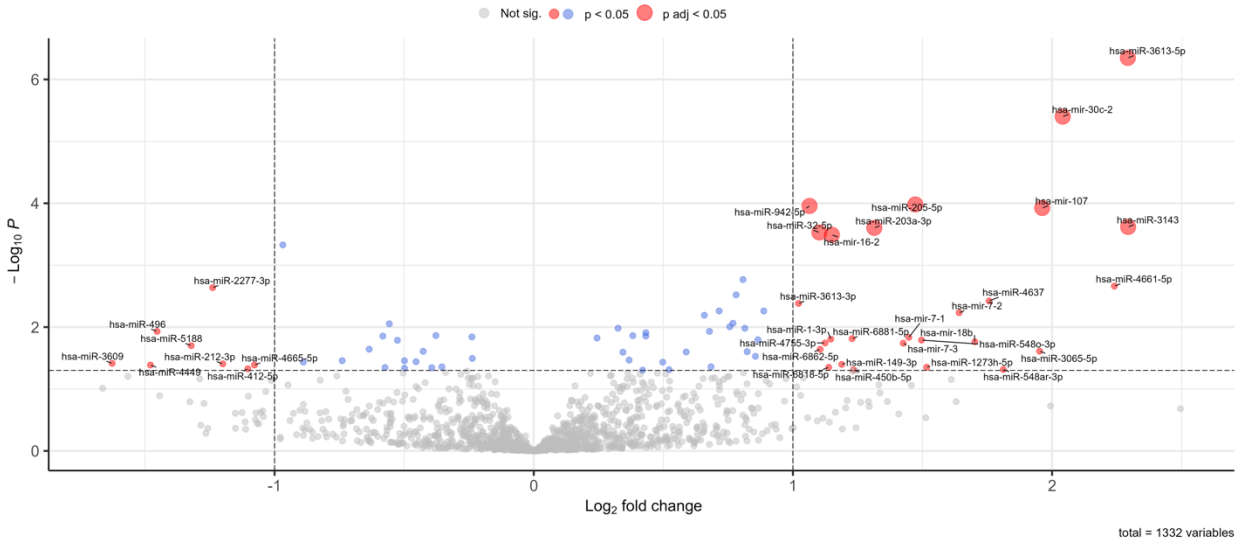


Figure 2. Volcano plot showing differential expression analysis between Control and MDD groups of 1332 microRNAs. In grey are microRNAs that do not pass any significance threshold. In blue are microRNAs that pass the nominal p-value but are below fold change (FC) of 1. In red, are microRNAs that pass the nominal p-value and are FC>1, with top DE miRNAs that pass the FDR enlarged circles.

miRNA ID	Log2 Fold Change	padj
hsa-miR-3613-5p	2.29	0.0006
hsa-mir-30c-2*	2.04	0.0026
hsa-mir-107*	1.96	0.0317
hsa-miR-205-5p	1.47	0.0317
hsa-miR-942-5p	1.06	0.0317
hsa-miR-203a-3p	1.31	0.0475
hsa-miR-3143	2.29	0.0475
hsa-mir-16-2*	1.15	0.0478

Table 2. The top DE miRNA by padj<0.05, with upregulated fold change in MDD compared to control group.

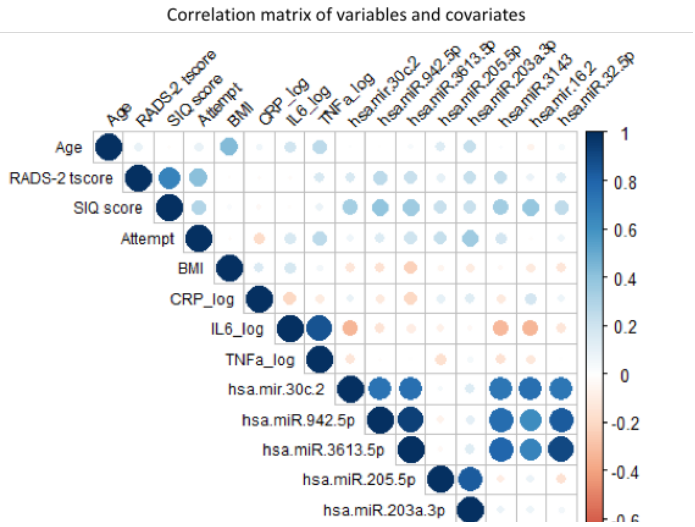


Figure 3. Correlation matrix of variables and covariates were computed using Pearson's correlation coefficient. Positive associations are depicted in cooler colors and negative associations are depicted in warmer colors.

Differential miRNA expression across key brain regions: substantia nigra, hippocampus, and frontal lobe

Exploratory analysis using the publicly available miRNA datasets DIANA-miTED (Figure 4A) and TissueAtlas (Figure 4B) revealed that the top DE miRNAs with mature sequences all have traces of expression in the regions of the human brain. Precursor sequences, referenced in Table 2, could not be included in the bioinformatics analysis because they are typically not represented in these datasets, which focus on mature miRNA sequences. miR-203a-3p and miR-3143 show the highest and lowest expression levels, respectively, suggesting their differing modulatory influences in brain tissue based on the region (Figure 4A). A breakdown by brain region shows remarkable concentration of miR-3613-5p, miR-942-5p, miR-32-5p and miR-3143 in the substantia nigra, an area well-known for its enrichment in dopaminergic cells (Figure 4B). miR-205-5p shows the highest expression levels among the measured miRNAs in the hippocampus, while miR-203a-3p has the highest expression in the frontal lobe, with moderate levels also observed in the hippocampus. Cell type signature analysis, as per DIANA miRPath, indicates that the top DE miRNAs have multiple associations with midbrain, PFC neurotypes, and glial cells during early development (Supplementary Table 2).

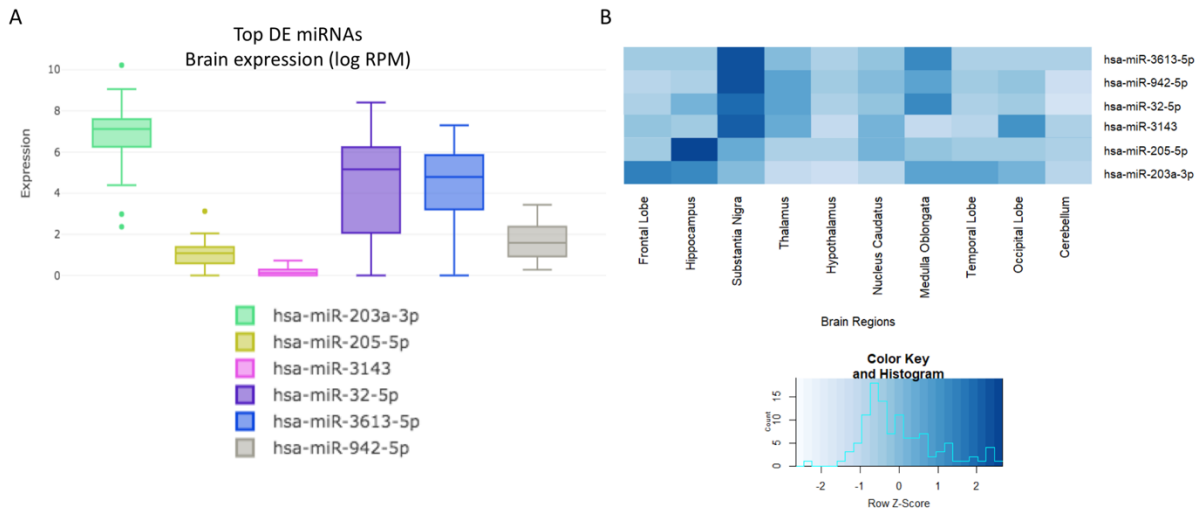


Figure 4. Exploring top DE miRNAs in DBS in other datasets. Using the microRNA Tissue Expression Database (miTED) we explored the potential distribution of these miRNAs in the brain. TissueAtlas dataset allowed to look further into the expression in the brain by specific brain tissue samples.

Expression of top miRNAs in DBS matches their distribution profile in serum and plasma

A heatmap depicting the top 20 miRNAs expressed in the DBS samples is shown in Figure 5A. To investigate the similarity between the expression of these miRNAs in the DBS samples and their levels in whole blood and its fractions (i.e. serum, white blood cells, and plasma), we extracted data from the DIANA-miTED. As shown in Figure 5B, the distribution of these miRNAs in DBS samples appears to more closely resemble the proportion of these miRNAs in serum, followed by plasma (see also Supplementary Figure 5 A and B).

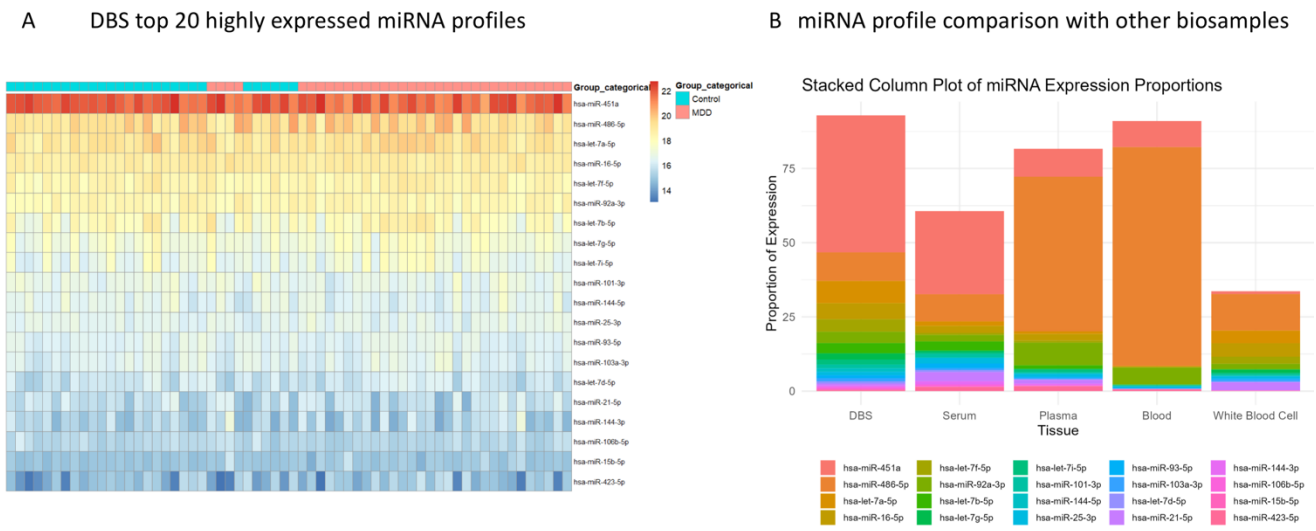


Figure 5: Exploring highly expressed miRNAs in DBS in other datasets. The heatmap of the top 20 expressed microRNAs in the current DBS samples. Based on the additional miTED data, we extracted expression profiles for miRNAs in other whole blood related samples, to compare the proportional similarities.

Clinical symptoms prediction analysis

To identify the potential of the top DE miRNAs to predict diagnostic outcome, we performed model selection employing stepwise selection and backward elimination methods to ensure reliability of the selection process. These analyses consistently highlighted miRNAs miR-203a-3p and miR-3143 to show the highest sensitivity and specificity. To evaluate their potential diagnostic utility, we constructed a logistic regression model with categorical diagnosis as the dependent variable and the expression levels of these two upregulated miRNAs as independent variables. The model's area under the curve (AUC) is 0.71, with a 95% confidence interval (CI) ranging from 0.58 to 0.84. The specificity and sensitivity of the model is 0.62 and 0.57, respectively (visualized as ROC in supplementary Figure 6).

MiRNA expression as a predictor of future self-reported depression severity

We performed linear regression analyses to determine the relationship between the expression levels of each of the top DE miRNAs and the RADS-2 t-scores that were recorded during a follow-up visit 9 months after the sampling session. The follow-up data were available for 23 participants with MDD diagnosis and 6 control participants. The linear model of MDD

severity modeled as a function of miRNA expression revealed a statistically significant positive association for miR-3613-5p and miR-942-5p (β = 3.556, adj r squared = 0.1517, p = 0.021; β = 5.836, adj r squared = 0.1496, p = 0.022, respectively) as shown in Figure 5A. None of the points reached Cook's distance above 1. Interestingly, none of the top DE miRNAs were associated with future anxiety score (quantified by MASC; Figure 5 C and D). This suggests that the potential predictive capacity of the top DE miRNAs is specific to the severity of depression symptoms. Associations between the severity of MDD and the expression levels of mir-30c-2 (p=0.059), mir-16-2 (p=0.077), miR-32-5p (p=0.079), and miR-3143 (p=0.095) were trending to the conventional statistical significance cut-off.

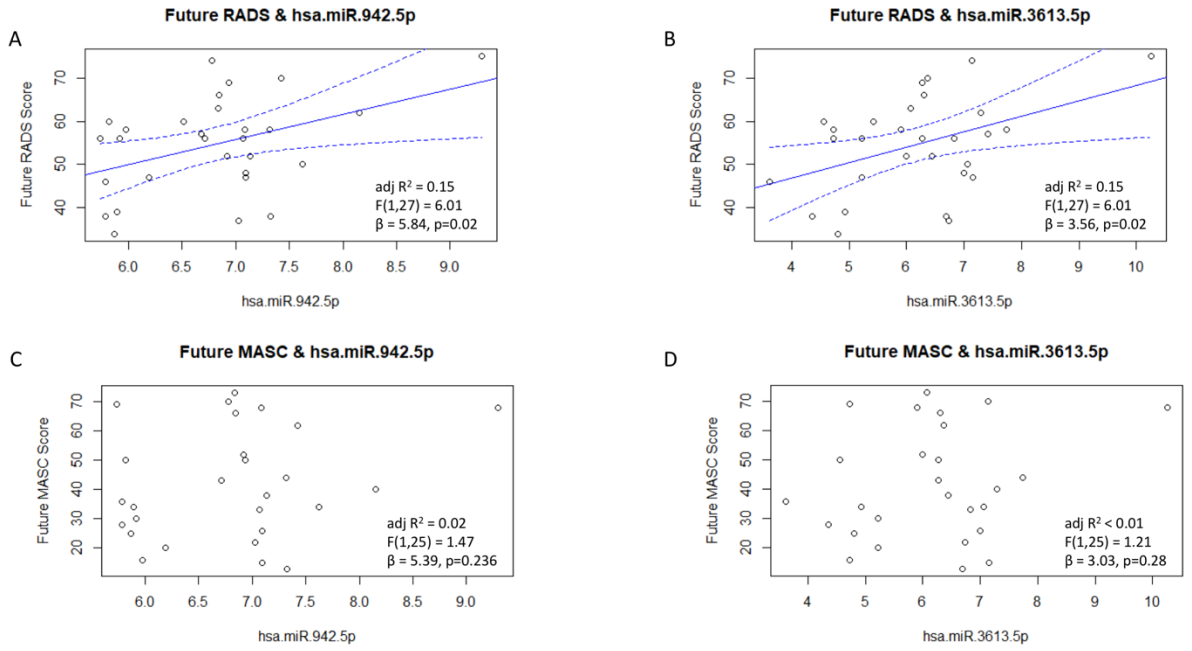


Figure 6. A & B: Linear models of miRNAs as predictive values for depression severity, measured with RADS t-scores. C & D: Linear models of miRNAs as predictive values for anxiety, measured with MASC t-scores, reveal a lack of prediction. No outliers were detected as per Cook's distance cutoff >1.

Predicted gene targets of top DE miRNAs associate with neurodevelopmental processes and mental illness conditions

Utilizing the microRNA Data Integration Portal (mirDIP), a comprehensive tool that amalgamates confidence scores from various prediction algorithms, we identified 958 gene

targets for the top DE miRNAs (Supplementary Table 3). We posited that common genes targets of at least two of the top DE miRNas (we refer to as “multi-miRNA target genes,") would be central nodes in biologically relevant processes. This criterion narrowed the list to 127 genes (Figure 7).

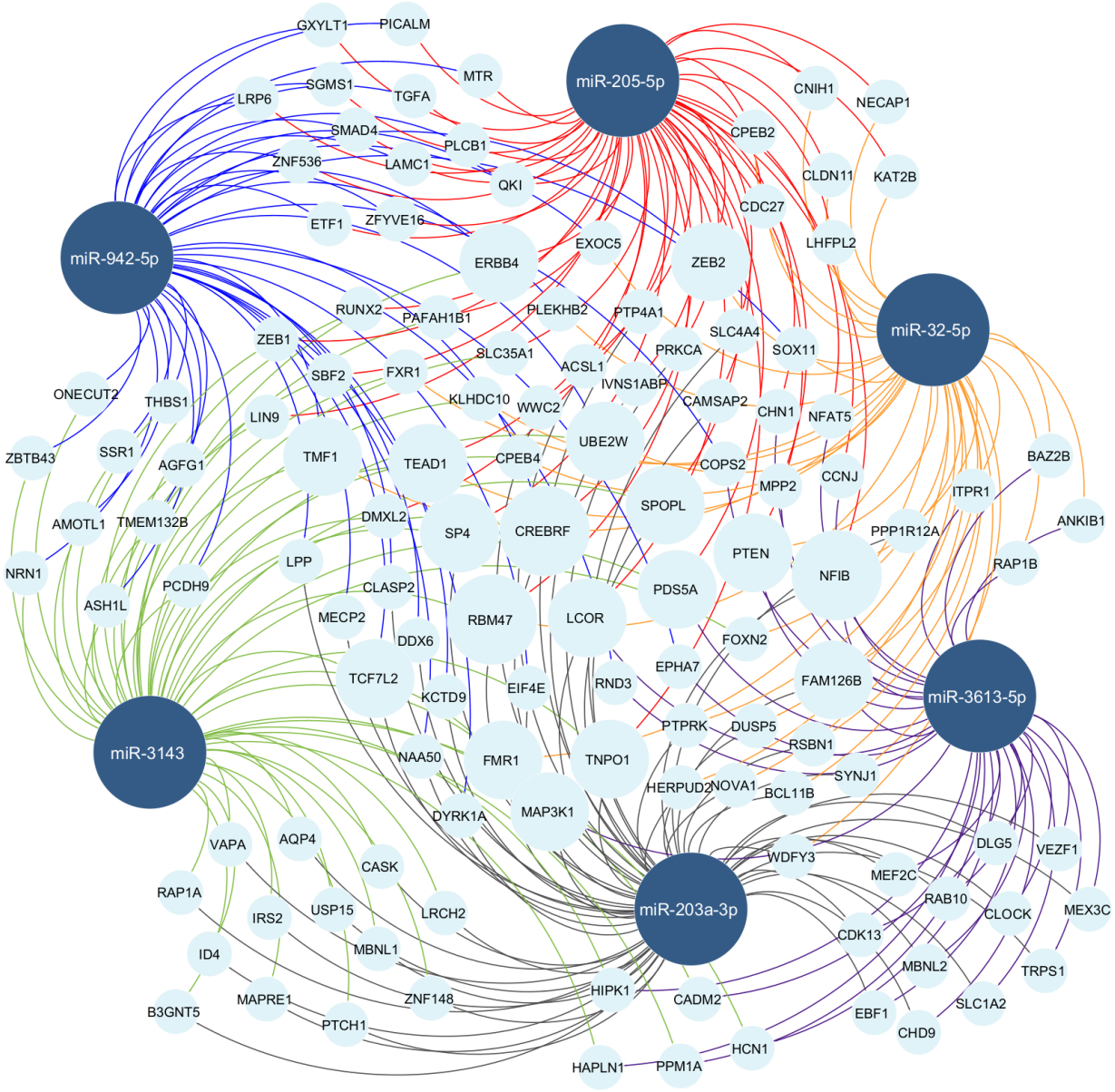


Figure 7. Illustration of the top DE miRNAs and their shared gene targets.

Gene ontology analysis (as per enrichGO) of these multi-miRNA target genes (full analysis output in Supplementary Table 4), shows associations with several key Biological Processes (BP), highlighted in Table 3, which capture broad themes including: cellular signaling,

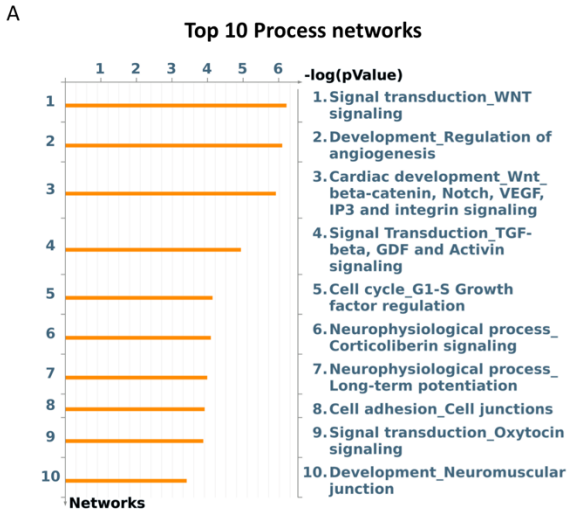
neurodevelopment and neuronal function, and behavioral and cognitive functions. Furthermore, Cellular Component (CC) ontology localized the target gene activity to the synaptic region. In supplementary Figure 7 we show how the GO terms cluster based on the similarity of their associated genes, with large nodes including: regulation of neuron differentiation, regulation of cell junction assembly and negative regulation of epithelial cell, regulation of long-chain fatty acid import across plasma membrane, epithelial cell development, regulation of mRNA splicing, and regulation of microtubule polymerization. Through regulation of a diverse array of gene targets, these miRNAs exert control over complex biological systems, ranging from neural development to cellular signaling pathways, thereby potentially influencing multiple facets of cognitive function.

For network-centric approach, we employed the integrated curated knowledge database MetaCore. Top (FDR <0.05) network associations from the enrichment analysis, consistently encompassed signal transduction, cardiovascular development and signaling, and neurophysiological processes (Figure 8A), in line with the gene ontology analysis. Notably, the disease-based analysis identified several psychiatric and depression related disorders (FDR<0.05; Figure 8B; see Supplementary Table 5 for all possible disease associations). These results elucidate potential biological networks linking top differentially expressed miRNAs with MDD diagnosis, simultaneously validating the efficacy of miRNA profiling in DBS-derived samples.

ONTOLOGY	Description	p.adjust	Count	geneID
BP	signal release	0.0024	14	CASK/CLOCK/FMR1/IRS2/ITPR1/MEF2C/PLCB1/RAP1A/RAP1B/SMAD4/SOX11/SYNJ1/TCF7L2/TMF1
BP	hippo signaling	0.0047	5	AMOTL1/DLG5/SOX11/TEAD1/WWC2
BP	cellular response to transforming growth factor beta stimulus	0.0047	10	MEF2C/ONECUT2/PPM1A/PTPRK/SMAD4/SOX11/THBS1/USP15/ZEB1/ZEB2
BP	modulation of chemical synaptic transmission	0.0051	13	CASK/EIF4E/EPHA7/FMR1/HCN1/MECP2/MEF2C/MPP2/PAFAH1B1/PLCB1/PTEN/RAP1A/RAP1B
BP	regulation of trans-synaptic signaling	0.0051	13	CASK/EIF4E/EPHA7/FMR1/HCN1/MECP2/MEF2C/MPP2/PAFAH1B1/PLCB1/PTEN/RAP1A/RAP1B
BP	regulation of dendritic spine development	0.0056	5	DLG5/FMR1/MEF2C/PAFAH1B1/PTEN
BP	multicellular organismal response to stress	0.0056	6	EIF4E/HCN1/MECP2/MEF2C/PTEN/THBS1
BP	positive regulation of hormone secretion	0.0063	7	IRS2/ITPR1/PLCB1/SMAD4/SOX11/TCF7L2/TMF1
BP	regulation of neuron differentiation	0.0070	8	BCL11B/DDX6/EIF4E/ID4/MEF2C/SOX11/ZEB1/ZNF536
BP	axon development	0.0098	12	BCL11B/CAMSAP2/CHN1/EPHA7/MTR/NFIB/PAFAH1B1/PICALM/PTCH1/PTEN/RAB10/SMAD4
BP	cell fate commitment	0.0112	9	BCL11B/ERBB4/MEF2C/ONECUT2/PTCH1/RAB10/RUNX2/SMAD4/TCF7L2
BP	vesicle-mediated transport in synapse	0.0112	8	CASK/FMR1/NECAP1/PICALM/PTEN/RAP1A/RAP1B/SYNJ1
BP	regulation of neurotransmitter secretion	0.0154	5	CASK/FMR1/MEF2C/RAP1A/RAP1B
BP	regulation of synaptic vesicle exocytosis	0.0167	4	CASK/FMR1/RAP1A/RAP1B
BP	central nervous system neuron differentiation	0.0355	6	BCL11B/ID4/NFIB/PAFAH1B1/PTCH1/PTEN
BP	forebrain development	0.0375	9	BCL11B/ERBB4/ID4/NFIB/PAFAH1B1/PLCB1/PTEN/SLC1A2/ZEB1
BP	behavioral fear response	0.0447	3	EIF4E/MECP2/MEF2C
BP	ERK1 and ERK2 cascade	0.0451	8	DUSP5/EPHA7/ERBB4/PRKCA/PTEN/RAP1A/RAP1B/SMAD4
BP	behavioral defense response	0.0456	3	EIF4E/MECP2/MEF2C
BP	learning or memory	0.0490	7	MECP2/MEF2C/PAFAH1B1/PICALM/PLCB1/PTEN/SYNJ1
CC	glutamatergic synapse	0.0057	11	DLG5/EIF4E/EPHA7/ERBB4/FXR1/HCN1/PAFAH1B1/PLCB1/RAP1A/RAP1B/SLC1A2
CC	neuron to neuron synapse	0.0075	10	CPEB4/DLG5/EPHA7/ERBB4/FMR1/FXR1/ITPR1/MPP2/PTCH1/PTEN

Table 3: Performing gene ontology analysis for multi-miRNA target genes (N=127) we identified many associations - here showing an excerpt of only the notable Biological Processes (BP; the pathways/processes to which that gene product's activity contributes) and Cellular Component (CC; where the gene products are active)

A



B

Notable diseases	FDR	# of Genes
Psychiatry and Psychology	6.850E-07	75
Mood Disorders	8.553E-07	28
Mental Disorders	9.093E-07	74
Depressive Disorder, Major	2.832E-06	23
Depressive Disorder	3.638E-06	23
Nervous System Diseases	3.864E-06	108
Neurocognitive Disorders	6.683E-06	52
Brain Diseases	8.816E-06	80
Neurodevelopmental Disorders	2.327E-05	34
Neurobehavioral Manifestations	3.476E-05	35
Bipolar and Related Disorders	1.301E-04	17
Child Development Disorders, Pervasive	1.323E-04	12
Cardiomyopathy, Hypertrophic	5.455E-03	6
Cardiovascular Diseases	1.274E-02	52
Substance-Related Disorders	1.399E-02	13

Figure 8: Notable MetaCore-based A) process networks and B) disease associations for multi-miRNA target genes (n = 127).

Discussion

Depression rates are globally on the rise, with adolescents being particularly vulnerable. Understanding the biological mechanisms that contribute to susceptibility during this critical developmental period is imperative. The necessity for early identification of at-risk individuals cannot be overstated, given that early interventions are demonstrably effective in altering the disease trajectory (Okuda et al., 2010; Merry et al., 2012; Habert et al., 2016; Beames et al., 2021). Our study offers a comprehensive analysis of blood miRNome profiles in psychiatrically healthy adolescents and those afflicted with depression, providing insights into the molecular and cellular processes that may be affected by the condition. Our methodological approach is notable for its combination of cost-effectiveness and suitability for future large-scale application.

Differential expression analysis shows a predominance of upregulated miRNAs in the MDD group, potentially denoting an active modulation of genes and pathways involved in the depression condition. Stringent statistical criteria identified nine miRNAs, miR-3613-5p, mir-30c-2, mir-107, miR-205-5p, miR-942-5p, miR-203a-3p, miR-3143, mir-16-2, and miR-32-5p, as the most upregulated in MDD compared to controls (Figure 1, Table 2). The overexpression of these miRNAs may result in an increase of RNA inhibition or degradation and/or in greater number of genes being actively targeted. To delve deeper into these likely spatiotemporally specific mechanisms, argonaute-crosslinking and immunoprecipitation techniques in the brain tissue can be employed to dissect miRNA-target interactions (Quévillon Huberdeau and Simard, 2019).

Existing literature, although limited, supports the association of two of the top DE miRNAs with depression. For example, miR-205-5p is also found to be upregulated in secreted exosomes in patients with depression (Zhang et al., 2018). A recent review (Musazzi et al, 2023) highlights the role of mature miR-16 in several stress induced behavioral abnormalities in animal models and response to therapeutic treatments. Emerging research in adolescent depression has begun to profile miRNAs in extracellular vesicles. However, methodological differences and less stringent statistical thresholds, compared to our study, likely contribute to the lack of replication of the top differentially expressed miRNAs. Ran et al. (2022) identified

three miRNAs that may mediate the link between childhood trauma and MDD vulnerability. Another study by Honorato-Mauer (2023) found two miRNAs to be downregulated in adolescent MDD, with a shared downregulation of a single miRNA in both MDD and anxiety disorders.

While all top DE miRNAs are expressed in the brain, their spatial distribution revealed region-specific expression patterns. There is a remarkable concentration of miR-3613-5p, miR-942-5p, miR-32-5p and miR-3143 in the substantia nigra (Figure 3), the midbrain region associated with the somatodendritic release of dopamine. This finding was corroborated through a separate cell type enrichment analysis (Supplementary Table 2), raising the possibility that these miRNAs are involved in motivation and reward function, which are altered in MDD (Hamilton et al., 2018; Delva and Stanwood, 2021; Eckstrand et al., 2022). Additionally, among the top DE miRNAs, miR-205-5p shows the highest expression in the hippocampus, another brain region highly implicated in MDD (MacQueen and Frodl, 2011; Whittle et al., 2014; O'Callaghan and Stringaris, 2019; Toenders et al., 2019; Schmaal et al., 2020). The dopamine and hippocampal circuitries are vulnerable to stress exposure during their ongoing maturation in adolescence, a factor that may contribute to the development or exacerbation of MDD (Gee et al., 2018; Gomes et al., 2020; Zhu and Grace, 2023). Whether the expression of the top DE miRNAs is dysregulated in these brain regions in MDD remains to be determined, such as with human postmortem brain tissues and targeted manipulations in animal models that can directly assess the impact on gene expression and behavior. Our findings mark a starting point for this inquiry.

The analysis of the aggregate expression of top DE miRNAs and their diagnostic outcome shows preliminary promise. The logistic regression model based on just two miRNAs, miR-203a-3p and miR-3143, achieved specificity of 62% and sensitivity of 57%. For future validation of the model, these data should be applied to train the diagnostic prediction model using a larger independent cohort dataset to assess the model's generalizability.

In addition, a linear model analysis revealed that blood levels of miR-3613-5p and miR-942-5p predict the severity of future self-reported depression symptoms (Figure 6). This association appears exclusive to depression, given that none of the DE miRNAs predicts future

anxiety symptoms. This points to a potential role for miR-3613-5p and miR-942-5p in mechanisms tied to the pathophysiology of depression, rather than those involved in general emotional distress. The specific link between peripheral miRNAs in adolescence and future depression symptoms could potentially serve as a tool for risk stratification.

Using bioinformatics within the framework of systems biology and narrowing our focus to genes with sequences targeted by two or more DE miRNAs, we identified over 100 genes (Figure 7). Ontologies of these genes span across different levels of neurodevelopmental processes, including axon development dendritic spine growth, and forebrain development (Table 3). As an instance, these genes include EPHA7, a target of both miR-942-5p and miR-3613-5p, which drives neuronal maturation and has been linked to neurodevelopmental deficits (Clifford et al., 2014; Beuter et al., 2016; Yan et al., 2022); BCL11B, a target of miR-203a-3p and miR-32-5p, a transcription factor critical in vital developmental functions, including long distance axon guidance and neuron differentiation (Lennon et al., 2017); ERBB4 a gene encoding a neuregulin receptor, targeted by miR-205-5p, miR-3143 and miR-942-5p, has been directly associated with depression pathology (Fiori et al., 2021; Wang et al., 2023). Thus, the link between the DE miRNAs and the dysregulation of ongoing maturational processes in the adolescent brain is through these genes, which can be investigated further as a network.

Notably, our gene ontology approach identified a significant enrichment of these genes with MDD and psychiatric conditions of adolescent onset (Figure 8), providing support for the idea that peripheral miRNAs in adolescence can serve as both markers and mediators of psychiatric risk. Cardiovascular associated illnesses are also among the notable conditions in which these genes play a role, which is in line with known comorbidities associated with MDD (Kendler et al., 2009; Barth et al., 2004; Schnabel et al., 2013). In this young cohort, the molecular link between these disorders would suggests an early strain on cardiovascular systems, which is supported by evidence of heart-brain interactions influencing brain activity (Hsueh et al., 2023) and the mutual impact of stress on both cardiovascular health and MDD (Topic et al., 2013).

The top DE miRNAs included mir-30c-2, mir-107, and mir-16-2 which are precursor sequences that can be spliced into various mature forms. In the case of mir-30c-2, for example,

mature isomiRs are miR-30c-2-3p or miR-30c-5p. Both forms may be functional, contingent on sequence stability (Gebert and MacRae, 2019). Based on the primary DE analysis for this cohort, detailed in Supplementary Table 1, we did not detect differences in expression for the mature forms corresponding to these precursor miRNAs between the groups. Further functional validation is needed to understand how these precursors are dysregulated upstream of the miRNA functional form. Consequently, we refrained from speculating on potential gene targets.

DBS sampling offers universal accessibility (Zhuang et al., 2022) and enhances the predictive power of miRNA-based studies in MDD. This can be further augmented by an incorporation of genotyping (Hollegaard et al., 2009), proteomics (Eshghi et al., 2020), and methylome profiling (Hollegaard et al., 2013), among other biological studies. While DBS has not been employed for psychiatric research until recently (Krammer et al., 2023), ample literature supports the use of serum or plasma for miRNA profiling. Our comparative analysis of miRNA expression levels in DBS against those in existing biofluid datasets (Figure 5) show that serum and DBS have similar proportional representation of miRNAs. Plasma also shows a notable number of shared top twenty highly expressed miRNAs. These findings suggest that DBS could serve as a reliable alternative to serum and plasma in studies investigating circulating miRNAs in healthy and disease conditions, with simplified logistical requirements of sample collection and storage. Given that the distribution in liquid blood was less similar to DBS samples, caution is warranted when comparing miRNAs between different types of peripheral fluids samples, as noted in existing literature (Wang et al., 2012; Diener et al., 2019).

The strength of our study is the focus on a population of adolescents diagnosed with depression, however it comes with a limitation of a smaller sample size. The sample size of the current study is sufficient for detecting statistical group differences, however it would greatly benefit from a larger study for replication and subtyping symptom profiles for even better classification of an individual during prediction analysis. Just as progress has been made through large-collaborative GWAS studies, that addressed sample size, population heterogeneity and phenotypic complexity, additional studies focused on miRNA function in adolescence will foster better insights. In the current study, the population sample is of educated, mid- to high- socioeconomic status, and we cannot rule out the impact of medication

history on the profiled miRNome. We also performed a separate differential expression based on sex, however no statistically significant miRNAs were identified. A further limitation note can be made in regard to the non-cell-specific nature of the DBS and it is therefore critical to interpret the results in the context of the biotype being studied.

Conclusions

This study stands as one of the very few to delve into the role of microRNAs in adolescent depression, leveraging the advantages of dried blood spot (DBS) samples for a novel and accessible approach. By profiling miRNAs in both psychiatrically healthy controls and adolescents diagnosed with MDD, our research offers insights into the molecular complexities of psychiatric disorders and lays the groundwork for earlier identification and intervention methods. Our methodological approach synthesizes widespread DBS sample accessibility, the predictive utility of miRNAs, and cutting-edge standardized techniques, setting a foundational framework for future multimodal studies. This investigation not only deepens our understanding of the biological mechanisms at play in adolescent-onset MDD, but also presents promising avenues for early detection.

MicroRNAs serve dual roles as epigenetic factors bridging environmental influences and genetic predisposition, and as potential messengers from the brain, reflecting regulatory changes in disease states in the peripheral blood. The robustness of our approach is validated by the coherence between various analyses and the alignment of overarching themes, linking circulating miRNAs to future symptoms and the gene ontology of MDD. Utilizing DBS in routine pediatric primary care would enable rapid and remote identification of at-risk individuals and even those at immediate risk for severe outcomes like suicide, fulfilling the critical need for objective markers.

Connecting statement chapter III to chapter IV

In the preceding chapter I focused on the assessment of peripheral miRNAs associated with MDD and explore associated signaling pathways. It was reassuring that synaptic localization and axon guidance appeared in the gene ontology analysis, as I expected these miRNAs to be involved in the nervous system pathways in association with the disease.

Peripheral markers provide representation of systems-wide changes associated with the disease. To probe into brain-specific mechanisms without the reliance on animal models, my next study uses genotype and morphological measures from community-based child cohorts and gene expression databanks. As I described in the *Introduction*, there is a critical functional trajectory for DCC in the brain as informed by studies involving major genetic abnormalities or animal models, however it is not yet known what its role may be in psychiatrically healthy children from a community-sampled cohort. To begin exploring the multimodal analysis and to address this gap, my goal was to examine the potential of genes co-expressed with DCC in the prefrontal cortex during early years with developmental outcomes. The decision for building DCC-specific expression-based polygenic score was driven by lines of evidence from our lab and that in the literature that DCC is critical in psychiatric outcomes in adulthood.

Studies have shown that the genetic influences during this period are likely to impact the way that the brain develops over time. In animal models we have shown that DCC is responsible for guiding corticolimbic innervations to the prefrontal cortex. We thus explored structural alterations associated with the DCC gene network activity in the PFC by linking SNPs in the genomes of two cohorts to downstream gene expression. My results focus on grand developmental outcomes - brain volume, because brain growth is directly related to developmental milestones and cognitive abilities.

CHAPTER IV

DCC gene network in the prefrontal cortex is associated with total brain volume in childhood

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Keywords: Netrin-1, guidance cues, polygenic risk scores, human brain development, expression-based polygenic risk score.

Abstract

Background: Genetic variation in the guidance cue DCC gene is linked to psychopathologies involving prefrontal cortex (PFC) dysfunction. We created an expression-based polygenic risk score (ePRS) based on the PFC DCC co-expression gene-network hypothesizing that it would associate with individual differences in total brain volume (TBV). **Methods**: The ePRS was calculated by the aggregate effect of genetic variants of DCC gene co-expression network in the PFC during the first decade of life. We assessed an association between the DCC ePRS and TBV in children in two community-based cohorts, the MAVAN and UCI projects. A conventional PRS was calculated based on a TBV GWAS for comparison. **Results**: Higher ePRS associates with larger TBV in 8-10 y.o. children ($p=0.043$, $\beta=0.212$; N=88). Conventional PRS at several different p-value thresholds did not predict TBV in this cohort. The replication analysis in an independent cohort of newborn children from a UCI study confirmed the association between the ePRS and newborn TBV ($\beta = 0.101$, $p = 0.048$; N=80). The genes included in ePRS demonstrate high level of co-expression throughout the lifespan and are primarily involved in cellular function regulation. **Limitations**: The relatively small sample size and age differences between main and replication cohorts. **Conclusions**: Our findings suggest that the PFC DCC co-expression network is critically involved in whole brain development across childhood. Genes comprising the score are involved in gene translation control and cell adhesion, and their expression in the PFC at different stages of life provides a snapshot of their dynamic recruitment.

Introduction

The prefrontal cortex (PFC) modulates executive functions, such as attention, decision making, and cognitive control (Fuster, 2015). The maturation of these behaviors parallels the structural refinement of the PFC, including the ingrowth of mesocortical dopamine (DA) axons, which continues until early adulthood (Gogtay et al., 2004; Luna 2009; Sturman and Moghaddam, 2011; Ordaz et al 2013; Larsen and Luna, 2018; Kalsbeek et al., 1988; Naneix et al., 2012; Willing et al., 2017; Reynolds et al., 2018; Flores and Hoops 2017). The gradual maturational trajectory of mesocortical DA connectivity, and of the PFC itself, is controlled by the guidance cue, Netrin-1, and its receptor, DCC, which determine spatiotemporal targeting of growing axons (Reynolds et al., 2018; Cuesta et al., 2020). Studies in rodents show that variation in Dcc gene expression leads to differences in cognitive flexibility, behavioral inhibition, vulnerability to stress induced depression-like behaviors, and altered sensitivity to drugs of abuse (Grant et al., 2007; Flores 2011; Manitt et al., 2013; Pokinko et al., 2015 Reynolds et al., 2018 Torres-Berrio et al., 2017; Hoops and Flores, 2017; Reynolds et al., 2019).

In humans, an autosomal dominant DCC mutation results in structural and functional alterations in connectivity of mesocortical pathways with associated decrease in novelty seeking behavior and cigarette use (Vosberg et al., 2018; Vosberg et al., 2019). An increasing number of meta-analysis of genome-wide association studies (GWAS) and post-mortem human studies show that altered DCC expression levels and the presence of specific genetic polymorphisms in the DCC gene associate with psychiatric conditions (see review Vosberg et al., 2019), most notably major depressive disorder (Manitt et al., 2013; Dunn et al., 2016; Okbay et al., 2016; Smith et al. 2016 Torres-Berrio et al., 2017; Zeng et al., 2017; Ward et al., 2017; Roberson-Nay et al. 2018; Aberg et al 2018; Barbu et al., 2019; Leday et al., 2018; Wray et al., 2018; Arnau-Soley et al., 2019; Strawbridge et al., 2019; Lee et al., 2019; Torres et al., 2020), schizophrenia (Grant et al. 2012; Yan et al., 2016; Smeland et al., 2018; Lee et al., 2019) and drug abuse (Khadka et al., 2014; Zanetti et al., 2016; Ward et al., 2017; Vosberg et al., 2018; Kichaev et al., 2019; Linnér et al., 2019). A study by the Cross-Disorder Group of the Psychiatric Genomics Consortium (Lee et al., 2019) revealed that a PFC-enriched network of genes prominently affects eight psychiatric disorders, with a DCC isolated single nucleotide

polymorphism (SNP) showing the highest pleiotropic association to all eight disorders. A separate analysis of integrated multi-omics data on genes expressed within the dorsolateral PFC singled out DCC to predict well-being, cognitive function and neuroticism (Li et al., 2020). The converging evidence suggests that DCC expression in the PFC is critical for neurodevelopment.

In this study, we go beyond investigating genetic mutations or contributions of SNPs to particular traits, and address whether variation in the function of the DCC gene network within the PFC associates with differential brain development in children in community-based samples. We utilized data of well-established child cohorts, the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) study (O'Donnell et al., 2014) and University of California, Irvine (UCI) project (Moog et al., 2018; Töpfer et al., 2019), and assessed general brain development, represented by total brain volume. Brain volume measure in children associates with future cognitive outcomes (McDaniel, 2005; Rapoport et al., 2001; Nave et al., 2019) and here is used as an indicator of prominent neurodevelopmental differences.

Methods

MAVAN cohort

Sample. We used data collected from a community-based birth cohort from the project called Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) project (O'Donnell et al., 2014), for which pregnant women, 18 years of age or older, were recruited in Montreal (Quebec) and Hamilton (Ontario), Canada. Exclusion criteria were severe maternal chronic illnesses, placenta previa, and history of incompetent cervix, impending delivery, or a fetus/infant affected by a major anomaly or born at a gestational age of 37 weeks. Magnetic resonance imaging (MRI) of the brain was acquired when the children were 8-10 years of age.

Genotyping. As described elsewhere (Silveira et al., 2017), using genome-wide platforms (PsychArray/PscyhChip, Illumina), autosomal SNPs were genotyped from 200ng of genomic DNA derived from buccal epithelial cells, according to manufacturer guidelines. Subject samples with low call rate (<90%), as well as SNPs with low call rate (<95%), minor allele frequency (MAF) <5% and SNPs violating Hardy-Weinberg equilibrium (HWE) with p<1e-40 were removed,

which resulted in 260 subjects and 242,211 SNPs. PLINK 1.9 (Chang et al., 2015) was utilized for quality control (QC) procedure. The remaining SNPs were submitted for imputation with Sanger Imputation Service using Haplotype Reference Consortium (release 1.1) panel (McCarthy et al., 2016) and post-imputation QC, which resulted in 20,790,893 SNPs with imputation accuracy above 0.80. Imputed dosage genotypes were recoded to hard-called genotypes using posterior genotype probability above 0.90. The polygenic scores were calculated on hard-called genotypes.

Because the subjects are potentially from ancestrally heterogeneous backgrounds (classified as population stratification) that may bias genetic associations, the population structure was examined using principal component analysis (Price et al., 2006; Patterson et al., 2006). Genotyped SNPs that had high linkage disequilibrium ($r2<0.20$) were pruned with a sliding window of 50 SNPs in increments of 5 using PLINK 1.9. We performed principal component analysis using SMARTPCA on this pruned dataset and generated a scree plot. Based on the inspection of the screeplot, the first three principal components (PCs) were the most informative of population structure and were included in all analyses as covariates.

Neuroimaging. We used high-resolution T-1 weighted neuroimaging scans and processed them using Multiple Automatically Generated Templates Brain Segmentation Algorithm (MAGeTbrain) (Chakravarty et al., 2013; Pipitone et al., 2014). Scans containing obvious artifacts (such as ghosting or blurring due to head motion) were excluded. Each subject's structural scan was segmented into gray matter, white matter and cerebral-spinal fluid (CSF) tissue classes. Gray matter and white matter were used to calculate total brain volume (TBV).

Replication cohort: University of California Irvine

Sample. A prospective longitudinal study of pregnant mothers and their offspring conducted at the University of California, Irvine, Development, Health and Disease Program (Moog et al., 2018; Töpfer et al., 2019) was used as a replication cohort (UCI cohort). Mothers were recruited during early pregnancy, and shortly after birth (Postnatal age at scan M = 27 days +/- 13 days) their newborns underwent an MRI scan of the brain during natural unsedated sleep.

Genotyping. We used the Illumina HumanOmniExpress BeadChip (Illumina) to describe genetic variation in our replication cohort. QC, imputations, hard-calling, and PCA were performed using the same pipelines as for MAVAN cohort. QC of genotype data was performed using PLINK 1.9, where samples with low call rate, variants with low call rate (< 95%), MAF < 5%, or HWE test p < 1e-25 were removed. A total of 584,711 genotypes and 142 samples passed QC. We used the Sanger Imputation Service to impute missing genotypes using the Haplotype Reference Consortium (release 1.1) panel. Following post-imputation QC and applying INFO score of 0.8, 25,060,157 SNPs were left for the downstream analyses. Similar to MAVAN, we recoded imputed dosage genotypes (posterior genotype probability above 0.90) and used hard-called genotypes for polygenic scores. We applied principal component analysis to the pruned dataset to describe population stratification in the UCI cohort and included the first three principal components in data analyses of the UCI cohort.

Imaging. MRI acquisition. Infants were scanned using Siemens 3.0T Scanner (TIM Trio, Siemens Medical Systems Inc., Germany) as previously described (Cherel et al., 2015; Moog et al., 2018). Tissue segmentation was performed using a neonate multi-atlas-based (https://www.nitrc.org/projects/unc_brain_atlas/) iterative expectation maximization segmentation algorithm as in the previous studies from the same cohort (Cherel et al., 2015). Brain tissue was classified as gray matter, white matter and CSF. The first two tissue types were used to calculate total brain volume (TBV), and all three tissue types were used to calculate intracranial volume (ICV), that was controlled for in the analyses.

All clinical and neuropsychological assessments were conducted in accordance with the relevant ethics committees' approval for the 3 sites (two sites in MAVAN – Montreal and Hamilton). Approval for the MAVAN project was obtained from McGill University, Université de Montréal, Royal Victoria Hospital, Jewish General Hospital, Centre hospitalier de l'Université de Montréal, Hôpital Maisonneuve-Rosemount, St Joseph's Hospital, and McMaster University; and one site in replication cohort – University of California Irvine, for which UCI IRB approved all assessments) at which the data were collected, and all participants' parents provided written informed consent.

Polygenic expression score

Following the protocol described by Silveira et al., (2017) and Dass et al., (2019), our polygenic expression score (ePRS) was created for DCC co-expression network with brain region and age specificity using publicly available genetic databases and genotype of a prospective study. We obtained a list of genes co-expressed with DCC (absolute correlation of 0.5 or more) in the human adult PFC using the BrainEAC database (http://www.braineac.org/). Then, we filtered this list to genes that are differentially expressed (1.5 folds or more) between the ages of 1.5 and 11 years versus adults using data from BrainSpan (http://www.brainspan.org) by combining dorsolateral PFC (dPFC), ventrolateral PFC (vPFC), medial PFC (mPFC), orbital frontal cortex (OFC). Resulting list consisted of 175 genes. It was screened using the NCBI tool to identify annotated SNPs in humans (https://www.ncbi.nlm.nih.gov/variation/view). The resulting SNPs were subjected to linkage disequilibrium clumping ($r2 > 0.2$) to yield a list of independent SNPs that were representative of the region. Then, based on the genotype data we used the count function of the number of effect alleles at a given SNP and weighed it by the slope coefficient from the GTEx regression model that predicted gene expression in the PFC tissue according to the genotype (https://www.gtex portal.org/home). As such, the polygenic expression score was calculated as a sum of the number of effect alleles multiplied by the effect size of association between the genotype at a given SNP and gene expression.

Gene ontology enrichment analysis

To understand biological functions of the 175 genes that our score comprised of, we performed gene ontology enrichment analysis using Metacore® (Thomson Reuters), a commercially available literature database that screens for main functional networks.

Gene expression developmental trajectory

To map the expression of the DCC co-expression network for different developmental periods, we plotted the co-expression levels in the PFC in the four selected regions (dPFC, vPFC, mPFC, OFC) based on available postmortem data (BrainSpan): childhood ranged between 4 months to 8 years (n= 11), adolescence 11-18 years old (n=4), adulthood 21 through 40 y.o. (n= 6). We created a gene expression correlation matrix at each developmental stage and plotted the resulting data using heatmaply package (Galili et al., 2018) genes were ordered in a consistent manner across timepoints (based on the correlation matrix calculated for childhood

period, the order was repeated for all other time stages) to help visualize changes in co-expression over time.

Comparison of a conventional PRS for brain volume versus the DCC ePRS: Unlike ePRS, which takes into account that co-expressed genes are part of the same network, polygenic risk scores (PRS) are used in population genetics to aggregate the information from a genome-wide association study and describe the cumulative, additive effects of a large number of SNPs and their contribution to variation in complex phenotypes (Wray and Goddard 2010), including psychiatric disorders (Wray et al., 2014). To identify whether a conventional brain volume PRS would also be associated with total brain volume differences in the MAVAN sample, we calculated the PRS using our accelerated pipeline (Chen et al., 2018; https://github.com/MeaneyLab/PRSoS). These scores were created for each subject based on a large brain volume genome-wide association study (GWAS) meta-analysis (Jansen et al., 2019). This GWAS was performed using data from the UK Biobank, Enhancing NeuroImaging Genetics through Meta-Analysis partnered with CHARGE, and Early Growth Genetics consortiums. We used the cumulative summary scores computed as the sum of the allele count weighted by the effect size across SNPs at different p-value thresholds according to the GWAS.

Statistical analysis:

In all the analyses we used the ePRS as a continuous variable to examine its main effect on the total brain volume. For descriptive statistics, we compared children from high and low genetic score groups (defined by a median split) in the main confounders.

We used the IBM SPSS Inc. (version 20.0 - 25.0; Chicago, IL, USA) and R (R Core Team, 2019) for data analysis. Statistical significance was considered at $p < 0.05$. Student's t-test and chi-square tests were used to compare baseline characteristics. Linear regression analysis was performed to investigate the association between genetic score (DCC ePRS) and total brain volume. Our analysis considered well-established variables that affect child neurodevelopment as possible confounders. In MAVAN the model was adjusted by age at scan, sex, z-score BMI, PCs and site (Montreal/Hamilton). Since the UCI replication cohort consisted of newborns, we used pediatric age, as well as the birth weight ratio (Kramer et al., 1999; Villar et al., 2014), sex, intracranial volume, and PCs to adjust the model. UCI was adjusted for intracranial volume to

account for head size, however this analysis is not included for MAVAN because intracranial volume and total brain volume are highly correlated in the sample; adjusting for ICV in MAVAN would pose the collinearity issue, which would inflate the variance. Pediatric age is defined as age at scan adjusted by gestational age at birth.

Results

The DCC gene co-expression network is dynamic across the lifespan

To "map" variation in gene expression of the PFC DCC co-expression network over the human lifespan, we utilized the BrainSpan gene expression database and compared the postmortem data specific to the PFC of donors of various ages. We aggregated the data for childhood, adolescent and adulthood periods, and calculated correlation matrices presenting them with retained gene order (Figure 1). The network's co-expression pattern is dynamic across postnatal ages: during childhood there is robust synchrony of gene activity, but in adolescence, when PFC undergoes substantial maturation, the correlation matrix of genes co-expressed with DCC shows no clear structure. By adulthood, gene expression appears to concentrate into specific hubs of activity.

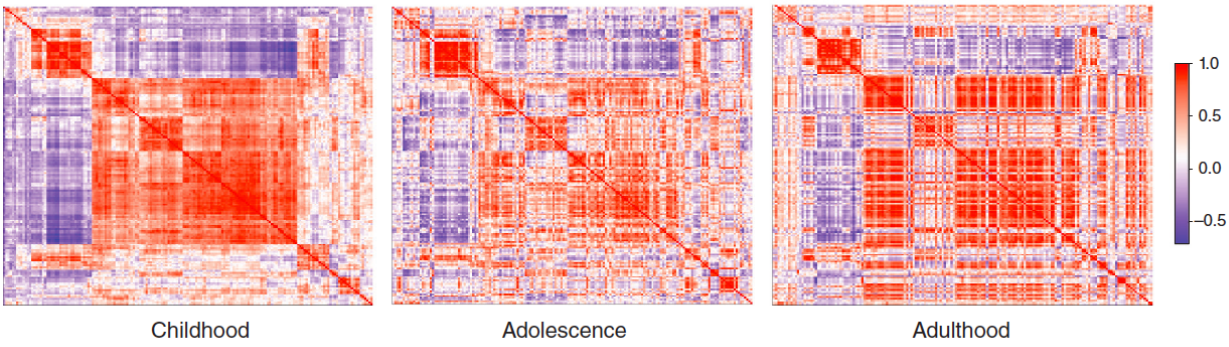


Figure 1. Using Euclidean matrix, we show expression levels of the DCC co-expression network in the PFC through three life stages (red = positive co-expression correlation; blue = inverse co-expression correlation; white = no correlation). The order of genes within the network has been retained for the different age periods. As indicated by large clusters of same-direction expression (red and blue), the network's co-expression pattern has robust synchrony of gene activity, with notable clustering of the pattern as the age increases.

DCC ePRS predicts individual differences in brain size

As shown in Tables 1 and 2, the subject demographics among the MAVAN and UCI cohorts were relatively homogeneous, with no differences between the low and high DCC ePRS groups regarding the confounders (p>0.05).

Table 1: Participant characteristics, MAVAN*

Characteristics	Total (*n* = 73)	Low ePRS (*n* = 39)	High ePRS (*n* = 34)	*p* value†
Male, *n* (%)	35 (47.9)	20 (51.3)	15 (44.1)	0.54
Age at scan, yr	9.27 ± 1.46	9.35 ± 1.41	9.17 ± 1.53	0.60
Gestational age, wk	39.18 ± 1.18	39.03 ± 1.16	39.35 ± 1.20	0.24
Birth weight, g	3265 ± 476	3223 ± 429	3314 ± 527	0.42
Breastfeeding duration, mo	7.37 ± 4.88	6.96 ± 4.79	7.81 ± 5.01	0.46
Smoking during pregnancy, *n* (%)	13 (18.1)	6 (15.8)	7 (20.6)	0.60
Maternal education, university degree or above, *n* (%)	38 (52.1)	20 (51.3)	18 (52.9)	0.89
Low income, *n* (%)	11 (16.9)	5 (13.9)	6 (20.7)	0.47

ePRS = expression-based polygenic risk score; MAVAN = Maternal Adversity, Vulnerability and Neurodevelopment.
*Data are presented as *n* (%) or mean ± standard deviation. Percentages were calculated based on the available data; for smoking during pregnancy, total *n* = 72, and for low income, total *n* = 65.
†We found no significant differences between children in the low and high ePRS groups.

Table 2: Participant characteristics, replication cohort (UCI)*

Characteristics	Total (*n* = 80)	Low ePRS (*n* = 44)	High ePRS (*n* = 36)	*p* value†
Male, *n* (%)	48 (60.0)	26 (59.1)	22 (61.1)	0.86
Corrected age at scan, d	21.08 ± 14.79	21.39 ± 16.27	20.69 ± 12.95	0.84
Gestational age, wk	39.18 ± 1.49	39.15 ± 1.67	39.22 ± 1.26	0.83
Birth weight, g	3336 ± 507	3361 ± 554	3305 ± 450	0.62
Breastfeeding duration, mo	6.41 ± 5.07	5.37 ± 4.89	7.7 ± 5.09	0.07
Smoking during pregnancy, *n* (%)	6 (7.5)	3 (6.8)	3 (8.3)	0.77
Maternal education, university degree or above, *n* (%)	27 (33.8)	12 (27.2)	15 (41.6)	0.13
Household income < $29 999/yr	26 (32.5)	14 (31.8)	12 (33.3)	0.28

ePRS = expression-based polygenic risk score; UCI = University of California, Irvine.
*Data are presented as *n* (%) or mean ± standard deviation. Percentages were calculated based on the available data.
†We found no significant differences between children in the low and high ePRS groups.

Next, we sought to understand how the PFC DCC co-expression network is associated with brain volume. We found a significant positive association between the ePRS and the total brain volume (gray and white matter) among the children in MAVAN cohort (p=0.043, β=0.212; N=88), where subjects with a higher genetic score tend to have a larger brain volume (Figure 2A).

We replicated this result in the UCI community-based prospective study with neuroimaging and genotype data available (see Methods for cohort information). Similar to MAVAN, the replication analysis (N = 80) revealed a significant association between the DCC

ePRS and newborn total brain volume (gray and white matter, adjusted by intracranial volume) (Figure 2B) (β = 0.101, p = 0.048). No outliers were identified. These findings provide evidence that the DCC co-expression gene-network predicts differences in brain morphology development.

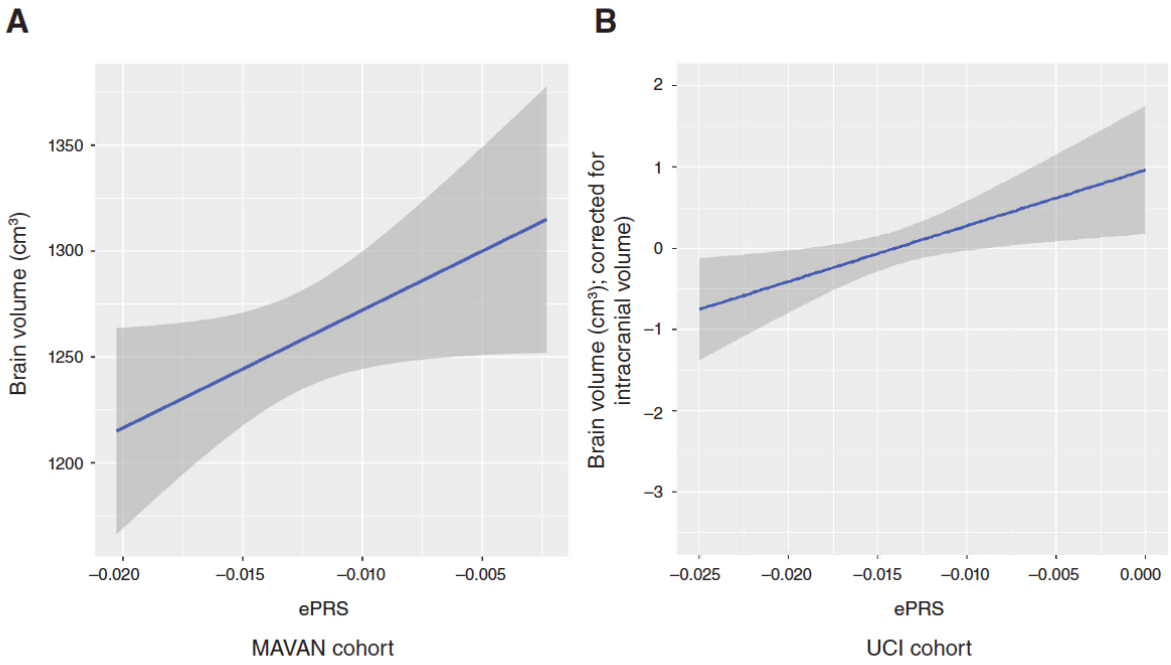


Figure 2. Morphological differences predicted by DCC expression-based polygenic risk score (ePRS). In (A) the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) cohort and (B) the University of California, Irvine (UCI) replication cohort, higher ePRS is associated with larger total brain volume.

Specificity of the ePRS

We investigated whether a conventional PRS (Wray et al., 2010; Wray et al., 2014) based on a brain volume GWAS (Jansen et al., 2019) is associated with differences in brain volume in the current cohort. We found no correlation between this polygenic risk score (for several p value thresholds) and the size of the brain volume in the MAVAN sample (Table 3). The DCC co-expression network score, is not only more biologically meaningful than the traditional polygenic risk score but it is also sensitive to predicting volumetric brain differences in the community-based samples of children.

Table 3: Conventional PRS association with total brain volume*

p value thresholds	β	*p* value	SNP count
PRS τ 0.0001	–0.113	0.31	2493
PRS τ 0.001	–0.055	0.61	6764
PRS τ 0.01	–0.184	0.12	23929
PRS τ 0.05	–0.145	0.24	65567
PRS τ 0.1	–0.189	0.13	103097
PRS τ 0.2	–0.151	0.25	161534
PRS τ 0.3	–0.144	0.28	208446
PRS τ 0.4	–0.152	0.25	248433
PRS τ 0.5	–0.156	0.24	282935

GWAS = genome-wide association studies; MAVAN = Maternal Adversity, Vulnerability and Neurodevelopment. PRS = polygenic risk score; SNP = single nucleotide polymorphism.
*Association between PRSs using the GWAS for total brain volume[63] and total brain volume in MAVAN children, with different PRS *p* value thresholds. We adjusted the regression models using the principal components of age, sex, site and population stratification.

The PFC DCC co-expression network is comprised of genes involved in mRNA translation

To examine the functional ontology of genes that comprise the DCC co-expression gene-network in the PFC, we screened for functional networks. The predominant enrichment was for the messenger RNA (mRNA) translation initiation and termination processes (Figure 3). Furthermore, gene ontology processes included cell adhesion, immune and inflammation functional networks, and Notch signaling.

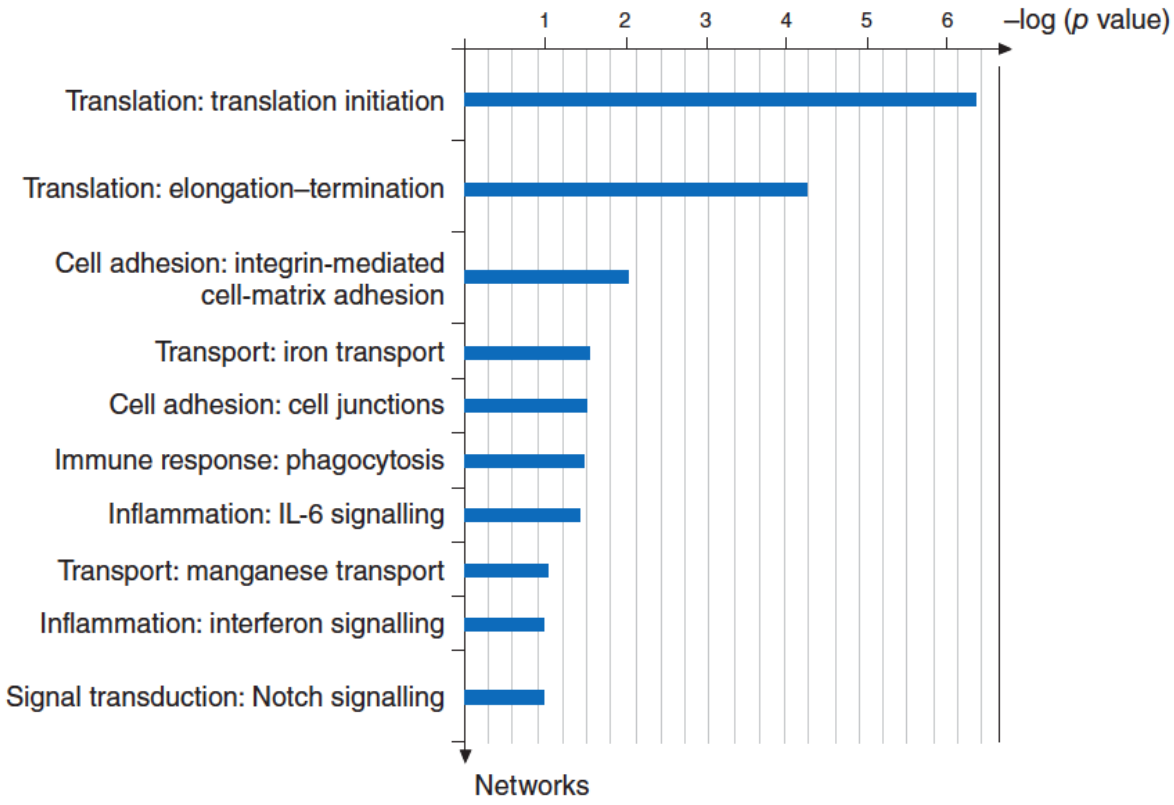


Figure 3. Using Metacore software, we analyzed the coexpression gene list for networks that depict main associations (top 10) with particular cellular processes. The y-axis shows the order of the networks by significance. IL-6 = interleukin-6.

Discussion

We generated an expression based polygenic risk score to determine whether variation in the function of the DCC gene network within the PFC predicts total brain volume in children. The PFC DCC ePRS is positively associated with brain volume in two separate community-based samples and is a stronger predictor compared to a conventionally computed PRS. Genes comprising the score are involved in cell function maintenance and their activity in the PFC during childhood, adolescence and adulthood provides a snapshot of their dynamic recruitment throughout the lifespan.

DCC receptors are implicated in the organization of long-distance axonal wiring across early life and adolescence (Livesey and Hunt 1997; Harter et al., 2010; Manitt et al, 2013; Reynolds et al., 2017). In humans, bialleic loss-of-function mutations within the DCC gene lead to broad disorganization of white-matter tracts, developmental split-brain syndrome, and

cognitive deficits (Jamuar et al., 2017; Marsh et al., 2018). DCC haploinsufficient subjects exhibit morphological and connectivity alterations, including mesocorticolimbic connectivity and volumetric alterations (Vosberg et al., 2018; Vosberg et al., 2019). DCC receptors have been shown to play a critical role in prefrontal cortex development (Reynolds et al., 2018, Hoops and Flores, 2017; Hoops et al., 2018; Manitt et al., 2013; Grant et al., 2007; Lee et al., 2019; Li et al., 2020). Our findings linking the PFC DCC co-expression network to total brain volume during normative childhood development suggests that early postnatal PFC maturation influences brain-wide development. It is also likely that similar DCC gene networks are expressed throughout brain regions, coordinating both local and overall neuronal networks.

The effect size between the PFC DCC ePRS and TBV is likely to be reflected on behavioral outcomes and vulnerability to psychiatric disorders. Brain volume is associated with intrinsic brain activity (Qing and Gong, 2016) and implicated with cognitive outcomes, including educational attainment and intelligence (Alemany et al., 2019; Cox et al., 2019). Furthermore, brain volume is associated with psychiatric conditions, including autism spectrum disorder (Nordahl et al., 2011, Sacco et al., 2015; Hazlett et al., 2017) schizophrenia (Mehler and Warnke, 2002; Steen et al., 2006), and depression (Steingard et al., 2002; Phillips et al., 2012; Pagliaccio et al., 2019). In forthcoming studies we are assessing whether the DCC ePRS differs between healthy individuals and those vulnerable to mental illness.

The biological processes modulated by genes within the DCC network include mRNA translation, cell adhesion, particle transportation, and inflammation. Based on the large clusters of genetic activity during childhood observed in the expression matrix and on the gene ontology analysis, it is likely that at this early age, the gene network regulates cellular fate and neural proliferation events. This idea is consistent with a recent analysis of genetic signatures of the human brain showing DCC among top 10% of genes that have a consistent transcriptional regulation pattern across 132 different brain structures (Hawrylycz et al., 2015). The DCC co-expression network is most likely part of a core network modulating biological processes across the entire brain that, according to our gene expression profile matrixes across different life stages, remains dynamically active into adulthood.

The different DCC network expression patterns observed in the childhood, adolescent and adult matrices suggest continuous recruitment and pruning of the network. Particularly interesting is the widespread reduction of synchronous gene expression during adolescence, which is most likely accounting for individual-based experience, and shaping the function of the network into adulthood. The importance of the changes in co-expression patterning across ages can be inferred from rodent studies showing that the function of DCC receptors varies according to maturational stage. During prenatal and early postnatal development, DCC is involved in myriad processes across the CNS, from driving migration of neural crest derived cells (Jiang et al., 2003) and participating in foetal telencephalic cortical plate development (Harter et al 2010), to guiding the growth of corticospinal tract axons (Finger et al., 2002; Furne et al., 2008) and retinal ganglion cell axons (Deiner et al., 1997). From juvenile age to adolescence, the role of DCC receptors appears to become more specialized, as they are primarily involved in controlling targeting decision of dopamine axons in limbic regions and in organizing their connectivity in local circuitries (Reynolds et al., 2018; Manitt et al., 2013, Hoops et al., 2018). In adulthood DCC receptors are involved in axonal sprouting, maintenance of already established synaptic connections, synaptic plasticity via the recruitment of nascent or immature synapses (Goldman et al., 2013, Horn et al., 2013; Glasgow et al., 2019; Manitt et al., 2009; Muramatsu et al., 2010; Yetnikoff et al 2010). The DCC co-expression network in the PFC may be involved in maintaining proliferative functions and establishing early global connections in childhood; fine-tuning network connectivity in adolescence; and maintaining local synaptic connectivity and function in adulthood.

A limiting factor in this study is the sample size (Steen et al., 2007), since MRI data collection is challenging in young subjects, the final number of processed imaging scans is small. However, the fact that our findings replicated in second sample of a community child cohort counteracts this limitation. It is important to note that only few child cohorts with neuroimaging and genotype data are available, and consistency in age between cohorts is difficult to achieve. The sample size of the gene-expression data from BrainSpan that we used to create developmental period-specific gene expression matrices is relatively small, limiting the interpretation of this result.

The use of brain volume as a phenotype has its benefits and limitations. In this study TBV allowed us to get a glimpse of global morphological differences according to the child's expression-based polygenic risk score. The population sample is that of a community cohort and thus the variation in brain volume among the individuals is expected to correspond to that observed in a healthy population. A higher association is likely to be identified in subjects with altered neurodevelopment. Using a gross measure as the TBV might mask some associations where developmental alterations are restricted to particular circuits. Additionally, our analysis does not discern between histological features, such as the ratio of neurons to glia, which can relate to variation in brain volume.

Conclusion

This study paves the path for research aimed to uncover pathways underlying brain characteristics associated with psychiatric conditions. Co-expression gene networks provide information on biological processes. DCC is an emerging hub gene associated with psychiatric illness, but the molecular pathways linking DCC dysfunction to pathological outcomes are only beginning to be elucidated. Our findings show that a network of genes co-expressed with DCC in the PFC and involved in basic cellular functions is associated with the development of brain volume and highlight the dynamic nature of the genetic contribution to brain development.

Chapter V: Discussion

Summary of main findings

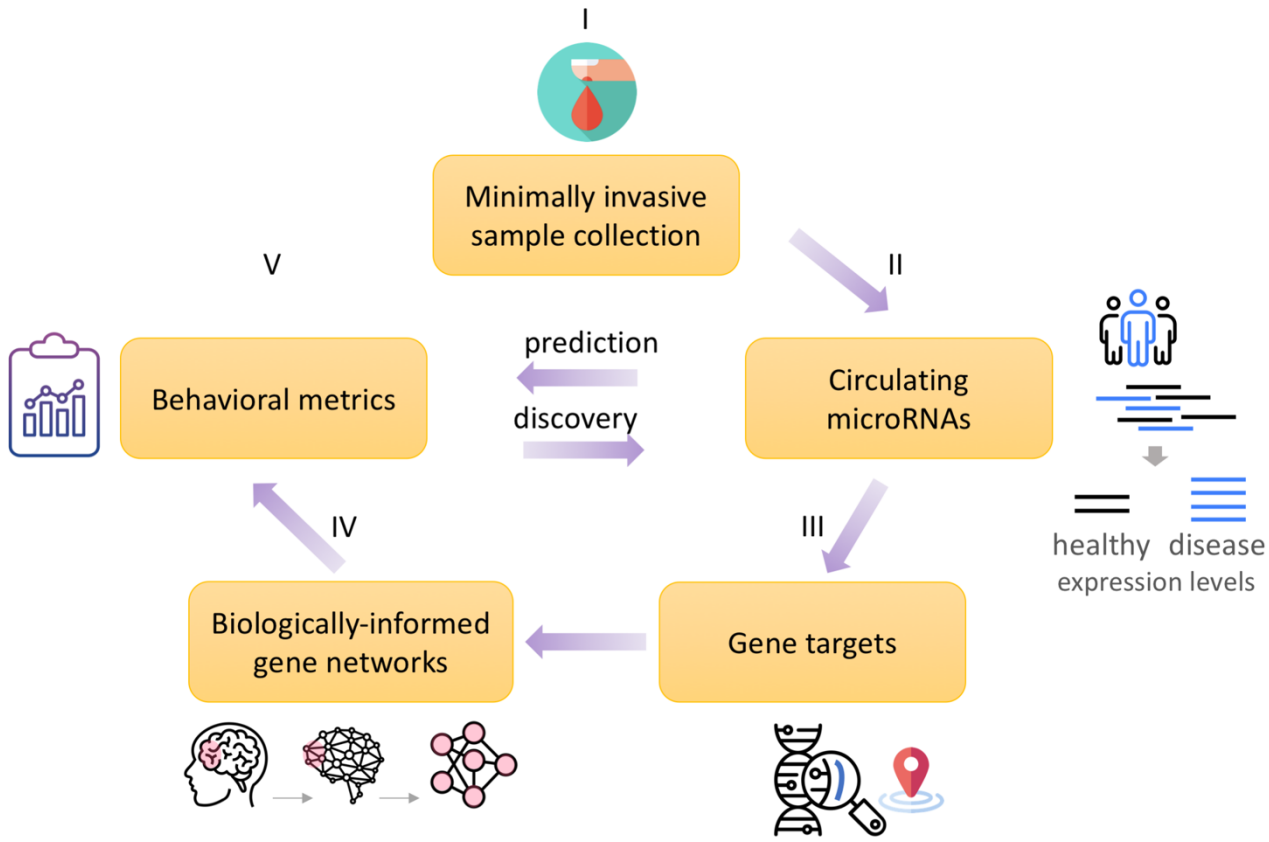
The overarching purpose of this thesis was to investigate markers that are implicated in psychiatric pathology of developmental origins. In my approach, I investigated both peripheral markers that could serve as indicators of vulnerability and gene activity within the brain. These investigations concentrated on specific temporal windows that encompass vulnerable points in neurodevelopment by employing complementary methods, each with its own strengths and significance. For one, peripheral markers serve as readily accessible metrics that reflect systemic changes and are indicative of epigenetically impacted pathways. Secondly, the study of genes as nodes of a network within the spatiotemporal context elucidates mechanisms potentially influencing early biological pathways related to cognition and mental health.

More specifically, my doctorate studies advance the scientific knowledge in three key aspects: (i) by providing a proof-of-concept methodological study advocating the feasibility of highly rigorous microRNA profiling in dried-blood spot clinical biosamples, (ii) uncovering several differentially expressed microRNAs between adolescents diagnosed with depression and healthy peers, and (iii) finding that DCC's role in healthy child development is associated with morphological outcomes. The focus on miRNAs in adolescent behavioral outcomes is part of such few studies that in a recent systemic review of the research landscape (Vázquez-Ágredos et al., 2022), researchers in Flores laboratory make up prominent cluster in the author-based network, including a little node of my own.

Collectively, I envisage my studies as a blueprint for identifying miRNAs as biomarkers of disease risk, illustrated in Figure 3. The approach begins with the collection of minimally invasive blood samples, from which miRNAs are isolated and contrasted between groups based on the diagnosis. Gene targets for these miRNAs are then predicted, and according to ontology processes of interest, biological hypothesis dictates the selection of genes for further network analysis. These networks can then be used to construct polygenic scores within the same individuals who provided the blood samples. This strategy allows for the linkage of peripheral markers to brain-specific pathways, revealing the underlying mechanisms of the disease. In the

final step, newly identified miRNA biomarkers can be validated across different populations and employed for clinical applications, such as patient stratification and risk prediction.

Figure 3. *A framework for establishing microRNAs as biomarkers of disease: I) Blood collection is minimally invasive, achieved*



through finger pricking and utilizing a dried-blood spot approach, suitable for vulnerable populations. II) These samples are then processed and prepared for small RNA sequencing to profile circulating microRNAs. Disease diagnosis is used to distinguish between healthy controls and affected individuals during the biomarker discovery phase. III) Computational tools analyze miRNAs with the most significant expression levels in the disease state, identifying potentially modulated genes and assessing their functions through gene ontology. IV) The identified ontology terms that are relevant to the disease aid in constructing gene networks. Using the participants genotypes, biologically informed polygenic scores are calculated to predict gene expression within tissue-specific contexts. V) These gene scores are employed to examine the associations between miRNA-modulated networks, symptoms and behavioral metrics, helping to map pathognomonic molecular pathways. Once molecular candidates for the disease are established, the framework can be adapted to skip directly from profiling miRNAs at a different time point or in an independent cohort (Step II) to assessing their predictive power for behavioral metrics (Step V).

Together, my findings build a strong methodological and biological knowledge foundation. In this chapter, I review takeaways for each aim, offering context while addressing limitations, discussing overarching implications, and outlining the next steps in the research.

5.1 Establishing methodological pipeline to isolate microRNAs from minimal blood volume

Starting with a scarcity of miRNA marker studies in psychiatry, my aim was to develop a workflow for profiling circulating miRNAs. Based on the expertise available in our lab, the initial idea was to focus on a select set of miRNAs identified from both our research and existing literature, followed by a qPCR analysis. As we assessed our options, it was clear that sequencing technology was more practical, and I seized the opportunity to conduct a pilot study using spare saliva and dried-blood spots (DBS) provided by Dr. Ian Gotlib and Dr. Tiffany C. Ho and to collaborate with bioinformatics scientist Dr. Nicholas O'Toole in Dr. Michael Meaney's laboratory for data analysis. We found distinct miRNA profiles between saliva and blood samples (Figure 4; unpublished), and that blood-based measure captured significantly more diverse profiles of miRNAs. For this pilot, we outsourced RNA extraction and sequence library preparation to a commercial company.

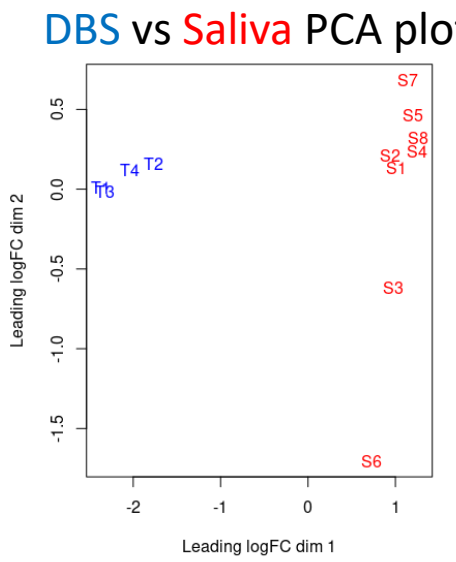


Figure 3. Transcript based principle component analysis between dried blood spot samples (blue) and saliva samples (red) over two dimensions. Clearly microRNA expression between the two biosamples is not comparable.

When proceeding with the full cohort samples, I came across a major roadblock (not just on the streets of Montreal), encountering quality control issues that the majority of the archived DBS samples posed to the standard servicing pipeline. With enormous support by Dr. Flores and invaluable guidance by Dr. Corina Nagy, which enabled me to push the work forward, I explored five RNA extraction protocols with different modifications in order to compare and maximize the yield. Using a kit with nanoscale sensitivity for single dried-blood spots, I confirmed that the issue was low recovery of ribosomal RNA upon which sample volume input quantities are normalized.

Working with Pascal Ibrahim in Drs. Gustavo Turecki and Nagy's labs, I arrived at the solution with an optimized ligation-based library preparation, which is indicated for exosomal miRNA sequencing. Honing our approach resulted in high-quality libraries and selective

transcript mapping to miRNAs, culminating in our publication in Biology Methods & Protocols. Open access to this work fosters scientific progress by encouraging ongoing refinement and improvement. Though this study may not be groundbreaking, it serves as a guide for future collaborations, especially within the Douglas Research Institute, which also operates as a psychiatric hospital and actively recruits research participants.

To introduce the main limitations of this method approach, I will discuss below the main features and considerations relating to miRNA cell source and degradation, highlighting the most recent advances in the literature.

The DBS-sourced miRNA sequencing approach offers distinct advantages, most notably the reduced need for excessive blood donations from patients. However, one primary limitation is the inability to pinpoint the cellular origin of the miRNAs. MicroRNAs are initially processed in the nucleus, exported into the cytoplasm in their precursor form, and finally matured before joining the RNA-Induced Silencing Complex (supporting Figure 2 in the Introduction). Once part of this complex, they circulate freely, are contained within extracellular vesicles, or are bound to proteins. The exact cellular composition of these circulating microRNAs—whether they originate from the nervous system, liver, or red blood cells—is still an open question. This challenge is not unique to DBS samples; many peripheral marker studies face similar issues when they do not employ cell-specific 'tags.' To address this, it may be useful to compare the transcriptional profile of DBS-derived miRNAs with those obtained from whole blood, plasma, and serum samples. Such a comparison could narrow down potential sources. Though this analysis was beyond the scope of my initial pilot study, it has been included in the larger cohort study.

Circulating miRNAs have been extensively studied in liquid biosamples like whole blood, plasma, and serum (Musazzi et al., 2023). While these samples allow for the subcategorization of miRNAs and the potential isolation of neuron-specific extracellular vesicles, they require larger blood volumes from donors and may introduce pre-analytical errors. Most miRNAs in plasma are derived from red blood cells and platelets (Patil et al 2022), limiting their specificity. Emerging methodologies, such as the field of research on vesicle-derived small RNA (Saeedi et al., 2021), aim to narrow down the source contributions of miRNAs. Yet, even with neuron-

specific markers, these techniques fall short of identifying the exact types of cells or brain regions from which miRNAs originate (Alexander et al., 2022). Exosomes can also stem from a variety of other cell types, including epithelial and endothelial cells, further complicating source identification. Therefore, while liquid-based samples offer some advantages in subcategorization, they come with their own set of challenges that make DBS-sourced miRNA sequencing a viable alternative, especially for large-scale or longitudinal studies.

Another uncertainty is whether after the collection of blood, the degradation has an impact on the miRNA levels, and if so, which ones and why. Studies in the literature using DBS samples show positive outlooks (Kahraman et al., 2017; Diener et al., 2019; Atneosen-Åsegg et al., 2019; Li et al., 2021), which bolster my confidence in the DBS, however rigorous technical analysis into the DBS specific dynamics of miRNA preservation, such as with a synthetic spike-in sequence, and comparing multiple spots within a person, are still needed. DBS is one of the oldest microsampling techniques, and there are continuous innovations into optimization and mitigation of limitations for biomedical applications that a single drop of blood can offer (Thangavelu et al., 2023). This methodology opens up novel possibilities for expanding at-home testing and child-friendly blood sampling; it enables the inclusion of a broader range of demographic variables and can work in tandem with downstream microRNA profiling (Enderle et al., 2016; Whittaker et al., 2021; Jacobson et al., 2022; Green et al., 2023; Zailani and Ho, 2023).

While our methodology emphasizes miRNA profiling, it does not include other non-coding RNA types like long RNAs or PIWI-interacting RNAs, which could also be crucial for disease understanding and can be minimally invasively measured. A study by Zhou et al. (2019) used serum droplets to profile extracellular RNA, bypassing RNA extraction to prevent material loss and proceeding directly to cDNA synthesis in cell lysis solution. A specific limitation of broad RNA profiling is the potential for length-based bias in sequences, as both library preparation and sequencing platforms tend to favor fragments of certain sizes. This could affect both sensitivity and specificity of the method. They also find that out of all profiled transcripts, miRNAs were particularly effective in differentiating disease outcomes, which for us reinforces the rationale for a targeted approach.

Together, while the DBS approach does have limitations, it's important to note that many of these challenges are common to all methods focusing on circulating miRNAs, which is a relatively young field.

Our study contributes valuable insights for planning and implementing future microRNA profiling research. This includes addressing challenges such as microRNA detection, quantification, sample integrity, and cost-effectiveness in early diagnosis. The method's minimal blood requirement makes it applicable to diverse populations, including infants and the elderly, without necessitating elaborate laboratory equipment. To mitigate the limitations of our targeted approach, the minimal sample input allows for the utilization of remaining DBS card samples to analyze other regulatory transcripts. This can be complemented by DNA methylation or genotyping studies, offering a comprehensive biological and genetic profile. The standardization of our protocol ensures high-quality isolation of microRNAs from DBS samples and profiling on a single nucleotide basis, paving the way for broader applications in peripheral marker studies.

5.2 Circulating microRNAs as markers of vulnerability to depression in adolescents

After successfully establishing a pipeline for isolating and measuring miRNA expression, I leveraged this methodology to explore its utility in identifying psychiatric pathologies. In the subsequent section, I discuss some of the key findings to date, provide an overview of ongoing research, and outline plans for future studies.

The miRNA profile comparison between adolescents diagnosed with depression and healthy controls in the TIGER cohort led me to identify several key microRNA markers. Curiously, all DE miRNAs were upregulated. To begin elucidating the pathways potentially impacted by this aberrant expression, I employed publicly available databases and bioinformatics tools. I first explored where these miRNAs can be localized in the brain, to focus on the cognitive context of these miRNAs. Interestingly, miR-205-5p, which has been previously identified in studies of peripheral markers for depression (Zhang et al., 2018), is highly expressed in the hippocampus (Chapter IV; Figure 3B), as well as grey matter and pituitary gland (not shown). Existing literature reveals that miR-205 is implicated in preventing defects

related to neurite outgrowth, particularly in the context of Parkinson's disease. Notably, it also shows differential expression in rodent brain across the lifespan (Cho et al., 2013) and exhibits sex-related differences during neurodevelopment (Szakats et al., 2023). Thus, with the supporting lines of evidence, miR-205 is a promising candidate marker for adolescent depression, and future work should assess its role in sex differences in MDD vulnerability.

The distinct expression patterns of the top DE miRNAs across various brain regions can be an indication of dynamic temporal modulation and specificity in biological pathways associated with different behavioral outcomes. For instance, miR-942 and miR-3613, both of which show high expression in the substantia nigra, also both selectively predict future vulnerability to self-perceived depression severity, but not anxiety. As the substantia nigra plays a significant role in dopaminergic circuits, future research should investigate the involvement of these miRNAs in the combined association of reward circuitry, such as the experience of loss of pleasure, and MDD (Bragulat et al., 2007; Strigaris et al., 2015). The nine DE miRNA signatures we detected do not necessarily represent the same aberrant systems, but rather the approach of profiling miRNAs in blood allows us to glimpse into systemic changes. Further investigation can then elucidate the specific roles of these miRNAs in the context of psychiatric pathology.

To elucidate the functional roles of the identified differentially expressed miRNAs, we employed computational tools for gene ontology term search and enrichment analysis, thereby mapping these miRNAs to relevant genes, pathways, and networks (Villoslada and Baranzini, 2012; Rao et al., 2013). It is worth noting that existing literature on miRNAs predominantly focuses on their oncogenic roles, which complicates the interpretation of miRNA function in other contexts. To refine my analysis, I selected gene targets that were shared by at least two of the top DE miRNAs. This stringent criterion strengthened the relevance of these miRNAs to functions related to the brain. The functional connection to glutamatergic synapse activity that we detected based on the gene ontology analysis (Chapter III, Table 3) is interesting in light of the TIGER study particularly, as one of the main aims of the cohort study is to investigate the anterior cingulate cortex (ACC) and glutamate metabolism in association to morphology related alterations in adolescent depression. The most recent finding suggests that peripheral pro-inflammatory markers were positively associated with glutamate in ACC, the relationship that

may be driving glutamatergic excitotoxicity induced developmental and functional alterations of the ACC, such as changes in affective functioning and cognitive control (Ho et al., 2021). In an ongoing analysis, we are looking to assess the potential association of the top DE miRNAs with morphological alterations seen in the developing brain and in psychopathology. Ultimately, the identification of these gene targets lays the groundwork for constructing polygenic network scores, bridging the gap between peripheral markers, brain-specific gene networks, and associated behavioral outcomes to deepen our understanding of disease etiology.

Beyond the current cohort, I have also begun profiling circulating microRNAs in children (N=170) which were selected to represent the spectrum of self-reported low, middle, and high scores on child depressive inventory. Although the children in this cohort do not have the clinical diagnosis of depression, I was interested in understanding 1) microRNAs that consistently associate with high depressive symptoms throughout development (childhood and adolescence) and 2) physiological profiling that may indicate the vulnerability prior to the surge in adolescent symptoms. My preliminary findings reveal a microRNA that surpasses the false discovery threshold when comparing children that scored low versus high on the CDI. This microRNA shows upregulation in both the community-based cohort and the TIGER diagnostic comparison. Thus, a microRNA that consistently associates with depressive symptoms in childhood and adolescence may be a marker of risk across development.

An additional valuable contribution of this work is the creation of the sequencing database, with fully disclosed methodology and raw data, which will be made open access to facilitate collaboration and further discovery. In addition to the split in microRNA trajectories (Beveridge et al., 2014), another interesting transcriptional event in the brain revealed to coincide with the time of adolescence suggests miRNA sequences undergo trimming in connection with brain maturation, which can only be detected via sequencing (Thomas et al., 2023). Our data set would make an important future direction of the research as it relates to human adolescence, but also for elucidating miRNA sequence alterations in association with psychiatric disorders. The possibilities of future investigations also include: miRNA-miRNA networks (Hill and Tran, 2022), isomiR (miRNA variants; Gómez-Martín et al., 2023), and exogenous miRNAs. In connection to inflammation and the innate immune system, an

interesting aspect of miRNAs is that they are used by viruses to interfere with host DNA (Cullen et al., 2009). The interplay between depression-induced immune dysfunction and viral exposure (Coughlin, 2012; Blank et al., 2016; Zhang et al., 2022; Yu et al., 2023) is a prospective study for miRNA research. Many sequencing mapping pipelines, such as the exeRcpt, allow for endogenous and exogenous small RNA mapping, hence this would be another novel approach to studying MDD and utilizing our dataset. The potential to assess for interactions across different layers of a biological system is vast with peripheral miRNA samples (Rao et al., 2013).

In terms of the limitations, given that MDD is observed on the global scale, validation and generalizability is critical in independent and populations of diverse backgrounds (Kieling et al., 2011; Zajkowska et al., 2021; Tan et al., 2022).

By capturing a snapshot of molecular states through circulating miRNAs, our study provides a glimpse into the molecular mechanisms underlying adolescent development and offers a promising route for early depression risk assessment. Utilizing dried blood spots as a biosampling technique ensures minimal invasiveness and allows for seamless clinical integration, aiding in early disease prediction and monitoring its progression. These methodological advantages, combined with our findings, have significant implications for understanding the epigenetic fingerprint and underlying biological mechanisms specific to adolescent-onset MDD.

5.3 From gene candidates to molecular processes in the PFC and global brain morphology

MicroRNAs modulate post-transcriptional landscape and exert regulatory function on genes to modulate biological processes. Thus, in addition to studying circulating miRNAs as systemic markers of illness, it is equally important to understand the developmental molecular mechanisms occurring directly in the brain. I was particularly interested in the DCC gene, which prior to my study was shown to be critical in early life development and to be altered in psychiatric conditions, with remaining questions as to how DCC may be implicated in early developmental trajectories that may potentially lead to cognitive deficits.

As in profiling systemic microRNAs, I was interested in investigating DCC within the context of biological networks during the ongoing neurodevelopmental period. At the time, I

had access to two community-based child cohorts with genotype and neuroimaging data, and I took the opportunity to assess the potential impact of the DCC co-expression network in the PFC in brain development of children. Parallel findings in the literature based on DCC mutation carriers showed that there are structural alterations including in long distance tracts between the brain and the spine (Welniarz et al., 2017), the corpus callosum connecting the two brain hemispheres (Marsh et al., 2017), and reduced anatomical connectivity and regional volume in mesocorticolimbic circuitry (Vosberg et al., 2018).

Implementing the methodological approach established by Dr. Patricia Pelufo Silveira, we built an expression based polygenic score (ePRS) of the DCC network in the brain and measured morphological changes that may be detected in children. To create the ePRS we identified a network of genes based on the DCC expression, specific to the prefrontal cortex, and inferred the gene activity by relying on the genotype of the community-based child cohort and gene databases. It was intriguing that the DCC ePRS associated with the global phenotype of brain volume, further reinforcing the DCC role in organizing and regulating white matter projections and also child's healthy development. It also appears that these differences persisted from early life as shown by the replication in newborns.

Serving as the foundational exploration, many more questions arise about healthy neurodevelopmental trajectories, such as which fiber projections drive the global change and how this ePRS related to adolescent morphological outcomes. These future studies would explore additional metrics acquired by using diffusion tensor imaging for white matter tract tracing, functional MRI for functional connectivity and behavioral measures (e.g., the Patient Health Questionnaire and tests measuring cognitive function highly associated with PFC function). As these cohorts precede the period of adolescence, the psychiatric presentations to emerge at this age are more likely to be related to attention deficit hyperactivity disorder (ADHD), rather than depression. In my limited analysis, I saw a glimpse of behavioral correlates in respect to inhibitory control a measured by Cambridge Neuropsychological Test Automated Battery at 72 months of age (Figure 5; high DCC expression group with longer SSRT, p=0.034,

F=4.55, unpublished). Indeed, a subsequent study in our lab has corticolimbic DCC ePRS score to impulsivity and to predict ADHD outcomes (Restrepo-Lozano et al., 2022).

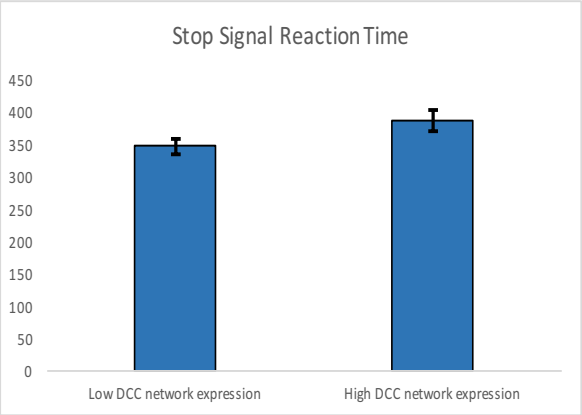


Figure 4. MAVAN cohort stop signal reaction time (ms) as a measure of inhibitory control, with lower DCC ePRS showing shorter time taken to inhibit or stop a prepotent response.

It is evident that ePRS studies should not be confined to community-based cohorts alone. Extending this approach to clinically-based cohorts will help investigate associations with the onset of depression symptoms and assess the significance of this network in psychiatric vulnerabilities. These studies will be imperative to begin understanding the impact of modulatory genes, as it offers a glimpse into evolving neurodevelopment. Regional brain abnormalities have been linked to adolescent depression, and the insight into these childhood trajectories are likely preceding or setting the stage for future maladaptive vulnerabilities. The disarray in adolescence observed in the gene expression that composed the ePRS (Chapter IV, Figure 1) is interesting as it echoes the global observations at this time in miRNA expression (Beveridge et al., 2014; Thomas et al., 2023). Interestingly, via miRNA-expression quantitative trait loci analysis, a single miRNA was recently found to associate with outcomes related to neocortical brain development, brain size and educational attainment (Lafferty et al., 2023).

There is an imperative need to understand evolving biological mechanisms underlying mental illness vulnerability to inform disease detection strategies. The ePRS approach is particularly strong in advancing our understanding of candidate genes within their spatiotemporal biological context and in extending insights gained from mechanistic studies of these candidate genes.

5.4 Limitations and continuation of work

My studies intertwine major research fields: depression disorder, adolescence and microRNAs. While innovative, the study has limitations, as it cannot cover all relevant factors in

these domains simultaneously. Future work should consider additional variables like the interplay between MDD and inflammation, hormonal changes in adolescence, and sex differences, as well as the cellular origins and isoforms of microRNAs.

At the early stages of establishing potential markers of risk, our research necessitates separating individuals into affected or healthy. This binary classification may simplify complex conditions like MDD and overlook subtle markers related to varied symptoms. Moreover, the study's focus on adolescence could miss other influential factors such as ethnicity, early life stress, and socioeconomic status. In TIGER, the demographic variables did not appear to strongly associate with any of the outcomes, and therefore were not included in the association models. However in a larger sample size this is unlikely to be the case and subgroups are likely to emerge in relation to the demographic factors. Our preliminary unpublished research is finding sexual dimorphisms in miRNA expression in rodent prefrontal cortex, hinting at the need to consider these variables in future investigations.

Future work that would tie chapters III and IV of my thesis together would be with the inclusion of the ePRS approach (in chapter V) through genotyping of the leftover DBS of the TIGER cohort. We have begun the process, already obtaining the approval from the ethics committee overseeing the main TIGER cohort study. The basis for the network construction would be the genes identified as targets of the top DE miRNAs. Similar to the DCC ePRS study, co-expression based networks would be inferred in the brains of adolescents, and allow us to trace back from the peripheral markers to the molecular pathways in the brain within the same individual in association with developmental and psychiatric outcomes. The limitation of indirect inference of brain activity would remain, but is the best proxy we can attain with current computational approaches.

Childhood psychiatric disorders also exist and merit a focus on their own. Childhood disorders likely have a different etiology compared to those that emerge in adolescence. My thesis encompasses participants aged 8 (with DCC ePRS replication done in newborns) to 19 (TIGER cohort) years old, with the younger participants being studied from the perspective of the longitudinal build up to adolescence. A plethora of studies have shown the link between individual trajectories from childhood to adolescence and adulthood (Luby et al., 2017; Luby et

al., 2019), however longitudinal investigations are best equipped to delineate the molecular dynamics that are occurring over time.

Lastly, in the interest of adolescence and development, brain maturation is affected by both positive and negative environmental exposures (Nelson and Gabard-Durnam, 2020). It is therefore equally important to study biological mechanisms underlying resilience. Resilience is an active process to the demands in the environment (Russo et al., 2012) and is deserving of equal investigation. One study reports that mechanisms underlying resilience continue to persist even during psychopathology and improve depressive symptoms over time (Wiglesworth et al., 2023). In the TIGER cohort, which was designed to follow up individuals across three longitudinal points, some individuals exhibited improvement in their symptoms. However, the small sample size hinders an adequate assessment of miRNA associated with improvement outcomes.

The study of biomarkers is an emerging field, and miRNAs are the focus of our studies for being abundant, readily measurable, and biologically important. However, the association between the miRNA markers and the clinical outcomes is just the first step in the long journey of arriving at a clinically applicable candidate (Holland, 2016). Subsequent efforts must validate the identified associations with clinically relevant metrics, which is a challenge in psychiatry (Belzeaux et al., 2017), however, it is essential to replicate and justify that the addition of these markers will result in a meaningful impact on patient care (Holland, 2016).

Finally, ethical and social considerations surrounding the use of biomarkers are valuable aspects to weigh in this line of translational research (Singh and Rose 2009; Furrer et al., 2023).

5.5 Conclusions

The pervasive impact of major depressive disorder on both individuals and society underscores the pressing need for innovative strategies to enable early prediction and deepen our understanding of the disease. Adolescents are a high-risk group for the development of depression, however our understanding of the biological trajectories that underly this vulnerability is rudimentary. This research addresses existing gaps by combining minimally invasive sampling with robust microRNA measurements, uncovering novel molecular links that enhance our understanding of circulating markers and developmental pathways associated with the risk of adolescent-onset depression. My research establishes a blueprint for bidirectional inquiry: from identifying molecular candidates based on current diagnosis and their potential mechanisms within the brains of living individuals to employing these tiny clues to predict disease outcomes and specific symptoms in at-risk patients. While important questions remain regarding the generalizability across different populations, source validation and mechanistic etiologies, the wholistic approach is essential for advancing psychiatric research. As we amass more evidence on disease identification, the ultimate objective is to facilitate personalized healthcare through easily measurable objective metrics, thereby improving both patient care and broader societal well-being.

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