Insulin Modulates the Effects of Early Life Adversity on Executive Functions Skills

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Abstract

While the co-morbidity between metabolic and psychiatric disorders is well-established, the mechanisms are poorly understood, and exposure to early life adversity (ELA) is a common developmental risk factor. ELA is associated with altered insulin sensitivity and poor behavioral inhibition throughout life, which seems to contribute to the development of metabolic and psychiatric disturbances in the long term. Here, we demonstrate that individual differences in insulin functioning interact with ELA to modulate mesocorticolimbic dopaminergic-related executive functioning behavior in children and adults. First, we showed that the genetic background linked to fasting insulin (FI) interacts with postnatal adversity to predict impulsivity in childhood, which is a DA-related behavior. Second, since impulsivity is an endophenotype linked to the development of substance use disorders (SUDs), we also demonstrated that the same FI genetic background interacted with adversity to predict SUD and addiction related behaviors in adulthood. Third, using the sex-specific FI genome wide association studies, we established that the FI genetic background interacts with ELA to predict executive function psychopathology throughout development in a sex-specific manner. Furthermore, using mendelian randomization, we were able to establish a causal association between FI and impulsive behavior in females exposed to adversity. Lastly, we performed an exploratory mechanistic study in rodents to investigate the effect of early adversity and insulin on dopaminergic axonal growth pathways. Since the Netrin-1 guidance cue system participates in the maturation of the dopamine circuitry via its interaction with its DCC receptor, we showed that insulin action on miR-218, which is a negative DCC regulator, could be mediating the metabolic effects of early adversity on DCC expression in the

medial prefrontal cortex of male rodents, which could potentially explain the resulting altered executive functioning behaviors. This work may contribute to the early identification of vulnerability to chronic adult diseases associated with early adversity, as well as inform on the mechanisms involved.

Résumé

La comorbidité entre les troubles métaboliques et psychiatriques est bien établie, mais les mécanismes sont peu compris. L'exposition à l'adversité au début de la vie est un facteur de risque développemental commun aux troubles métaboliques et psychiatriques. L'adversité précoce est associée à une altération de la sensibilité à l'insuline et à une faible inhibition comportementale tout au long de la vie, ce qui semble contribuer au développement des troubles métaboliques et psychiatriques à long terme. Ici, nous démontrons que le fonctionnement de l'insuline interagit avec l'adversité précoce pour moduler le comportement des fonctions exécutives liées au système dopaminergique mésocorticolimbique chez les enfants. Premièrement, nous avons montré que le fond génétique lié à l'insuline à jeun (IF) interagit avec l'adversité postnatale pour prédire l'impulsivité durant l'enfance, qui est un comportement lié au systeme dopaminergique. Deuxièmement, comme l'impulsivité est un endophénotype lié au développement de troubles liés à l'utilisation de substances, nous avons également démontré que le même bagage génétique lié à l'IF interagit avec l'adversité pour prédire les comportements liés à l'utilisation de substances et aux dépendances à l'âge adulte. Troisièmement, en utilisant les études d'association pangénomiques FI spécifiques au sexe, nous avons établi que le fond génétique FI interagit avec l'adversité précoce pour prédire les troubles des fonctions exécutives tout au long du développement d'une manière spécifique au sexe. De plus, en utilisant la randomisation mendélienne, nous avons pu établir une association causale entre FI et le comportement impulsif chez les femelles exposées à l'adversité précoce. Enfin, nous avons réalisé une étude chez les rongeurs afin d'étudier les mécanismes liés à l'adversité précoce et de l'insuline sur les voies de croissance

axonale des neurones dopaminergiques. La nétrine-1, un facteur qui aide a guider la croissance de l’axone des neurones, participe à la maturation des projections dopaminergiques via son interaction avec son récepteur DCC (deleted in colorectal cancer). Nous avons demontré que l'action de l'insuline sur miR-218, qui est un régulateur DCC négatif, pourrait médier les effets métaboliques de l'adversité précoce sur l'expression du DCC dans le cortex préfrontal médian chez les rongeurs males. Cela pourrait éventuellement expliquer les comportements de fonctionnement exécutif altérés qui en résultent dans les deux sexes. Ces travaux pourraient contribuer à l'identification précoce de la vulnérabilité aux maladies chroniques chez l'adulte associées à l'adversité précoce, ainsi qu'à informer sur les mécanismes impliqué.

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Contributions to original knowledge

The first part of this thesis work that contributes to original knowledge is the refined polygenic risk score (rPRS) method [1], as well as the resulting refined score as a putative biomarker of childhood impulsivity. The rPRS method allows for a refinement of the original polygenic risk score where the process leads to the selection of SNPs from a GWAS constructed in adults to a collection of SNPs relevant for children for the same trait. For our specific study, we used this for the fasting insulin (FI) GWAS. We start by using 100 different thresholds between p value 0 and 1 from the FI GWAS and found the threshold that best predicts insulin levels in ALSPAC Children (PRS Threshold 0.24). Then, we ran a correlation for each SNP within the 0.24 PRS threshold to find which SNPs were significantly predicting (p less than or equal to 0.05) the peripheral insulin levels. Lastly, using the SNPs that significantly predicted peripheral insulin levels from step 2, we calculated the rPRS in our test cohort, the MAVAN cohort. This method allowed us to identify the genetic background associated with FI in children even though the GWAS was performed in adults and there is no existing FI GWAS for children. The refined score for high fasting insulin was associated with childhood impulsivity in the presence of adversity, as shown in our first study [1]. Because this genetic score represents risk for high FI in children and is associated with childhood impulsivity, the refined score can be viewed as a putative biomarker of childhood impulsivity. As such, the score can be used for further analyses involving the cross section of metabolic and executive function disorders.

As part of this thesis work, we created a sex specific Impulsivity GWAS. For the purpose of our analyses, we wanted to investigate the possible shared genetic

architecture of FI and impulsivity and investigate the interaction effect of fasting insulin and adversity on impulsivity. Because there is no impulsivity GWAS in literature, we performed a sex-specific GWAS for impulsivity using the UK Biobank data. Genotyping data in the UKB cohort was available for 487,409 subjects. We restricted our analysis only to the participants who identified themselves as “Caucasians” so it would be comparable to the FI GWAS we used. The impulsivity phenotype was defined using Mean time to correctly identify matches (ID 20023), obtained at initial assessment visit (2006-2010) during a reaction time test based on the card-game “Snap”. This GWAS provides sex-specific genetic information on impulsive behavior that will further executive function research.

Another contribution to original knowledge is the interaction model we used for two-sample mendelian randomization (MR). Because two-sample MR requires an exposure GWAS and an outcome GWAS, it is traditionally only used for main effects. Since we wanted to inspect the interaction effect of early life adversity and fasting insulin on impulsive behavior, we created two GWASs using data from the UK Biobank where 1) individuals exhibited impulsivity and had exposure to adversity and 2) individuals exhibited impulsivity and did not have exposure to adversity. Then we ran two separate MR analysis between the FI GWAS and the two GWASs we created in the UKB. By comparing the results between the MR analyses, we were able to investigate the interaction effect. To the best of our knowledge, this model has not been used before to inspect gene by environment interaction through MR.

To the best of our knowledge, this thesis work is the first to show that there is a significant interaction effect of high FI rPRS and with postnatal adversity on impulsivity in

children [1] and adulthood addiction. Additionally, there is a significant interaction effect of high FI rPRS and adversity, prenatally and postnatally, on several executive function traits, throughout development in males and females separately. A significant association was established through MR, in which fasting insulin levels were associated with impulsivity in females exposed to adversity, implying causality as per MR assumptions. In HEK293 cells, we suggest that insulin modulates miR-218 levels which is a negative DCC regulator. Lastly, in male rodents, we showed that prenatal adversity leads to changes in Netrin-1 protein levels in newborns and DCC protein levels respond to insulin in adulthood.

Contribution of authors

This thesis was written by me with editing from Dr. Patricia Silveira. Details on manuscript chapters can be found below.

Study 1: Early life adversity, fasting insulin, and childhood impulsivity (Chapter III)

The published manuscript was written by me with editing from Lawrence Chen, Dr. Carine Parent, Irina Pokhvisneva, and Dr. Patricia Silveira. Statistical analyses were supervised by Dr. Patricia Silveira and Irina Pokhvisneva and conducted by Zihan Wang, Sachin Patel, Lawrence Chen, and me. The entire project was planned and supported with Dr. Patricia Silveira, Dr. Robert D. Levitan, and Dr. Michael Meaney.

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Study 4: Early life adversity, insulin, and netrin-1/DCC in the PFC DA pathway (Chapter VI)

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List of abbreviations

Abbreviation	Full Form
ABCD	Adolescent Brain Cognitive Development
AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
ADHD	Attention deficit hyperactivity disorder
AdLib	Ad libitum
ALSPAC	Avon Longitudinal Study of Parents and Children
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BA	Brodmann area
BDI	Beck depression inventory
BPM	Brief problem monitor
cAMP	Cyclic adenosine monophosphate
CANTAB	Cambridge neurophysiological test automated battery
CBCL	Child behavior checklist
CNS	Central nervous system
condFDR	Conditional false discovery rate
conjFDR	Conjunctional false discovery rate
DA	Dopamine
DAT	Dopamine transporter
Dcc	Deleted in colorectal cancer mRNA
DCC	Deleted in colorectal cancer protein
DDC	Dopa decarboxylase
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
DRD5	Dopamine receptor D5
DSM4	Diagnostic and Statistical manual of Mental Disorders 4th Edition
ELA	Early life adversity
EF	Executive function
EPDS	Edinburgh postnatal depression scale

ePRS	Expression based polygenic risk score
FBS	Fetal bovine serum
FDR	False discovery rate
FI	Fasting insulin
FR	Food restriction
GABA	γ-aminobutyric
GEE	Generalized estimating equations
GEO	Gene expression omnibus
GLUT	Glucose transporter
GUSTO	Growing Up in Singapore Towards healthy Outcomes
GWAS	Genome wide association study
HbS	Hemoglobin S
HEK	Human embryonic kidney
HOMA-IR	Homeostatic model assessment for insulin resistance
HPA	Hypothalamic pituitary adrenal
HRC	Haplotype reference consortium
HTR2A	5-Hydroxytryptamine receptor 2A
HWE	Hardy-Weinberg equilibrium
IED	Intra and extradimensional shift
IGF	Insulin-like growth factor
iPSYCH	Lundbeck Foundation Initiative for Integrative Psychiatric Research
IR	Insulin receptor
IRS	Insulin receptor substrate
IST	Information sampling task
IUGR	Intrauterine growth restriction
IVW	Inverse-variance weighting
LD	Linkage disequilibrium
LTD	Long-term depression
LTP	Long-term potentiation
MAF	Minor allele frequency
MAVAN	Maternal Adversity, Vulnerability and Neurodevelopment

MAOB	Monoamine oxidase B
MGD	Mouse Genome Database
miR	Micro ribonucleic acid
MR	Mendelian randomization
MR-PRESSO	Mendelian randomization pleiotropy residual sum and outlier
mRNA	Messenger ribonucleic acid
NAcc	Nucleus accumbens
NMDA	N-methyl-D-aspartate
NIH	National Institute of Health
Ntn-1	*Netrin-1* mRNA
PA	Proportion affected
PC	Principal component
PDGFR	Platelet-deprived growth factor receptors
PET	Positron emission tomography
PFC	Prefrontal cortex
PGC	Psychiatric Genomics Consortium
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PheWAS	Phenotype wide association study
PoI	Proportion of interaction
PND	Postnatal day
PPVT	Peabody picture vocabulary test
PRS	Polygenic risk score
QC	Quality control
qPCR	Quantitative polymerase chain reaction
Q-Q	Quantile-quantile
RGD	Rat Genome Database
RoS	Region of significance
rPRS	Refined polygenic risk score
RT PCR	Real-time polymerase chain reaction
SAGE	Study of Addiction, Genetics, and Environment

SD	Standard deviation
SDQ	Strengths and difficulties questionnaire
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
SSAGA	Semi-Structured Assessment for the Genetics of Alcoholism
SST	Stop signal task
STAI	State-trait anxiety inventory
SUD	Substance use disorders
TH	Tyrosine hydroxylase
UKB	UK Biobank
VIF	Variance inflation factor
VTA	Ventral tegmental area
VMAT2	Vesicular monoamine transporter 2

Chapter I. Introduction

Environmental conditions present during early developmental stages have a dramatic influence on the health and disease patterns of an individual over the course of their life [2-9]. These changes induced by early life adversity (ELA) have been commonly linked to alterations in executive functions [10-13], contributing to the development of both physical [14, 15] and mental health issues [16-20] in the long term. These effects occur through direct signaling on brain receptors, modifying genomic and non-genomic processes during development, and defining both the behavioral phenotype as well as influencing disease risk over the life course [21]. Insulin merits special attention as studies have indicated that insulin acting on neuronal receptors regulates synaptic plasticity and monoaminergic neurotransmission [22, 23]. Literature also shows that poor growth during fetal development, resulting from prenatal adversity, impairs pancreatic beta-cell development [24] hampering insulin secretion in the periphery and subsequent central insulin levels, given that the majority of insulin in the brain comes from the periphery. Specifically in dopaminergic neurons, insulin modulates dopamine (DA) related executive functions such as response to reward, impulsivity, decision-making, and cognition [25]. In this thesis, we will focus on exploring the link between early adversity, individual differences in insulin function, and DA related executive functions.

Rationale and Objective

Our main objective through the following studies was to investigate how variations in insulin function modulate the effects of early life adversity on executive functions. To do so, we performed the following studies with their corresponding rationale.

The thesis will begin with a comprehensive review of the literature where we will outline the effects of insulin signaling in the brain, which are different from insulin's well-known role in the periphery. This comprehensive review outlines the effects of insulin on several central neurotransmission systems and brain regions, as well as the comparison and relationship between insulin function and glucocorticoid programing. While insulin's role in the VTA and striatum has been established within the literature when it comes to DA neurotransmission, insulin's role in the prefrontal cortex (PFC) is still being explored. The review below will focus on insulin's established role in the nucleus accumbens and VTA in the context of DA neurotransmission and this section will inform about the research that has been completed on insulin's role in the PFC in the same context. The PFC is a region with high insulin receptor expression and insulin sensitivity in the region is associated with altered synaptic plasticity [26]. Intranasal insulin enhances functional connectivity to parts of the PFC when examined through human imaging studies [27]. Additionally, gene expression studies in post-mortem brain tissue have linked insulin-signaling expression genes with dopamine-expression genes in the PFC [27]. Current recording of a single cell in a slice from the pyramidal layers of the PFC previously incubated with 20 nM of insulin showed an increase in tonic current [28]. These findings associate insulin signaling with altered PFC activity, especially in the area where DA neurotransmission takes place. Research on insulin's impact on DA signaling have largely focused on individuals exposed to adversity or metabolic disorders as these conditions are associated with altered insulin signaling. Obese individuals experiencing insulin resistance show lower expression of PFC DA expressed genes [29] marking insulin as a key player in cognitive functions related to DA. Our lab has also previously shown that

prenatal adversity, through a model of food restriction, causes changes in DA signaling in the medial PFC of rodents in response to a stimuli [30]. This altered dopamine transmission was accompanied with impulsive behavior suggesting the role between prenatal stress and altered executive functioning. The literature review will explain the effects of early life adversity on executive functions in greater detail in addition to expanding on the effects of early life adversity on insulin signaling. Considering that insulin signaling impacts DA neurotransmission and that adversity affects both executive functions and insulin signaling, it is important to inspect whether insulin is mediating the relationship between early life adversity and DA-related executive functions.

Early life adversity, fasting insulin, and childhood impulsivity

Considering that the genetic background represents individual variation in biological processes, our first objective was to develop a model predicting the executive function behavior impulsivity in relation to biological/genetic markers of elevated fasting insulin levels and a history of early life adversity exposure in humans. We chose impulsivity as our primary executive function outcome because, as mentioned above, previous lab work in rodents exposed to adversity exhibited impulsive behavior [30] and we wanted to investigate if these observations from model organisms could be reflected in humans. In this study, we assessed the relationship between a polygenic risk score (PRS) for high fasting insulin levels and the actual measured insulin levels in a child discovery sample (ALSPAC), and used the most highly associated score to predict impulsive behavior in response to early adversity in the target sample MAVAN [1].

We focused on the fasting insulin genome wide association study (GWAS) from the Meta-Analyses of Glucose and Insulin-related traits Consortium [31, 32]. A GWAS is

an experimental design that is used to detect associations between the genetic markers represented by single nucleotide polymorphisms (SNP) and a phenotype in samples from human populations [33]. SNPs are single base-pair variations in the DNA sequence which arise in high frequency in the genome [34]. SNPs are relevant in these studies because they can have functional impact by causing amino acid changes, changes to mRNA transcript stability, chromatin modifications and changes to transcription factor binding affinity [35]. GWASs allows for a better understanding of the genetics associated biology, through SNPs, behind a phenotype. GWASs consider linkage disequilibrium (LD) which is a property of SNPs explaining the degree to which an allele of one SNP is correlated with an allele of another SNP within a population [36]. Repeated random recombination events of alleles over generations break apart segments of contiguous chromosome until all the alleles are in LD or are independent. This linkage between genetic markers on a population scale is known as linkage disequilibrium. GWASs are designed to identify the SNPs present at a significantly higher frequency in individuals with the phenotype when compared to individuals without the phenotype, after accounting for LD [37]. It is important to keep in mind that an association between a genetic variant, such as a SNP, on a chromosome and a trait does not imply causation or explain the mechanism behind the association. However, establishing an association is the first step to discovering the underlying mechanism.

GWASs have shown that while certain diseases are monogenic, several phenotypes and diseases, such as fasting insulin, are polygenic and therefore, multiple genetic variants act in conjunction resulting in the phenotype. To account for the polygenic nature of the fasting insulin trait, we create the polygenic risk score (PRS). The PRS is

calculated using only the SNPs most associated with the trait of interest, identified through the GWAS, depending on the level of significance assigned. Details of which SNPs were included from the FI GWAS are provided in the chapters below with each study. A novel approach was used in our study, the refined PRS (rPRS), to ensure we were using the SNPs associated with fasting insulin in children while using a GWAS performed in adults. The rPRS provided us with a way of ensuring the SNPs used in the calculation were relevant for both children and adults to inspect whether the biological aspects related to high fasting insulin levels interacted with early life adversity to predict impulsivity in children.

To further inspect the pathways underlying the mechanism within this relationship, we used enrichment analysis. Several tools and software are available to perform genetic enrichment analyses and they all function similarly [38]. Using information from published literature in addition to open-source information, these enrichment tools are able to provide existing associations to genetic markers of interest in one place. By either providing the list of SNPs that we used within our rPRS calculations or the list of genes that those SNPs mapped to, we are able to discover which other biological pathways, networks, and diseases they have been previously associated with. This information can be crucial in guiding the research in the right direction, to go from associations to the biological mechanisms governing the diseases.

Early life adversity, fasting insulin, and adulthood addiction

Impulsive behavior consists of multiple traits: 1) lack of perseverance, which can lead to the incomplete tasks; 2) lack of planning, which results in acting without thinking; 3) sensation seeking, which refers to the tendency of trying new and stimulating activities

or sensation; 4) negative urgency, which refers to negative emotions resulting in the tendency to act rashly [39]; and 5) positive urgency, which represents to positive emotions resulting in the tendency to act rashly [40]. Impulsivity measures are either measuring one of these traits or a combination of these traits to determine whether an individual exhibits impulsive behavior. In the literature, it has been established that impulsivity can be characterized as a behavioral endophenotype mediating the risk of addictive behavior [41]. For example, sensation seeking has been shown to predict drinking frequency and positive urgency has shown to predict drinking quantity and problems [42]. When specifically examined through impulsivity traits listed above in association with alcohol use, the following was found: 1) negative urgency and lack of planning had the strongest effects on alcohol dependence, 2) lack of perseverance had the strongest effect on drinking quantity; and 3) sensation seeking had the strongest effect on binge drinking [43]. Although it is important to identify the impulsivity-related trait and the aspect of alcohol when examining the association, these studies establish that impulsivity is associated with addiction to alcohol. When looking at impulsivity in childhood, the same traits can be used and are known to be characterized by attention deficit hyperactivity disorder (ADHD) [44, 45]. Childhood ADHD, which is associated with impulsivity traits of lack of planning, lack of perseverance, positive urgency, and negative urgency, has been significantly associated with alcohol problems in adulthood [46]. Similarly, childhood impulsive behavior, including attention problems, hyperactivity, and problems with behavioral restraint has also been associated with gambling problems [47]. In fact, non-impulsive children were shown to have lower gambling problems, further solidifying the association between childhood impulsivity and adulthood addiction [47]. Furthermore,

childhood ADHD has also shown prevalence to food addiction in obese individuals in comparison to obese individuals with adulthood ADHD [48]. This prompts the idea that childhood impulsivity might not necessarily map to impulsivity in adulthood but could still be relevant in addiction behaviors in adulthood.

Therefore, after establishing the association between exposure to early adversity, high fasting insulin levels, and impulsive behavior in childhood, we wanted to inspect whether the same association would exist with addiction in adulthood. Additionally, as lifetime stress and adversity are linked to heightened impulsivity [49-51], we wanted to use our interaction model to assess adulthood addictions in association with childhood impulsivity. Therefore, we used the same tools and resources as the previous study to inspect the association in two adult cohorts: SAGE and UKB.

Early life adversity, fasting insulin, and sex-specific executive functions

As we established an association between the genetic background of fasting insulin and impulsive behavior, we wanted to inspect the shared genetic architecture between fasting insulin and impulsivity. To do so, we used a technique recently developed by Andreassen et al [52] called conditional and conjunctional false discovery rate (FDR). This technique relies on the premise that genes must be affecting multiple traits at one time and by combining independent GWASs from traits that might have a common genetic architecture, SNPs from related disorders would be revealed to explain heritability of co-morbidities. By combining GWAS statistics from two disorders, there is increased power in discovering genes associated with common biological mechanisms. It is important to note that ancestry and geography can have significant impact on GWASs because different ancestries have distinct allele frequencies [53]. Differential linkage

disequilibrium among differing populations can also lead to false-positive SNPs when local ancestry is ignored [54]. Therefore, it is important to use GWASs based in the same ancestry for this technique. By using the technique by Andreassen et al, summary statistics from two independent GWASs are used to identify SNPs demonstrating pleiotropic relationships between the fasting insulin GWAS and the impulsivity GWAS and the ADHD GWAS. This statistical approach is based on the observation that all SNPs should not be treated as exchangeable. Instead, a SNP with large effects in two associated phenotypes has a higher probability of being a true non-null effect, meaning it also has a higher probability of being replicated in independent studies [52]. The conditional FDR approach improves the detection of genetic variants associated with the primary trait by reranking the test statistics of the primary phenotype, fasting insulin in this case, based on the strength of the association with the secondary phenotype, impulsivity or ADHD in this case. The conjunctional FDR extends on the conditional FDR by identifying SNPs that are null for both phenotypes of interest at the same time when both phenotypes' p-values are smaller than the observed p-values [52]. This technique was especially important for us because it aimed to provide a clearer picture of the polygenic nature of both fasting insulin levels and executive function behaviors. Furthermore, metabolic and psychiatric conditions often occur in conjunction with each other, leading one to believe that they must share some genetic architecture. In our specific case, identifying the SNPs responsible for both phenotypes of interest would narrow down the genetic markers involved within the mechanism.

While exploring the genetic architecture shared by fasting insulin and executive functions is important, we also wanted to keep inspecting the interaction effect between

early life adversity and fasting insulin on executive functions. Therefore, with this study, we decided to expand our research beyond impulsive behavior and examine multiple executive functions. We also wanted to use this study to inspect sex-specific interactions now that we had established that there was an association between early adversity, fasting insulin, and impulsivity. Additionally, we wanted to investigate the interaction effect throughout development, so we focused on cohorts with data on children, adolescents, and adults. The tools used for this analysis were the same as the previous two studies where an rPRS was calculated for each cohort using the sex-specific fasting insulin GWASs [32]. However, here we examined multiple executive functions as outcomes through a phenotype wide association study (PheWAS) which accounted for multiple testing by applying an FDR correction to minimize type 1 errors [55]. PheWAS is created by analyzing the association between a SNP and multiple phenotypes. Our PheWAS is created by analyzing the interaction effect between the fasting insulin rPRS and early life adversity on executive function outcomes. This approach gave us the opportunity to show that impulsivity is not the only executive function behavior impacted by early life adversity and fasting insulin.

The last part of this study uses mendelian randomization (MR) to assess the causal effect of an exposure on a disease [56], which we used to inspect the causal relationship between fasting insulin and variations in executive function in individuals exposed to childhood adversity. MR studies employ the principles of randomized clinical trial design. In a randomized controlled trial, participants are randomly placed in the drug or placebo group and then the outcomes are compared between the groups to investigate whether the drug was effective or not. MR is similar because the Mendel law states that genetic

alleles are distributed randomly in individuals, meaning that populations already consist of randomized groups in terms of their genetic makeup. Therefore, we can use MR to inspect associations between an exposure, for example high fasting insulin, with the presence of an outcome, for example impulsive behavior. We added the component of early adversity within this study to inspect the interaction effect between adversity and high fasting insulin and whether a causal association exists between them on impulsive behavior, in a sex specific manner.

Early life adversity, insulin, and netrin-1/DCC in the PFC DA pathway

Considering the effects of insulin signaling on the mesocorticolimbic pathway function, we assumed that it was integral to investigate if the Netrin-1 system, which participates in maturation and adult function of DA circuitry via DCC receptor signaling and is closely related to insulin, can be a key component in this process, resulting in reorganization of DA synaptic circuitry and therefore, affect DA-related executive functions. The guidance cue Netrin-1 participates in the developmental organization of neural networks as a bifunctional cue, either attracting or repelling extending axons and dendrites [57]. Attraction to Netrin-1 depends on the activation of DCC (deleted in colorectal cancer) receptors, while repulsion is mediated by UNC5 (uncoordinated) receptors [58, 59]. Responses to Netrin-1 can be modulated by regulating the availability of DCC and/or UNC5 receptors at the cell surface. Thus, the selectivity with which Netrin-1 organizes neuronal connectivity is determined by the spatiotemporal expression of DCC and UNC5. Subtle alterations in their expression, at different developmental periods, results in discrete remodeling of specific neuronal circuits [60]. The proposed project is focused on DCC receptors. Netrin-1 is expressed in terminal fields of dopamine neurons, including

the nucleus accumbens and the prefrontal cortex [61]. Because DCC receptors are highly and conspicuously expressed by VTA dopamine neurons in humans and rodents, guiding DA neurons to their targets in the VTA and PFC [59], we decided to measure DCC and Netrin-1 protein expression levels in an intrauterine growth restriction animal model, as a model of adversity, at birth and in adulthood. In adult rodents, we also inspected the changes in DCC and Netrin-1 levels when exposed to insulin. Additionally, we inspected if the microRNA regulator of DCC, miR-218, was affected by insulin exposure. With these findings, we aimed to better understand the possible mechanism linking early life adversity, insulin signaling and sensitivity, and DA-related executive functions dominated by the PFC.

Chapter II. Comprehensive review of the literature

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Early adversity and insulin: neuroendocrine programming beyond glucocorticoids

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Abstract

Exposure to direct or contextual adversities during one's early life programs the functioning of the brain and other biological systems, contributing to the development of physical as well as mental health issues in the long term. While the role of glucocorticoids in mediating the outcomes of early adversity has been explored for many years, less attention has been given to insulin. Beyond its metabolic effects in the periphery, central insulin action affects synaptic plasticity, brain neurotransmission, and executive functions. Knowledge about the interactions between the peripheral metabolism and brain function from a developmental perspective can contribute to prevention and diagnosis programs, as well as early interventions to vulnerable populations.

Keyword(s): insulin, early adversity, executive function, brain

Brain-body connections shape developmental processes

Environmental conditions present during early developmental stages have a dramatic influence on an individual's health/disease trajectory over their life course. Exposure to direct or contextual adversities, such as violence or abuse, family dysfunction, socio-economic difficulties, malnutrition, or parental stress can hinder the development of the brain architecture and other biological systems, leading to increased risk for diseases later in life [1]. These changes, induced by early life adversity, have been commonly linked to alterations in executive functions (e.g., impulse control, cognitive flexibility, memory) [2, 3] contributing to the development of both physical and mental health issues in the long term. Advances in neuroendocrinology have established that there is a reciprocal communication between the brain and the body via hormonal and neural pathways. Hormonal feedback from neuroendocrine, immune, and metabolic

systems to the brain regulates homeostatic functions in the hypothalamus. The same feedback affects emotions, behavior, and cognition in response to internal and external signals. These effects occur through direct signaling on brain receptors in cortical and subcortical structures, modifying genomic and non-genomic processes during development, and defining both the behavioral phenotype as well as influencing disease risk over the life course.

Among the many peripheral signals that act on the central nervous system, insulin merits attention considering the widespread distribution of its receptors in the brain [4]. Insulin has an essential role on peripheral glucose homeostasis and energy metabolism, but it is insulin's central action as a neuromodulator that is critically involved in the regulation of synaptic plasticity and **monoaminergic neurotransmission** (see **Glossary**). While the role of glucocorticoids in mediating the outcomes of early adversity has been explored for many years, less attention has been given to insulin. Glucocorticoids and the insulin axis interact with each other at different levels, in such a way that the well-known alterations in the **hypothalamus-pituitary-adrenal axis** induced by early adversity modify glucose metabolism, insulin secretion, and insulin signaling (**Table 1**). Acting on the adipose tissue, liver, skeletal muscle, pancreas and to a lesser extent on bone, gut, and brain, long-term glucocorticoid exposure leads to metabolic dysregulations with hyperglycemia, and insulin resistance, ultimately, contributing to the development of cardio-metabolic disease [5-8].

Despite this close relationship between glucocorticoids and the insulin axis on the development of chronic adult disease related to early life adversity (**Box 1**), insulin signaling has many independent roles in the growing child's health and in the developing

brain. Insulin influences physical health, neurodevelopment, behavior, and executive functions in physiological conditions and in response to early adversity. This review focuses on the function this hormone has on neurodevelopmental processes, as well as deviances from a healthy developmental trajectory induced by environmental adversity.

Central action of insulin

Insulin is one of the primary hormonal regulators of metabolism in animals. This hormone is composed of small polypeptides secreted by beta islet cells in the pancreas and regulates glucose uptake by cells in most peripheral tissues. The central nervous system uses non-insulin sensitive glucose transporters, **GLUT**-1 and GLUT-3, for the majority of glucose uptake, leaving insulin to play a neuroregulatory role in the brain [4]. Although most of the insulin in the brain comes from the periphery, a few studies suggest its synthesis also occurs in the brain (e.g., in rats [9]). Pancreatic insulin is transported into the brain via a specific, saturable carrier located on capillary endothelial cells [10], which has been suggested to be the insulin receptor (**IR**) itself [11].

The IR has two α-subunits, which are extracellular and include a ligand binding site and two cytoplasmic β-subunits. There are two isoforms of the IR: a long isoform called IR-B involved in the metabolic effects of insulin and predominating in adult peripheral tissues such as muscle, liver, kidney, and fat; and a short isoform called IR-A that binds to IGF-2, resulting in receptor activation and influencing hippocampal neurogenesis. While astrocytes express both IR-A and IR-B, neurons only express IR-A, even though IR density in general is much higher in neurons than in glia. Both IR isoforms are expressed throughout the brain in areas including the ventral tegmental area (VTA), striatum, hippocampus, hypothalamus, amygdala, and prefrontal cortex (PFC) [4].

IR central anatomical distribution overlaps with neurotransmitter systems [e.g., dopamine (DA), serotonin, γ-aminobutyric (GABA), glutamate] that are major players in the mechanism of neuronal communication. A wealth of studies demonstrate the importance of insulin signaling in many central nervous system functions, such as synaptogenesis, synaptic plasticity, neuroprotection, memory, and cognition, including **long-term potentiation and depression** (**LTP** and **LTD**) [12], attention, sensitivity to reward, inhibitory control [13], energy balance, and eating behavior [14]. Insulin and its receptor play a key role in the dynamics of dendrite formation, spine density, neurite growth, and neuronal development [15]. Disruptions to their function, induced by early adversity, can have potent effects on neurodevelopment. When these changes occur during **critical periods** of development, they can have '**programming**' effects, leaving persistent marks in individuals' physiology and defining their health/disease patterns in the long term. Insulin can affect the development of different neurotransmitter systems, especially those with protracted developmental periods like the **mesocorticolimbic** dopaminergic pathway (**Box 2**), as well as modulate the function of these systems during one's life-course.

Therefore, there is a large range of adult disorders that could emerge from early adversity- induced dysfunctions in central IR-mediated processes, due to alterations in IR activation, diminished insulin availability, or malfunction of downstream intracellular signaling [4]. These include mood disorders [16], schizophrenia [17], and neurodegenerative processes such as Alzheimer's and Parkinson's diseases [18], suggesting a functional overlap between brain insulin dysfunction and altered brain neurotransmission in the pathogenesis of these conditions.

The modulation of central neurotransmission by insulin

Dopaminergic system

IRs are expressed by dopaminergic neurons in the midbrain, including the VTA and substantia nigra [19]. At the end of the 1970s, studies started demonstrating that glucose and insulin could modulate the number of DA receptors, the firing of DA neurons, and the release and turnover of striatal DA [20, 21], in addition to suggesting that this modulation was an important feature of psychopathologies like schizophrenia. A more recent postmortem gene expression study demonstrated that in individuals with bipolar disorder, major depression disorder, and schizophrenia, a lower expression of DA-related genes [**dopa decarboxylase (DDC)**, **tyrosine hydroxylase** (**TH)**, **vesicular monoamine transporter 2** (**VMAT2)** , DRD1, DRD2, DRD5, and **monoamine oxidase B (MAOB**)] was fully mediated by a lower expression of IR-related signaling genes [INSR, **insulin receptor substrate 1 (IRS1**), and IRS2], suggesting an impaired IR function in these cases [22].

Beyond gene expression, insulin seems to increase the capacity of DA reuptake through the dopamine transporter (DAT) by activating the **phosphatidylinositol (PI) 3-kinase (PI3K)** in rat synaptosomes and human cell cultures [23]. It was also shown that insulin reduces electrically evoked exocytotic [(3)H]DA release in rat nucleus accumbens (NAcc) slices and in medial PFC slices [24]. Concomitantly, insulin reduces premature responses in the five-choice serial reaction time task and enhances the stimulatory effect of peripheral cocaine administration on impulsivity when injected directly into the NAcc [24]. This suggests that the presynaptic action of insulin regulates cocaine-sensitive monoamine transporter function in the NAcc and, consequently, impulsivity. Direct

intracerebroventricular infusion of insulin results in an increase in DAT mRNA levels [25], suppressing DA-related behavioral outcomes such as sucrose intake and sucrose self-administration [26], which indicates that insulin modifies the rewarding value of sucrose. These studies in rats suggest that insulin can change the molecular response of DA within the mesocorticolimbic pathway, affecting the associated behavioral phenotype.

At the behavioral level, while insulin injection in the rodent NAcc shell leads to sweet flavor-preference conditioning [27], intra-VTA insulin inhibits food anticipatory behavior and conditioned place preference for food, suggesting that insulin may attenuate the salience of food-related contexts or cues in this brain area [28]. Moreover, as VTA DA neurons are heterogeneous [29], it remains to be established if insulin modulates subpopulations of VTA DA neurons depending on their projection targets. Finally, as insulin suppresses excitatory inputs to the VTA yet increases DA firing rate, it is possible that insulin has a differential action on tonic versus burst firing, such that tonic DA release is increased while phasic bursts are suppressed [30] (**Figure 1**).

A mouse model of brain-specific knockout of IR is linked to age-related anxiety and depressive-like behavior; this is due to altered mitochondrial function, aberrant monoamine oxidase (MAO) expression, and increased DA turnover in the mesolimbic system [31]. Loss of IR in astrocytes is associated with increased depressive-like behavior, which is accompanied by impaired DA release from brain slices, particularly in the NAcc [32]. These studies in mice suggest that the modulation of DA neurotransmission by insulin action on its receptor is linked to the development of phenotypes related to mood disorders such as depression and anxiety-like behaviors.

In humans, there is evidence for the modulation of DA by insulin. Insulin sensitivity index estimated from a glucose tolerance test was negatively correlated with ventral striatum D2 receptor availability measured by positron emission tomography (PET), whereas fasting insulin was positively associated with D2 availability in the right insular cortex [33]. Lower β-cell function (estimated using data from a glucose tolerance test) was related to stronger preference for an immediate and smaller monetary reward over delayed receipt of a larger one (greater delay discounting), although no relationship was found with striatal D2 receptor binding [34]. Intranasal insulin administration can improve memory and mood in healthy men and women, behaviors linked to DA neurotransmission [35]. Intranasal insulin is also linked to differential resting-state activity in brain regions involved in reward processing [36], decreased food palatability ratings [37] and attenuation of visual processing of food images [38], without altering peripheral glucose sensitivity [39]. Taken together, these findings suggest that impaired insulin signaling in the brain, even in individuals who are non-diabetic, can have an important effect on behaviors that are associated with dopaminergic neurotransmission and with the development of different psychopathologies in the long-term including depression, anxiety, cognitive decline, and Alzheimer's disease [16, 18] as well as in the co-morbidity between these diseases and insulin resistance/type 2 diabetes [40].

Lastly, there is a tight connection between insulin and netrin-1, one of the guidance cues involved in the development of the mesocorticolimbic DA pathways [41]. Netrin-1 is involved in pancreatic morphogenesis and tissue remodeling, regulating fetal islet cell migration and stimulating in vitro insulin secretion by promoting β-cell Ca (2+) influx and **cyclic AMP (cAMP)** production [42]. Plasma netrin-1 levels are decreased in patients

with type 2 diabetes and correlate negatively with insulin resistance measures [43]. In cultures of rat Schwann cells (glial cells that are part of the myelin sheath from peripheral nerve fibers), netrin-1 enhances migration through the activation of **PI3K**, an essential component of the cellular insulin signaling cascade [44]. Although not directly investigated to date, it is tempting to think that insulin could signal changes in the environment to the developing brain by modulating axonal guidance cues like netrin-1 and promoting neuroadaptations of the DA pathways. These could have noticeable consequences on discrete changes in executive functions, but also long-term effects on psychopathology risk.

Serotoninergic system

The placenta produces serotonin that accumulates in the embryonic forebrain during the early phases of telencephalic development, until serotonergic raphe neurons progressively start to synthesize and uptake serotonin [45]. The brain expresses seven types of serotonin receptors (5-HT1-7) comprising a total of 14 subtypes [46]. Serotonin neurons from the dorsal and/or medial raphe nuclei innervate the entire forebrain and midbrain and are considered important in modulating several neurobiological functions especially those relating to emotional states [47]. Serotonin neurons express IR mRNA and peripheral injection of insulin increases both plasma and brain tryptophan levels, favoring serotonin synthesis [47, 48]. In humans, serotonin transporter binding is diminished in the diencephalon of insulin-resistant subjects [49]. Although the limited available data suggest that insulin directly increases serotonergic neuronal activity, there is a possibility that insulin modulates serotonin neurotransmission by acting on other neuronal subpopulations.

GABAergic system

GABA is the main inhibitory neurotransmitter in the adult brain. However, GABAergic synaptic transmission is excitatory in early life, exerting widespread trophic effects and undergoing a switch during development from being excitatory to inhibitory. There are two main types of GABA receptors: the ionotropic GABA-A receptor and the metabotropic GABA-B receptor. Heterogeneity on the distribution of their subfamilies and localization at the synaptic cleft, or extrasynaptically/perisynaptically, defines their role in tonic versus fast, phasic inhibition. GABAergic neurons develop early in the cortex during embryonic development, but in humans, the system continues developing into the first few years of infancy and possibly until adolescence. GABAergic cortical interneurons are the first neurons to generate network-driven activity in the developing brain. Interneuron-generated network-driven patterns modulate the proper development of synapses among cortical neurons, priming unorganized silent neurons to shift into functional circuits [50].

A recent hyperinsulinemic-euglycemic clamp study in mice showed that peripheral insulin upregulates the expression of multiple subunits of GABA-A receptors in the hypothalamus [51]. Insulin also regulates the tonic GABA-activated synaptic and extrasynaptic current density in hippocampal dentate gyrus granule cells and CA3 pyramidal neurons, reducing spontaneous neuronal firing during maturation of the young mouse brain as well as in a model of Alzheimer's disease [52]. Similar modulatory effects of insulin on GABA also occur in the rat amygdala [53] and PFC [54]. In patients with type 2 diabetes, a relationship between elevated medial PFC GABA concentrations (measured using magnetic resonance spectroscopy) and poorer episodic memory performance was

observed, suggesting that abnormal GABA levels in the medial PFC are linked to the episodic memory decline that occurs in patients with type 2 diabetes [55].

Glutamatergic system

The neurotransmitters glutamate and GABA are involved in the balance between brain excitation and inhibition, having important roles in neuronal migration, synaptogenesis, synaptic plasticity, and modulation of memory and learning processes. Beyond their individual roles, glutamate and GABA receptors are colocalized in many brain regions and their steady interaction is a key factor for normal brain development. There are three types of ionotropic glutamate receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) [56]. In primary cultured neurons, acute insulin stimulation induces the expression of NMDA receptor subunits [51]. IR signaling enhances NMDA-mediated glutamatergic neurotransmission in the hippocampus and modifies AMPA receptor surface expression with effects on LTP/LTD depending on the activation of specific subunits [57]. Hippocampal-specific insulin resistance induced by a lentiviral vector leads to alterations in the expression and phosphorylation of glutamate receptor subunits, altering LTP and impairing hippocampal-dependent learning in rats [58]. IR are also expressed by NAcc medium spiny neurons in rats, where insulin influences glutamatergic excitatory transmission via pre- and postsynaptic mechanisms [59]. In cultures of rat cortical neurons, insulin modulates glutamate excitotoxicity [60]. In sum, the modulation of glutamatergic neurotransmission by insulin is involved in synaptic plasticity, memory formation, motivational systems, and excitotoxicity/neuronal survival.

Early life adversity, insulin, and brain neurotransmission

In pediatrics, a healthy childhood can be estimated by means of adequate age-appropriate growth and neurodevelopment [61]. Different aspects of the child's environment can affect these two parameters, such as the psychosocial context, the family function, or individual physiological or pathophysiological changes. From a biological perspective, insulin function is involved in both growth and development [62, 63] as well as in the adaptive responses to changes in the environment [64]. Stressful conditions or adversities happening early in life, either pre- or postnatally, can affect glucose homeostasis and insulin function in the short and long-term (**Box 1**).

Alterations in dopaminergic neurotransmission are seen in animal models of pre- and postnatal adversity, with varied impact on executive function-related behaviors. For example, in rodents exposed to intrauterine growth restriction (IUGR, induced by malnutrition during pregnancy), there is systemic hyperglycemia/hyperinsulinemia [65], as well as alterations in the expression of genes or proteins related to dopaminergic signaling in the VTA, NAcc, and PFC (TH, pTH, D1, D2) [66-69]. These metabolic and neurochemical effects are accompanied by changes in cognitive flexibility, sensitivity to reward, and poor inhibitory control [66, 67, 69-71]. Chronoamperometric measures of DA release in response to sweet food were not affected in the orbito-frontal cortex, but were blunted in the medial PFC [70] and NAcc of IUGR animals, being completely reversed by a peripheral injection of insulin [68]. There is a decrease in suppressor of cytokine signaling 3 (**SOCS3**) protein in the VTA in these animals, which is a marker of altered insulin sensitivity in this brain area [68]. All these findings suggest that the modulation of DA by insulin is linked to the behavioral alterations in adult rats exposed to prenatal adversity. Prenatal stress in rodents also affects the development of serotonin raphe

neurons as well as the long-term expression of serotonin receptors in limbic structures [45].

Interference with the early postnatal experience in rodents also has effects on DA-related behaviors and neurotransmission, while leading to increased insulin resistance later in life [72, 73]. Both brief and longer periods of maternal separation events in the first few days of life are linked to alterations in sensitivity to reward in adulthood in rats [74, 75], as well as changes in DA metabolism in the NAcc [74, 75], in the expression of D1 and D2 receptor on projection neurons [76], and in the excitability and diameter of dendritic spine heads of dopaminergic neurons in the VTA [77]. In mice, stress early in life alters the transcriptomic patterns across the reward circuitry in males and females [78] and reprograms accumbal D2 medium spine neurons to increase the susceptibility to chronic social defeat stress in adulthood via histone methylation modifications [79]. Serotonin signaling is critically involved in long-term molecular adaptations related to the brain glucocorticoid programming effects in response to variations in the rearing conditions in rats [80]. Early life stress can also accelerate or delay critical periods of development, reflecting GABA circuit maturation and the brain's excitatory/inhibitory balance, which may be linked to the development of cognitive disorders [81].

Childhood maltreatment is a form of severe early life adversity, being a classical risk factor for developing adult cardio-metabolic diseases in humans, including insulin resistance and type 2 diabetes [1], and has been associated with several effects on brain and behavior. A study using PET described that severe physical or sexual abuse accompanied by unstable family arrangements in childhood were associated with elevated DA function in the associative striatum in adulthood [82]. Another study using

simultaneous electroencephalography-functional magnetic resonance imaging in young adults shows that early life adversity leads to hyporesponsiveness during reward anticipation and hyper-responsiveness when receiving a reward, a pattern that correlated with lifetime attention deficit hyperactivity disorder (ADHD) symptoms [83]. However, less extreme forms of early life adversity also elicit long-term effects [84], where even alterations in the maternal metabolic context can be seen as a form of adversity (**Box 3**).

Childhood contextual stress exposure is associated with differences in the serotonin receptor 2A gene methylation, which were also related to post-traumatic stress and depressive disorder symptoms in a sample of children [85]. A post-mortem study described decreased NMDA receptor binding in the dorsal prefrontal, dorsolateral prefrontal, and anterior cingulate cortex of individuals exposed to childhood adversity, suggesting that early life stress can cause glutamate excitotoxicity with NMDA receptor downregulation and/or neuron loss [86]. Methylation in a regulatory region of the ionotropic glutamate receptor NMDA type subunit 2B gene was associated with exposure to childhood adversity in a sample of adults, although no association was found between the epigenetic marker and depression status [87].

While a systematic review of the association between epigenetic modifications of the serotonin transporter gene and adverse exposures in humans seems to corroborate findings from experimental models in rodents, a wide heterogeneity in the revised literature prevents the establishment of a definite conclusion [88]. Dated candidate gene approaches have suggested that serotonin-related polymorphisms interact with early adversity modifying the risk for stress-related psychopathology [45]. A recent study [89] filtered the genetic markers identified in a genome-wide association study (GWAS) for

adult high fasting insulin levels by selecting those most highly associated with peripheral insulin levels in children, to calculate a polygenic risk score (PRS). This fasting insulin PRS interacted with early life adversity and predicted childhood impulsivity at 3 years of age in an independent cohort. Interestingly, the markers composing the high fasting insulin PRS are mapped into genes associated with DA D2 receptor signaling, suggesting that individual variations in insulin function are related to differential effects of childhood adversity on executive functions, via DA-related mechanisms [89]. Finally, high fasting insulin genetic markers that predict childhood impulsivity in response to adversity were also significantly enriched in the accelerated cognitive decline GWAS, which may suggest that these genes are also important for long-term effects on cognition [89]. This study is an example of novel functional genomics investigations, which carry the promise to illuminate these relationships from a genome-wide perspective [84, 90] (**Figure 2, Key figure**).

Concluding remarks

Metabolic factors (like insulin) acting on the brain very early in life can modify the development of different neurotransmitter systems and influence both executive functions and the risk of physical and mental diseases later in life. Basic science studies directly exploring these relationships at the molecular level in response to pre- or postnatal adversity are needed (see **'Outstanding Questions'**). Multilevel integration of models (animal, clinical, observational) and data modalities (gene expression, gene variants, neuroimaging, behavior, biomarkers) in the context of big data analysis from a life-course perspective is a promising avenue to identify the mechanisms involved in the response to adversity long-term consequences. For example, we have recently described that

biologically informed polygenic scores reflecting individual differences in the mesocorticolimbic and hippocampal IR coexpression gene networks have a better prediction of child impulsivity and cognitive performance, as well as risk for addiction and Alzheimer's disease in comparison with conventional polygenic scores for ADHD, addiction, and dementia [91]. Insulin modulation of brain neurotransmitter systems may be the key to discover the impacts of early life stress on neurodevelopmental processes and executive function and consequences on the risk for psychopathology in adulthood. As we recognize the important roles that insulin has on the development of neuropsychiatric conditions like major depression, dementia, and Alzheimer's disease, it is essential to understand the role that these developmental aspects have on the establishment of risk for these diseases, as well as for the highly prevalent comorbidity between psychiatric and metabolic conditions. Knowledge about the interactions between the peripheral metabolism and brain function from a developmental perspective, can contribute to early prevention and detection as well as early intervention programs for vulnerable populations.

Tables and Figures[1]

Table 1. Chronic glucocorticoid effects on glucose homeostasis, insulin secretion and signaling

Tissue	Glucose homeostasis	Insulin secretion	Insulin signaling	References
Adipose tissue	Inhibit glucose uptake and utilization	-	Increase insulin sensitivity in subcutaneous adipose tissue	Review in [5-8]
	Stimulate gluconeogenesis via increased lipolysis		Increase lipolysis contributing to adipose tissue redistribution and insulin resistance in muscle and liver	
Skeletal Muscle	Inhibit glucose uptake and oxidation	-	Reduction of insulin signaling at different points of the insulin receptor intracellular cascade	
	Reduce glycogen storage		Accumulation of triglycerides in fibers, increasing insulin resistance	
	Increase protein degradation to favor gluconeogenesis			
Liver	Stimulate gluconeogenesis	-	Increase insulin-stimulated hepatic lipogenesis leading to steatosis and insulin resistance	
	Increase glycogen storage		Decrease insulin binding to its receptor	
			Impairment of insulin receptor function and signaling cascade	
Pancreas	Decrease β-cell sensitivity to glucose	Inhibit insulin	-	

[1] All tables and figures for this chapter were obtained from an open-source paper, therefore, they does not require copyright approval.

		secretion from β-cells		
	Decrease upstream oxidative glucose metabolism	Induce β-cell hyperplasia		
	Reactive oxygen species generation, β- cell damage			
	α-Cell stimulation with enhanced glucagon action and consequent hyperglycemia			
Brain	Abnormalities in cerebral glucose metabolism	-	Decrease insulin uptake/transport into the brain	
			Decrease insulin receptor expression in certain regions like the hippocampus and hypothalamus	

Figure 1. Cellular effects of insulin in the ventral tegmental area (VTA) and nucleus accumbens (NAcc). Insulin acts on insulin receptors (InsR) in dopaminergic neurons from the VTA, reducing somatodendritic dopamine (DA) concentrations by upregulating dopamine transporter (DAT) in addition to suppressing excitatory inputs by increasing phosphatidylinositol 3-kinase (PI3K) signaling. In the NAcc, insulin also acts on cholinergic interneurons, increasing their burst firing and enhancing DA release from the presynaptic neuron originating from the VTA. Insulin can also bidirectionally modulate synaptic transmission onto NAcc core medium spiny neurons, increasing excitatory synaptic transmission at low concentrations and suppressing evoked excitatory synaptic transmission in these accumbal neurons at higher concentrations via activation of insulin growth factor 1 receptors. Insulin receptor action on astrocytes in the NAcc stimulates ATP exocytosis. This figure was created using BioRender (https://biorender.com/).

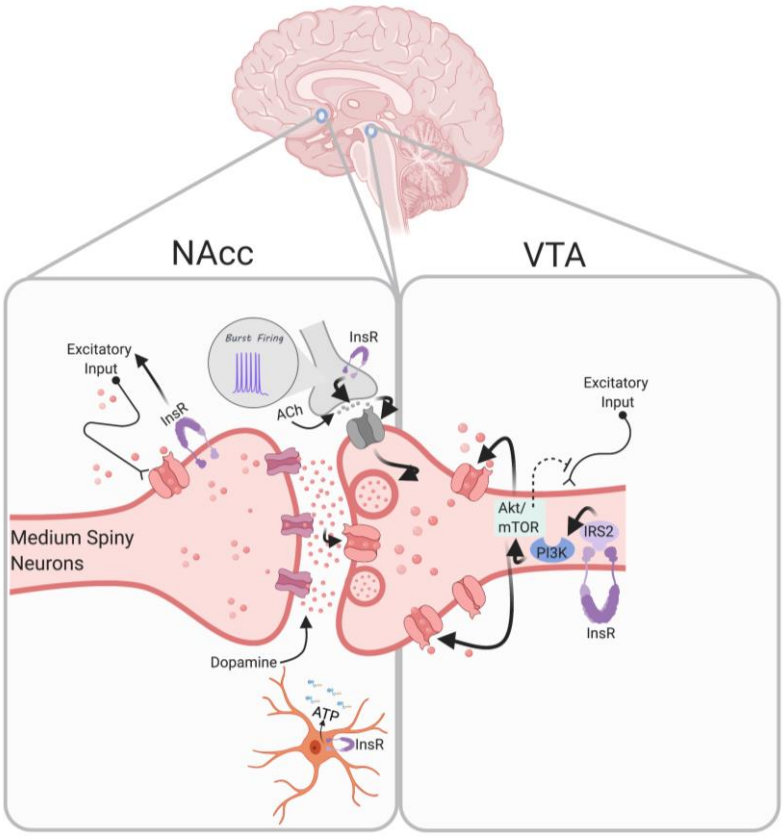
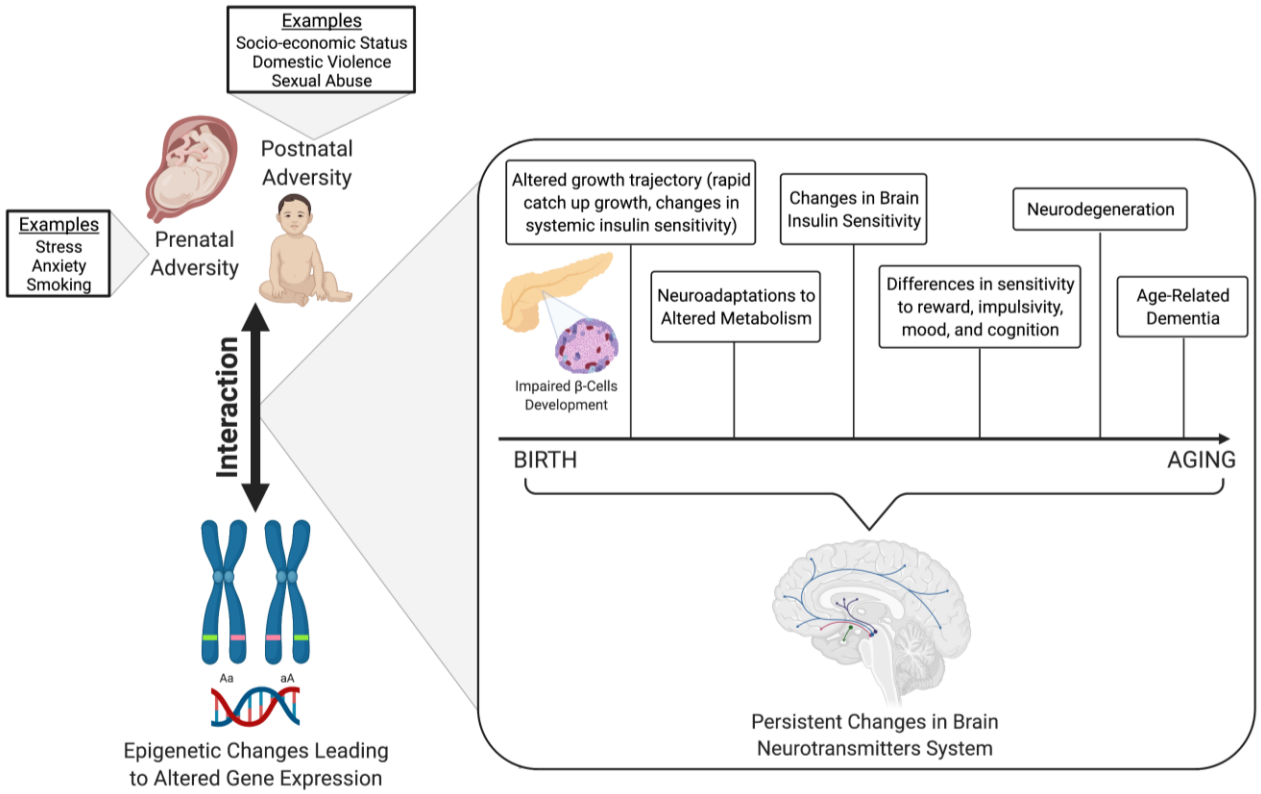


Figure 2. Key figure. Life-course perspective of the programming effects of insulin on neurodevelopment. The interaction between early life adversity (pre- and/or postnatal) and the genetic background modifies gene expression and the effects of this interaction define the individual's health and disease trajectory over the life course. One of the main immediate effects of adversity occurring in a critical developmental period is the resulting impairment in optimal growth in general but also, specifically, pancreatic development, compromising insulin production and signaling. This triggers a cascade of adaptive metabolic and neurobiological mechanisms affecting behavior and the risk for mental and physical illness throughout the lifetime. This figure was created using BioRender (https://biorender.com/).



Supplementary Information

Boxes

Box 1: Stress and insulin – glucocorticoids and beyond

In response to stress, the HPA axis, the **sympathoadrenal** system, and the **proinflammatory cytokines** (TNF-α, IL-1 and IL-6) act synergistically to alter energy metabolism, resulting in an increase in gluconeogenesis, glycogenolysis, and insulin resistance. Long-term exposure to these mechanisms during chronic stress is implicated in the dysregulation of metabolism over time, as elevated glucocorticoids and prolonged sympathoadrenal activation promote visceral accumulation of adipose tissue and insulin resistance. At the same time, a systemic low-grade inflammation acts as an additional chronic stressor, prolonging the cycle.

Upon chronic stress exposure, glucocorticoids act on the adipose tissue reducing the expression of the glucose **GLUT1** transporter and the translocation of GLUT4 to the plasma membrane, diminishing the insulin-induced glucose uptake. They also decrease insulin receptor substrate (**IRS1**) phosphorylation and protein expression. In the liver, glucocorticoids dramatically decrease protein phosphorylation of important insulin signaling molecules like IR, IRS1 and PI3K, inhibiting insulin signaling and consequently stimulating the expression of its neoglucogenesis related target genes. In skeletal muscles glucocorticoids reduce PI3K activity and IR tyrosine phosphorylation, decreasing the level of **AKT** phosphorylation and diminishing insulin signaling. Glucocorticoids also affect insulin transport into the brain, as well as reduce IR activity, with reduced insulin-stimulated phosphorylation of the IR and decreased total AKT and total GLUT4 protein

expression. Interestingly, glucocorticoids also severely impair insulin secretion by the pancreas, affecting the proliferation and survival of beta cells.

When stressful events occur during a **critical period** of development, they can have long-term **programming** effects, persistently modifying these molecular relationships between glucocorticoids and insulin, similarly to what happens in chronic stress. This is an important mechanism by which early life adversity leads to lifelong risk for chronic diseases like metabolic syndrome and psychopathology. Moreover, insulin itself also has long-lasting developmental programming effects on its target organs that are independent of glucocorticoid actions (see review main text). As opposed to initially thought, pancreatic beta cells have a dynamic development in terms of their capacity for insulin secretion and proliferation, with immature secretory function and a high rate of proliferation at birth, progressively increasing secretory capacity and reducing the ability to replicate. Like any other developmental process, pancreatic development can also be affected by the conditions existent during early life. The primary role of pancreatic beta cells is to produce insulin and therefore, impairment of such cells hampers insulin secretion in the periphery and subsequent central insulin levels, given that the majority of central insulin comes from the periphery. In sum, insulin signaling acts both in conjunction as well as independently from glucocorticoids, having long-term programming effects on health and neurodevelopment in response to early adversity.

Box 2: The prolonged development of dopaminergic pathways

Dopamine (DA) is widely distributed in the central nervous system. There are multiple DA receptors separated into two families: D1 (which includes D1 and D5

receptors) and D2 (D2, D3 and D4 receptors). D1 stimulation activates adenylyl cyclase (AC) activity whereas D2 activation inhibits AC, increasing protein kinase A (PKA) activity. Enhanced PKA activity elevates synaptic plasticity, stimulates neuronal development, and increases DA synthesis. Low PKA signaling is known to be the cause of several brain degenerative diseases including Alzheimer's and Parkinson's disease, suggesting that PKA could play a neuroprotective role.

DA neurons are found in three main locations: First, the ventral midbrain, divided into a) substantia nigra pars compacta, that innervates the dorsolateral striatum and caudate putamen forming the nigrostriatal pathway, involved in the control of voluntary movement and body posture and b) the ventral tegmental area (VTA), that projects to the ventral striatum (NAcc, amygdala and olfactory tubercle) and the prefrontal cortex modulating cognitive and emotional/rewarding behaviors. Second, a group of cells on the diencephalon that projects to autonomic areas of the lower brain stem, spinal cord, hypothalamus, and to the pituitary gland and amygdala, playing a role in neuroendocrine functions (gonadotropin-releasing hormone and prolactin secretion). Lastly, a small group is found in the telencephalon (olfactory bulb periglomerular interneurons and retina amacrine interneurons) that makes local connections.

Studies in mice suggest that the first mesocorticolimbic DA neurons appear by mid gestation [92]. The different mesocorticolimbic DA cell groups have been reported to be generated at slightly different time points, with a rostrolateral to caudomedial gradient during neurogenesis. Right after these neurons are formed, they start extending neurites in the direction of their migratory pathways, beginning their long way towards their projection areas in the forebrain in a unique developmental process that will be completed

only by early adulthood [41], with final axon density levels and increase in dopamine synapses onto prefrontal pyramidal neurons. During neural development, growing axons find their targets by responding to the coordinated actions of proteins called guidance cues, that form signaling pathways that conduct growing axons to their intended targets [93]. The extent and organization of the mesocortical dopamine axon growth early in life determines the organization of the local PFC circuitry and cognitive function in adulthood [41]. As a result of this protracted developmental trajectory of the prefrontal cortex with progressive changes in dopamine innervation, the dopaminergic neurotransmitter system is especially vulnerable to the effects of environmental adversity during critical periods of development. This explains why the effects of early adversity often involve behaviors related to the function of dopamine signaling, such as inhibitory control, cognition, and sensitivity to reward, with long-term impact on the risk for diseases such as mood disorders, ADHD, addiction, and dementia.

Box 3: Maternal diabetes – when the mother's metabolism is the early adversity

Exposure to a non-optimal fetal environment can also be considered a form of early life adversity. An important example is maternal diabetes mellitus (gestational or pre-existing). In healthy pregnancies, there is a physiological expansion of the adipose tissue in early gestation, followed by insulin resistance and lipolysis in late pregnancy, promoting the use of fatty acids as energy substrates in the mother and saving glucose and amino acids for the growing fetus. These mechanisms are exacerbated in cases where the pregnant woman has had pre-existing obesity or diabetes, with consequent fetal overnutrition [94]. The excess of glucose from maternal blood being transferred by the

placenta to the fetus leads to fetal adaptations including reactive fetal hyperinsulinemia and excessive oxidative stress.

The consequences of this exposure to the offspring are diverse and contemplate health, metabolic, and neurodevelopmental outcomes [95]. Common acute consequences are complications during labor due to macrosomia and difficulties in the physiological adaptation to life ex-utero, such as hypoglycemia, changes in heart rate variability, and respiratory distress. There are also long-term effects of maternal diabetes during pregnancy, including accelerated fetal cardiac growth and altered cardiac development and function, altered hypothalamic circuit formation with elevated body weight and glucose intolerance later in life, and altered glial cell development [94, 96].

Normally, the fetus can cope with the excess of glucose, maintaining normal glycemia, but having higher levels of circulating insulin. Such increased exposure to insulin during prenatal period induces not only anabolic effects (growth leading to macrosomia and fat accumulation), but also programs the metabolism to function in an altered state, increasing the risk for diabetes in the long term [96]. This metabolic programming occurs in insulin-targeted tissues such as fat, liver, and pancreas. Since the brain is another target for insulin, it is also impacted, with increased risk for autism and ADHD [97].

It is important to note that the clinical presentation of maternal diabetes covers a wide range of phenotypes, from maternal gestational diabetes (altered glucose tolerance test in mid to late gestation), to different degrees of maternal type 1 and type 2 diabetes mellitus. While these presentations share most of the consequences described above, there are some specificities. For example, fetal malformations are less frequent in cases

of gestational diabetes than in cases where diabetes preceded the pregnancy. Another example is fetal growth restriction as opposed to macrosomia in cases of severe maternal diabetes with vascular compromising and placental insufficiency. Insulin is an important modulator of fetal growth and alterations such as hyperinsulinaemia and hyperglycaemia dramatically affect fetal growth and development, as well as short and long-term offspring morbidity.

Glossary

Akt: Serine/threonine-specific protein kinase involved in cellular processes such as glucose metabolism, cell migration, cell proliferation, apoptosis, and transcription.

cAMP: Cyclic adenosine monophosphate is a second messenger used for intracellular signal transduction, such as transferring effects of hormones like glucagon and adrenaline into cells which cannot pass through the plasma membrane.

DDC: Dopa Decarboxylase is the encoded protein that catalyzes the decarboxylation of L-3,4-dihydroxyphenylalanine (DOPA) to dopamine, L-5-hydroxytryptophan to serotonin and L-tryptophan to tryptamine.

GLUT (1,3, 4): Glucose transporters are membrane proteins that facilitates the transport of glucose across the plasma membrane through facilitated diffusion. GLUT1, in adults, can be found in the endothelial cells of the blood-brain barrier. GLUT3 is mostly expressed in neurons where it is the main glucose transporter isoform. GLUT4 is expressed in adipose tissues and striated muscle.

HPA Axis: The hypothalamic-pituitary-adrenal axis describes the interaction between the hypothalamus, pituitary gland, and adrenal glands. Its main function is to respond to stress by secreting corticotropin-releasing hormone and adrenocorticotropic hormone into the bloodstream.

IR: Insulin receptor is a transmembrane receptor activated by insulin, IGF-1, and IGF-II. Neuronal IR signaling has been linked to energy homeostasis, reproduction, and the development of neurodegenerative diseases.

IRS1: Insulin receptor substrate 1 is a gene that encodes a protein which is phosphorylated by insulin receptor tyrosine kinase. Mutations in this gene are associated with type II diabetes and susceptibility to insulin resistance.

Long-Term Potentiation and Depression: Patterns of synaptic activity that produce a long-lasting increase (potentiation, or LTP) or decrease (depression, or LTD) in signal transmission between two neurons.

MAOB: Monoamine oxidase B is a protein that catalyzes the oxidative deamination of biogenic and xenobiotic amines and involved in the metabolism of neuroactive and vasoactive amines in the central nervous system and peripheral tissues.

Mesocorticolimbic: A system, extending from the ventral tegmental area to the nucleus accumbens and prefrontal cortex, which comprises a dopamine projection implicated in reward, motivation, and reinforcement learning.

Monoaminergic neurotransmission: Describing a relation to the monoaminergic system, which is composed by the neurotransmitters dopamine, noradrenaline, and serotonin.

Phosphatidylinositol (PI) 3-kinase: Central enzyme in a signaling pathway that mediates cellular responses to insulin and other growth factors. This enzyme phosphorylates the **3** position of phosphatidylinositol-4,5-bisphosphate to produce phosphatidyl-inositol-**3**,4,5-trisphosphate (PIP_3) at the plasma membrane.

Proinflamatory Cytokines: Signaling molecule secreted from immune cells to initiate the inflammatory response against pathogens mediating the immune response.

Programming: Programming effects leave persistent marks on the individuals' physiology and define his or her health and disease patterns in the long term as shown

through studies which explain that fetal exposure to maternal depression during pregnancy has persistent effects on the metabolism of the young adult offspring.

SOCS3: Suppressor Of Cytokine Signaling 3 is a cytokine-inducible negative regulators of cytokine signaling. Studies of the mouse counterpart of this gene suggest that it plays a role in the negative regulation of fetal liver hematopoiesis and placental development.

Sympathoadrenal Axis: The sympathoadrenal system is a physiological connection between the sympathetic nervous system and the adrenal medulla and is involved in an organism's physiological response to outside stimuli.

TH: Tyrosine hydroxylase is the rate-limiting enzyme of catecholamine biosynthesis; it uses tetrahydrobiopterin and molecular oxygen to convert tyrosine to DOPA.

VMAT2: Vesicular monoamine transporter 2 is an integral membrane protein that transports monoamines, such as dopamine, norepinephrine, serotonin, and histamine from cellular cytosol into synaptic vesicles.

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Chapter III. Early life adversity and polygenic risk for high fasting insulin are associated with childhood impulsivity

Preface

As described in the previous chapter, early life adversity alters executive function behaviors later in life. One such executive function is impulsivity, a behavior which can be characterized as the preferences for a small and immediate reward instead of a delayed larger reward [62]. Impulsivity is a trait that constitutes several executive function disorders, making it an ideal behavior to test when inspecting executive functioning. Specifically, adversity has shown to affect impulsivity early in life [63] and in adulthood [64] but the mechanism through which adversity impacts impulsive behavior is still being explored in literature. Early life adversity is also known to impact insulin sensitivity early in life, leading to brain insulin resistance later in life. Prenatal adversity predisposes an individual's body to allocate resources for survival, leaving certain organs deprived such as the pancreas causing long term changes in insulin signaling [65]. Insulin signaling in the brain affects executive function behavior as described in the last chapter. Therefore, in our first study, we wanted to inspect the relationship between early life adversity, insulin, and the executive function impulsivity. Considering that the genetic background represents variations in biological function, we decided to use the genome wide association study for fasting insulin to investigate whether insulin mediates the relationship between early life adversity and impulsivity. Through this study, we will be able to reach a better understanding of the mechanisms behind which adversity impacts impulsive behavior alterations in which insulin signaling plays a role.

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Early life adversity and polygenic risk for high fasting insulin are associated with childhood impulsivity

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Abstract

While the co-morbidity between metabolic and psychiatric behaviors is well-established, the mechanisms are poorly understood, and exposure to early life adversity is a common developmental risk factor. Early life adversity (ELA) is associated with altered insulin sensitivity and poor behavioral inhibition throughout life, which seems to contribute to the development of metabolic and psychiatric disturbances in the long term. We hypothesize that a genetic background associated with higher fasting insulin interacts with ELA to influence the development of executive functions (e.g., impulsivity in young children). We calculated the polygenic risk scores (PRS) from the genome-wide association study (GWAS) of fasting insulin at different thresholds and identified the subset of single nucleotide polymorphisms (SNPs) that best predicted peripheral insulin levels in children from the ALSPAC cohort [N = 467; $p_{t\text{-}initial}$ = 0.24 (10,296 SNPs), $p_{t\text{-}refined}$ = 0.05 (57 SNPs)]. We then calculated the refined PRS (rPRS) for fasting insulin at this specific threshold in the children from the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) cohort and investigated its interaction effect with adversity on an impulsivity task applied at 36 months. We found a significant effect of interaction between Fasting Insulin rPRS and adversity exposure predicting impulsivity measured by the Snack Delay Task at 36 months [β = -0.329, p = 0.024], such that higher PRS [β = -0.551, p = 0.009] was linked to more impulsivity in individuals exposed to more adversity. Enrichment analysis (MetaCore®) of the SNPs that compose the Fasting Insulin rPRS at this threshold was significant for certain nervous system development processes including dopamine D2 receptor signaling. Additional enrichment analysis (FUMA®) of the genes mapped from the SNPs in the Fasting Insulin rPRS showed enrichment with the accelerated cognitive

decline GWAS. Therefore, the genetic background associated with risk for adult higher fasting insulin moderates the impact of early adversity on childhood impulsivity.

Keywords: ALSPAC, MAVAN, Fasting Insulin, Early Life Adversity, Impulsivity

Introduction

Early life adversity (ELA) increases the risk for adult chronic disease, including psychopathology, metabolic, endocrine, and cardio-metabolic conditions [1-6]. Neuroimaging studies have associated certain prenatal adversities with altered structural and functional trajectories in brain development [1, 2, 7-10]. Since certain areas of the brain continue developing until late adolescence, the brain is also highly sensitive to postnatal adversity. Childhood adversity has been linked to long term behavioral outcomes and neurobiological consequences: emotional problems [11], aggressive behaviors [12], changes in brain electrical activity [13, 14], cognitive functions [15], and executive functions [16]. The mechanisms contributing to the development of these phenotypes involve gene by environment interactions resulting in behavioral differences (e.g., attention, impulsivity, food preferences). However, not all individuals exposed to adversity develop these alterations. Responses to early adversity exposure have individual differences that are mostly driven by the genetic background.

At the neuroendocrine level, ELA is linked to alterations in responsivity to stress while also altering insulin sensitivity at different ages. Stressful conditions or adversities happening early in life, either pre- or postnatally, can affect glucose homeostasis and insulin function in the short and long terms. Some adversities are associated with a higher risk for insulin resistance and diabetes, such as: maternal/paternal history of diabetes [17, 18], exposure to gestational diabetes [19], socioeconomic status [20], placental

insufficiency [21], cigarette smoking [22], maternal malnutrition [23], and chronic stress [24, 25]. Such adverse events associated with both growth and metabolism [26-28]. These events also alter responses to subsequent stressors [29-31] and induce chronic inflammation [32, 33], both of which modify glucose homeostasis and insulin sensitivity [34, 35]. Beyond acute effects on brain development and child behavior [36-38], long-term effects of adversity increase the risk for both metabolic diseases [39, 40] as well as psychopathologies later in life [41-43].

Insulin is one of the primary hormonal regulators of metabolism in animals with several different functional roles [44]. Although most peripheral tissues depend on insulin signaling to acquire glucose, such is not the case with the brain as insulin is not needed for glucose transport into neurons [45]. However, brain insulin does play a role as a neuroregulatory peptide [46-48] acting in different brain areas such as the ventral tegmental area, striatum, hypothalamus, hippocampus, olfactory bulb, and prefrontal cortex [49]. Insulin within these areas modulates the development and expression of different executive function behaviors [45], such as attention, inhibitory control, and working memory. Insulin has also been shown to reduce activity in the prefrontal areas that control behaviors such as inhibitory control of eating [50]. Furthermore, abnormal insulin levels and function are seen in Alzheimer's patients where insulin impairments have been linked to learning deficits and memory formation impairments [51].

Considering that the genetic background represents variations in biological function, our objective was to develop a model that predicts an executive function behavior, impulsivity, as a function of the interaction between biological markers of elevated fasting insulin levels and early life adversity in children. To do so, we first assessed the

relationship between a polygenic risk score (PRS) derived from genome-wide associations with high fasting insulin [52] and the actual peripheral insulin levels measured in children from the ALSPAC cohort [53, 54]. The genome-wide association study (GWAS) of fasting insulin (Fasting Insulin GWAS) was performed in adults where the insulin measured was collected from individuals following a fasting period [52]. Since our study inspects the role of insulin in children, we used the ALSPAC cohort's data on peripheral insulin levels to identify the polygenic markers most highly associated with peripheral insulin levels in children. We further refined these markers to only include SNPs that significantly predicted peripheral insulin levels in ALSPAC. Because brain insulin levels are not readily measured or available, a genetic marker reflecting peripheral insulin levels in children was used to inspect insulin's role in neurodevelopmental behaviors. Using the SNPs identified in the discovery cohort ALSPAC, we calculated a refined polygenic risk score (rPRS) in an independent cohort [Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN)] to investigate the interaction between the genetic background associated with fasting insulin in children and early life adversity to predict childhood impulsivity.

Methods

Participants

We used data from two prospective birth cohorts, one based in England (Avon Longitudinal Study of Parents and Children – ALSPAC) [55] and the other in Canada (Maternal Adversity, Vulnerability, and Neurodevelopment – MAVAN) [56] to analyze the gene by environment interaction effects on cognitive neurodevelopment outcomes.

The Avon Longitudinal Study of Parents and Children (ALSPAC): The ALSPAC cohort included pregnant women from the county of Avon, UK [53, 54] (N = 14,541) with expected delivery dates between April 1991 and December 1992. Additional recruitment (N=913) was done during later phases, bringing the total sample size to 15,454. Participants provided informed written consent to participate in the study. Consent for biological samples had been collected in accordance with the Human Tissue Act (2004). Ethics approval for the study was obtained from the ALSPAC Ethics and Law Committee and the local research ethics committees (a full list of the ethics committees that approved different aspects of the ALSPAC studies is available at http://www.bristol.ac.uk/alspac/researchers/research-ethics/). Data were collected during clinic visits or with postal questionnaires. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool at http://www.bristol.ac.uk/alspac/researchers/our-data/. For the purpose of our analysis, we included children of 8.5 years old (an age closer to the outcome measure in the MAVAN cohort), whose mothers had a pregnancy duration between 37 and 42 weeks, a maternal age at delivery greater than 18 years, a child birthweight greater than 2 kg, child alive at 1 year of age, and we only included singleton pregnancies in the analysis. *Figure 3* describes the subset of the sample for the purpose of the analyses in the ALSPAC cohort. There were 467 subjects with complete data available for the analyses.

Maternal Adversity, Vulnerability, and Neurodevelopment (MAVAN) Project [56]: The study MAVAN is a birth cohort that followed up children from birth up to 6 years of age in Montreal (Quebec) and Hamilton (Ontario), Canada, and has 630 recruited

participants [56]. Mothers aged 18 years or above, with singleton pregnancies, and fluent in French or English were included in the study. Several maternal chronic illnesses, including placenta previa and history of incompetent cervix, impending delivery, a fetus/infant affected by a major anomaly, or gestational age <37 weeks composed the exclusion criteria. Approval for the MAVAN project was obtained by the ethics committees and university affiliates (McGill University and Université de Montréal, the Royal Victoria Hospital, Jewish General Hospital, Centre hospitalier de l'Université de Montréal and Hôpital Maisonneuve-Rosemount) and St. Joseph's Hospital and McMaster University, Hamilton, Ontario, Canada. Informed consent was obtained from all participants. *Figure 4* describes the criteria and selection of MAVAN sample for the purpose of our research. There were 101 subjects with complete data available for the analyses.

Genotyping

Avon Longitudinal Study of Parents and Children: Children in the ALSPAC cohort were genotyped using the Illumina HumanHap550 quad chip genotyping platform by the Wellcome Trust Sanger Institute, Cambridge, UK and the Laboratory Corporation of America, Burlington, NC, US [57]. Standard quality control (QC) procedure was applied: participants with inconsistent self-reported and genotyped sex, minimal or excessive heterozygosity, high levels of individual missingness (>3%), and insufficient sample replication (IBD < 0.8) were excluded. Also, SNPs with call rate < 95%, MAF < 1%, or not in Hardy-Weinberg Equilibrium (HWE) ($p < 5 \times 10^{-7}$) were removed. Following the QC, the genotyping data was imputed using Impute v3 and Haplotype Reference Consortium (HRC) imputation reference panel (release 1.1), which resulted in 38,898,739 SNPs available for analysis.

The population structure of ALSPAC cohort was described using principal component analysis [58, 59], which was conducted on the genotyped SNPs with MAF > 5% with the following pruning parameters for linkage disequilibrium: 100-kilobase sliding window, an increment of 5 SNPs, and variance inflation factor (VIF) threshold of 1.01. To account for population stratification, the first ten principal components were included in the analysis.

Maternal Adversity, Vulnerability and Neurodevelopment: Genome-wide platforms (the Infinium PsychArray v1 or the PsychChip v1.1/v1.2, Illumina, Inc.) were used to genotype 229,456 autosomal SNPs of buccal epithelial cells of children in MAVAN, according to the manufacturer's guidelines. SNPs with a call rate < 95%, MAF < 5%, or not in HWE ($p < 1 \times 10^{-30}$) were removed. Afterwards, imputation using the Sanger Imputation Service (McCarthy et al., 2016) and HRC as the reference panel (release 1.1) was performed and SNPs with an info score > 0.80 were retained for the analysis, resulting in 16,249,769 autosomal SNPs.

Similar to the ALSPAC cohort, the population structure of the MAVAN cohort was evaluated using principal component analysis of all autosomal SNPs that passed the QC and not in high linkage disequilibrium ($r^2 > 0.2$) across 50-kilobase region and an increment of 5 SNPs [59]. Based on the inspection of the scree plot, the first three principal components (PCs) were the most informative of population structure and were included in all subsequent analyses.

Polygenic Risk Scores

The rPRS procedure was administered in this study to inspect the interaction between genetic markers for fasting insulin and ELA to predict impulsivity in children using a GWAS constructed from adult data.

Avon Longitudinal Study of Parents and Children: The fasting insulin polygenic risk score (PRS) was calculated using the Fasting Insulin GWAS, shown in *Figure 5* (N = 108,557) from the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) [52]. Prior to any PRS calculation, the GWAS was subjected to LD clumping with r^2 of 0.2 and ALSPAC cohort as a reference dataset. PRS at 100 different GWAS p-value thresholds were calculated for each subject in the ALSPAC cohort as a sum of the risk alleles count weighted by the effect size described in the GWAS for each SNP [60, 61]. Using ALSPAC as a discovery cohort, we identified the threshold at which the PRS best prediction of peripheral insulin levels in children at age 8.5 years. The strongest (R^2 = 0.039) and most significant (p = 0.071) association in children at age 8.5 years was identified to be with a PRS at $p_{t\text{-initial}}$ = 0.24 threshold (consisting of 10,296 SNPs) as shown in *Figure 6*. To further refine the PRS, a process explained through *Figure 7*, we ran a linear regression analysis for each SNP within the 0.24 threshold PRS to find which SNPs were significantly associated ($p_{t\text{-refined}} < 0.05$) with the peripheral insulin levels. There were 57 SNPs significantly associated with peripheral insulin levels within the SNPs included in the 0.24 threshold. The list of these SNPs can be found in *Table 2* with their corresponding *p*-values from the Fasting Insulin GWAS [52]. These 57 SNPs included in the rPRS were ranging in *p*-values from 0.000123 to 0.238 in the original GWAS [52], however, they were all significantly associated with the peripheral insulin levels in children (all *p*-values < 0.05). This finding confirms that SNPs associated with

adult risk for high fasting insulin may not be the same as SNPs associated with children risk for high fasting insulin.

Maternal Adversity, Vulnerability and Neurodevelopment: The 57 SNPs within the PRS that we discovered to be associated with peripheral insulin levels in the ALSPAC cohort were used to construct a PRS in MAVAN. The PRS was standardized. Since the SNPs were selected through a refinement process of a PRS that was created through conventional means, we henceforth refer to this PRS in the MAVAN cohort as the refined PRS (rPRS). The rPRS was calculated similarly to the PRS scores in ALSPAC, as a weighted sum of 57 SNPs.

Early Life Characterization

To investigate the interaction between the PRS and early life adversity, we estimated adversity exposure using a cumulative score involving different environmental variables [62] for each individual in the MAVAN cohort as described by Silveira et al. [62] and de Lima et al. [63]. The adversity score was created by combining several markers of adversity. The following instruments were included as markers in the score: 1) *The Health and Well-Being Questionnaire* [64] to obtain information on how often and to what degree the woman lacked money for basic needs [65] using the *Daily Hassles Scale*, on chronic stress with the romantic partner [66], on conjugal violence [67, 68], on anxiety during pregnancy [69], on birth size percentile, and on gestational age; 2) *Smoking during pregnancy*; 3) *Household gross income [70]*; 4) *Child Health Questionnaire* [71] to assess acute, chronic conditions and hospitalizations; 5) *Maternal mental health* through the Beck Depression Inventory (BDI) [72], Edinburgh Postnatal Depression Scale (EPDS) to screen for postpartum depression [73], and State-Trait Anxiety Inventory (STAI) to

measure psychological components of state and trait anxiety [74]; 6) *Attachment* through the Preschool Separation-Reunion Procedure (PSRP) applied at 36 months [75] [76]; 7) *Family Assessment Device* to assess family functioning based on the McMaster Model of Family Functioning [77]. For every item with a continuous score, we used either the 15th or the 85th percentile as the cut-off to add a point to the adversity score. Presence of each component yields one point, and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

Behavioral Outcomes

In the Snack Delay Task at 36 months [78-81], the children placed their hands flat on a table in front of them and restrained themselves from eating a single M&M candy from under a glass cup placed on the table. The children were told to delay eating until the research assistant rang a bell. The test was conducted over four distinct trials (using delays of 10, 20, 15, and 30 seconds). Halfway through each trial delay, the experimenter lifted the bell but did not ring it. The children received a behavioral score for each trial ("behavior code") based on attempts to eat the candy before the bell rang. Coding ranged from 1 to 7, as displayed in *Table 2*. For each trial, the ability of the children to wait for the M&M was also recorded (snack delay latency to eat: 1 = child keeps hands on the table during the entire time either before OR after the bell is lifted and 2 = child keeps hands on the table during the entire time before AND after the bell is lifted). This latter score (1 or 2) and the behavioral code score (ranging from 1 to 7) culminated to provide a total performance score (ranging from 2 to 9) for each of the four trials. A "global cooperation score" rated the ability of the child to engage and complete the task (0 = the

child is unwilling or unable to engage in the task; 1 = the child is unwilling or unable to complete the task because of feeling tired, angry, irritable, or sick, or does not have the capacity to understand the instructions; 2 = the child does all the trials but has comprehensive or motivational difficulties, or is passive or inhibited, and 3 = the child understands the task well and participates). Children with a global score of 3 were included in the analysis. The Snack Delay Task was applied to children in the MAVAN cohort at the age of 36 months.

Statistical analysis

Data analyses were carried out using R [82]. Baseline comparisons between low and high PRS groups were done in the MAVAN cohort. Mean differences of the main confounding variables were assessed using the Student's *t*-test for independent samples if they were continuous variables or the chi-square test if they were categorical variables. Significance levels for all measures were set at $\alpha < 0.05$.

We used linear regression models to test the association between the outcome of impulsivity, evaluated using the Snack Delay Task in this case, and the predictors including adversity exposure, rPRS, and their interaction term with sex and the first three genomic PCs as covariates. In summary, we ran the following three linear regression models:

1. Outcome ~ sex + PC1 + PC2 + PC3 + Adversity Score
2. Outcome ~ sex + PC1 + PC2 + PC3 + rPRS
3. Outcome ~ sex + PC1 + PC2 + PC3 + Adversity Score$\times$rPRS

To identify the form of interaction between the rPRS and the adversity score, we used Roisman's method [84, 85] of simple slopes analysis and examined the regions of

significance (RoS) to determine the range of values of the predictor for which regression of the outcomes on the moderator (rPRS) is statistically significant. To explore the form of interaction, Roisman also recommends the use of two metrics designed to help identify between diathesis-stress and differential susceptibility models: the proportion of interaction (PoI) index and the proportion affected (PA) or the percentage above index. Both metrics show a preponderance of differential susceptibility when greater than a certain threshold. As a sub-analysis to handle the missing cases for the adversity score, we imputed the data with hot-deck imputation (hot.deck package in R) [86], assuming missing at random mechanisms. We imputed all adversity score components, calculated the extended adversity score on an additional 98 subjects, and repeated the linear regression analysis on the imputed datasets of 199 subjects each, reporting the pooled estimates from 30 imputed sets.

Enrichment Analysis

Enrichment analyses for gene ontologies were performed using MetaCore™ (Clarivate Analytics) on the SNPs that compose the Fasting Insulin rPRS. Furthermore, gene-based enrichment analyses were performed in FUMA (https://fuma.ctglab.nl/) [87-89] after mapping the SNPs composing the Fasting Insulin rPRS to genes with the biomaRt package in R [90, 91]. We also used GeneMANIA [92] to determine if the genes were part of a network. Specifically, the gene list derived from the Fasting Insulin rPRS is entered in GeneMANIA. GeneMANIA then extracts linked mRNA expression data from the Gene Expression Omnibus (GEO) and connects co-expressed data to form functional association networks. The node sizes represent gene scores indicating the number of paths that start at a given gene node and end up in one of the query genes.

Results

Baseline characteristics

Baseline comparisons between low and high rPRS groups were performed in the MAVAN cohort. No differences were found for the main confounding variables in the MAVAN cohort, as shown in *Table 4*. Participants' characteristics for ALSPAC cohort are reported in *Table 5*. *Table 6* details the degree of missing data for each component of the adversity score.

Interaction between Fasting Insulin PRS and the adversity score associates with impulsivity in MAVAN

We performed a linear regression analysis to investigate the interaction effect between the refined genetic score (rPRS) and adversity exposure on the Snack Delay Task in the MAVAN cohort applied at 36 months, adjusted by population stratification PCs and sex. A significant interaction effect was observed, as displayed in *Figure 8*, between Fasting Insulin rPRS and adversity exposure on impulsivity measured by the Snack Delay Task [β = -0.329, p = 0.024]. Simple slope analysis at $\pm$1 SD rPRS showed that higher early life adversity is linked to more impulsivity in children with higher rPRS [β = -0.551, p = 0.009]; there was no effect of adversity on impulsivity in the low rPRS group [β = 0.139, p = 0.348]. The region of significance is to the right side of the red line in *Figure 8*, which suggests that the association between impulsivity and the rPRS is significant in children highly exposed to adversity. We also analyzed the form of the interaction according to Roisman [84]. The RoS, as well as the proportion of interaction PoI (0.984) and PA (0.782) are consistent with the diathesis-stress model.

To obtain a distribution of the statistics of interest (interaction coefficient) and its confidence interval, we applied a nonparametric bootstrap, which resulted in estimated beta = -0.329 (SE=0.1764) and 95% confidence interval (-0.6729, -0.0202) for the effect of interaction between rPRS and adversity on Snack Delay Task in MAVAN.

The main effect of the refined genetic score (rPRS) on the Snack Delay Task in MAVAN [N = 101] applied at 36 months, adjusted by PCs and sex, was not significant [β = -0.190, p = 0.126]. The main effect of adversity on the Snack Delay Task in MAVAN [N = 101] applied at 36 months, adjusted by PCs and sex, was also not significant [β = -0.131, p = 0.171]. When performing imputations on the missing cases of the adversity score (final N=199), the effect of the interaction between rPRS and adversity score on Snack Delay outcome was no longer statistically significant [β = 0.082, p = 0.168].

Enrichment analysis on Fasting Insulin PRS

Enrichment analyses (MetaCore™) of the SNPs that compose the Fasting Insulin refined genetic score (rPRS) show that this subset of SNPs was significant for several nervous system development processes, as shown in *Figure 9.* Enrichment analysis (FUMA, https://fuma.ctglab.nl/) of the genes mapped by the SNPs that compose the Fasting Insulin rPRS showed that these genes were significantly differentially upregulated in the following brain specific tissues, as shown in *Figure 10*: hippocampus, frontal cortex Brodmann area 9 (BA9), anterior cingulate cortex Brodmann area 24 (BA24), and the hypothalamus. Furthermore, these genes also had a significant GWAS enrichment for accelerated cognitive decline after conversion of mild cognitive impairment to Alzheimer's disease (*FDR adjusted p-value* = 0.013). Using GeneMANIA [93], we discovered that this

set of genes was part of a single co-expression network in *Homo sapiens*, as shown in *Figure 11*, indicating their shared involvement in biological processes [94].

Discussion

The purpose of this study was to explore whether the genetic background associated with higher fasting insulin interacts with early life adversity to predict impulsivity, tested using the Snack Delay Task, in children. We demonstrated that the calculation of a refined polygenic risk score, consisting of SNPs most associated with peripheral insulin levels in children and representing the risk for high fasting insulin levels early in life, can be derived from the GWAS of fasting insulin in adults. This refined polygenic score interacted with early life adversity exposure to predict impulsivity in children in the MAVAN cohort. Additionally, we observed that the SNPs composing the Fasting Insulin rPRS and their mapped genes were significantly correlated with various nervous system development processes.

Instead of using an arbitrary *p*-value threshold to calculate the polygenic risk score, we calculated the PRS at one hundred different thresholds in the independent cohort ALSPAC as a training sample. To calculate the PRSs, we applied the PRSoS tool [95]. For each threshold, we explored the association between PRS and peripheral insulin levels within the ALSPAC cohort. This technique allowed us to identify the threshold of 0.24 to best predict peripheral insulin levels in children [96]. To further refine this PRS, we associated each SNP within the subset obtained from the 0.24 threshold PRS with peripheral insulin levels in the discovery cohort ALSPAC and selected only the SNPs that significantly predicted the peripheral insulin levels in children to calculate our final rPRS. This refinement was necessary as the genetic markers for fasting insulin levels in adults

may not be comparable to the genetic markers for fasting insulin levels in children. Since there is no GWAS available to identify the SNPs most associated with risk for fasting insulin levels in children, we took an alternative approach, the rPRS, that allowed us to identify a subset of SNPs associated with fasting insulin levels in children. Usually, analyses identifying which PRS threshold should be used are based on the greatest proportion of variance explained in the outcome, which would be the Snack Delay Task in this study. That does not take into account which PRS threshold is best predicting the phenotype composing the PRS itself, making our approach distinctive. Another strength in our approach is that we used a training sample to identify the best PRS threshold to use in our test sample. Subsetting the list of SNPs further adds to our distinctive methodology because we can be confident that the genetic background of fasting insulin used within the analysis is in fact correlated with actual peripheral insulin levels in children. The rPRS is a better predictor than peripheral insulin levels because the genetic background represents a more stable characteristic than the fluctuant insulin levels, which oscillate diurnally and may not be an accurate representation of a child's fasting insulin levels later in adulthood. By using the rPRS, which was calculated using the GWAS related to adults fasting insulin levels, we obtained a more accurate representation of the risk of a child to develop high fasting insulin. There is no overall effect of either adversity score or rPRS, but there is an interaction effect of adversity and rPRS on impulsivity at 36 months. Specifically, the effect of adversity on impulsivity was seen for individuals with a higher rPRS for fasting insulin.

Although the results of the interaction between rPRS and adversity were no longer significant after imputing the missing cases for the adversity score, we want to emphasize

that the adversity score is a composite measure computed based on several different tools and assessments, including total scores of instruments (e.g. BDI) or complex behavioral tasks such as the Attachment profile assessed through detailed coding of filmed interactions (Strange Situation Task) (*Table* 6). Therefore, as much as imputations can be technically performed, we are not convinced that the imputed data can capture the multifaceted feature of our unique composite score, so our main analysis is focused on complete cases. Our missing data was mostly related to unit-level non-response (no information was collected for the respondent on a specific survey/instrument/questionnaire) rather than item non-response (the respondent was missing one or two questions of the survey/instrument/questionnaire) [97]. Unit-level non-response can be more challenging to impute with confidence [98]. Relaxing the complexity of the adversity score mentioned above and considering the missing components of the score as missing items in the dataset, the hot-deck imputation was applied. While imputation is useful and necessary to support analysis and summarization, the imputation model should be properly specified, which we believe is difficult to achieve in this particular case. Some of the variables that compose the adversity score, for example the Attachment security information, are derived from a laboratory procedure designed to capture the balance of attachment and exploratory behavior under conditions of increasing moderate stress [99], a unique measure that is hardly comparable to any other measure available in the dataset. Finally, we assumed a missing at random mechanism for the missing data, although adversity itself could be associated with the missingness pattern [100, 101]. Therefore, the results of this sub-analysis should be considered with caution.

The gene ontology enrichment analyses, done through MetaCore™, showed that the SNPs composing the Fasting Insulin rPRS are associated with nervous system development. Some of the enriched processes should be highlighted, such as the neurophysiological process of dopamine D2 receptor signaling in the central nervous system. The dopamine system has been linked to impulsive behavior in animal models and human studies [102, 103]. This finding is interesting because it suggests a potential neurodevelopment pathway to support the relationship between the genetic background linked to insulin, ELA, and dopamine. Previous animal models from our laboratory have demonstrated that animals exposed to ELA showed a pronounced aversion to delayed rewards in addition to an increase in the medial prefrontal cortex D2 levels [104]. Additionally, our laboratory has shown that animals that have experienced ELA have a delay in dopamine release in the nucleus accumbens in response to palatable food, but insulin administration reverts this delayed effect [105]. These studies show that the relationship between ELA and dopamine is moderated by insulin. The set of fasting insulin SNPs identified in this present study could lead to further insight on the genetic background linking dopamine to impulsivity. Since the mechanism involving this association is still unknown, our findings bring us a step closer to this understanding.

Enrichment analyses in MetaCore™ revealed that the rPRS is enriched for single-stranded RNA binding. This suggests that the SNPs composing the rPRS play a crucial role in post-transcriptional regulation of gene expression [106] and hence are key in gene by environment interaction effects. The genes mapped to the SNPs in the rPRS are also differentially upregulated in the frontal cortex BA9, which is known to be involved in several executive functions such as short-term memory, inductive reasoning, working

memory, and planning [107]. These findings, in addition to the genes being enriched for the accelerated cognitive decline GWAS, suggest that the genetic background associated with fasting insulin can impact several neurodevelopment executive functions, impulsivity being one of them, as well as risk for cognitive decline later in life. This aligns with studies that identified insulin receptors at hippocampal glutamatergic synapses, suggesting a role of insulin in neurotransmission, synaptic plasticity, and modulation of learning and memory, while its inhibition is described in Alzheimer disease and related animal models [108].

There are limitations within our study. Our discovery cohort and our testing cohort both largely consist of White/European ancestry, allowing us to identify the SNPs required within one cohort and testing the hypothesis within another cohort that has a similar population structure. Unfortunately, we cannot be certain that this subset of SNPs will be relevant for a different ancestry. Different ancestries have distinct allele frequencies [109] and this could result in peculiarities in the interaction between the genetic background and the environment. In fact, differential linkage disequilibrium between ancestral populations can produce false-positive SNPs when local ancestry is ignored, meaning that gene expression traits have differences as a function of genetic ancestry [110]. Furthermore, several studies showed genomic differences when investigating multi-ancestry genomic analysis [111, 112]. In addition to ancestry, culture can impact one's behaviors, especially those related to executive functions like impulse control. There have been several examples of gene-culture interactions such as the cultivators in West Africa whose agriculture, which consisted of malaria-carrying mosquitos, showed preference for the hemoglobin S (*HbS*) 'sickle-cell' allele to provide protection from malaria [113].

Similarly, Polynesians being exposed to cold stress and starvation during their long open-ocean voyages may have resulted in positive selection for thrifty metabolism leading to type 2 diabetes susceptibility in present day Polynesisans [114]. This gene-culture evolution emphasizes that one's lifestyle and environment have lasting impact and could be responsible for the differences seen in gene-environment interactions. Unfortunately, to the best of our knowledge, there is currently no Fasting Insulin GWAS available in a different ancestry for us to address this limitation within our work. Future studies including a discovery cohort with peripheral insulin information in children and testing cohort of similar population structure in children are warranted.

These results together confirm that both early life adversity and the biological machinery associated with higher insulin levels are important factors influencing impulsivity in children. Our analyses showed that the genetic background associated with high fasting insulin levels moderates the effects of adversity on childhood impulsivity. This reinforces the idea that insulin signaling, which is implicated in metabolism and child growth, also plays a role in neurodevelopment. Previous studies have shown that impulsivity is a core feature of both psychopathology and metabolic diseases [115-117]. Therefore, the interaction described here could be the basis to explain the co-morbidity associated with ELA exposure. Our results align with Hari Dass et al. [118], which used a biologically-informed polygenic score based on insulin-related gene networks to predict both childhood impulsivity and risk for dementia later in life.

In conclusion, our present findings provide support for the impact of exposure to early life adversity in interaction with the genetic profile associated with high fasting insulin in predicting executive functions such as impulsivity in children. This research can be

highly impactful as it provides insights into the vulnerability to executive function disorders early on in an individual's life. The biological mechanisms that we discovered to be involved in these processes can inform the development of early interventions and more efficient management of such health outcomes.

Tables and Figures[2]

Table 2: Single nucleotide polymorphisms (SNPs) included in the refined PRS for MAVAN

SNP	P-value
rs7574670	0.000123
rs13225097	0.000846
rs196808	0.005017
rs6885750	0.010573
rs4841679	0.011172
rs870870	0.018874
rs2665316	0.019226
rs4405319	0.031478
rs11724118	0.045920
rs10804992	0.046243
rs6552502	0.058479
rs397234	0.058594
rs11898925	0.061942
rs1866816	0.061986
rs7807790	0.062096
rs2965106	0.062112
rs275146	0.062816
rs7155790	0.065848
rs9840453	0.067170
rs11693862	0.073821
rs1492377	0.074815
rs1377315	0.083513
rs4686837	0.088538
rs4803789	0.089959
rs10520768	0.090182
rs2295308	0.093524
rs7598551	0.094168
rs7332334	0.101271
rs728586	0.103540
rs4779876	0.103930
rs1935492	0.103989
rs7983099	0.112130
rs923554	0.118880

[2] All tables and figures for this chapter were obtained from a paper published in Frontiers. Under the Frontiers Terms and Conditions, authors retain the copyright to their work. All Frontiers articles are Open Access and currently distributed under the terms of the **Creative Commons Attribution License**, which permits the use, distribution and reproduction of material from published articles, provided the original authors and source are credited, and subject to any copyright notices concerning any third-party content. For earlier articles, please check the online page for license details. Therefore, copyright approval was not needed.

rs1885414	0.123295
rs9863801	0.126196
rs11891202	0.135653
rs4382157	0.136635
rs5753103	0.150747
rs17035960	0.151128
rs884972	0.151753
rs10002944	0.154237
rs10145606	0.156256
rs892114	0.159405
rs6543408	0.178625
rs4766912	0.184907
rs7138803	0.185122
rs2544164	0.197849
rs12219445	0.198058
rs1664256	0.199221
rs11603179	0.201429
rs11820303	0.211331
rs2341647	0.217823
rs6888754	0.219617
rs9808140	0.221567
rs1058065	0.223190
rs12731669	0.231006
rs7243066	0.238047

Table 3. The Snack Delay Task was performed at 36 months in the children from the MAVAN cohort. Each child was asked to place their hands flat on a table and to restrain themselves from eating a single M&M candy from under a glass cup placed on the table in front of them. The children were instructed to delay eating until the research assistant rang a bell. The test was conducted over four distinct trials (using delays of 10, 20, 15, and 30 seconds) and the scores of each trial were added together for the final cumulative score.

Snack Delay	
1	Eats before bell is lifted
2	Eats after bell is lifted
3	Touches candy before bell is lifted
4	Touches candy after bell is lifted
5	Touches cup or candy before bell is lifted
6	Touches cup or candy after bell is lifted
7	Waits for bell to ring before touching cup or candy

Table 4: Participants' characteristics in MAVAN.

Sample descriptive	Total (n = 101)	Low PRS (n = 50)	High PRS (n = 51)	*p*
Sex - male	44.6% (45)	42.0% (21)	47.1% (24)	0.756
Maternal age at birth (years)	30.51 (4.65)	30.17 (4.36)	30.84 (4.94)	0.470
Gestational age (weeks)	39.32 (1.17)	39.34 (1.27)	39.29 (1.08)	0.846
Birth weight (grams)	3326 (458)	3371 (472)	3281 (443)	0.325
Duration of breastfeeding (months)	7.23 (4.82)	6.91 (4.83)	7.54 (4.83)	0.511
Smoking during pregnancy	11.9% (12)	10.0% (5)	13.7% (7)	0.786
Maternal education – University degree or above	62.4% (63)	68.0% (34)	56.9% (29)	0.342
Low income at 36m	11.2% (11)	12.2% (6)	10.2% (5)	1.000
Self-reported ethnicity (Caucasian)	78.1% (75)	80.9% (38)	77.1% (37)	0.2

Numbers are presented as mean (SD) or percentage (number of participants). Comparison between Low/High PRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

Table 5: Participants' characteristics in ALSPAC.

Sample descriptive	Total (n = 467)
Sex - male	52.5% (245)
Maternal age at birth (years)	29.89 (4.44)
Gestational age (weeks)	39.76 (1.18)
Birth weight (grams)	3532 (482)
Breastfeeding at 3 m (yes)	51.5% (240)
Smoking during pregnancy (yes)	19.1% (89)
Maternal education – University degree or above	18.7% (84)
Low Socioeconomic Status (SES) measured at 2 years, 9 months	37.3% (174)
Self-reported ethnicity (White)	99.8% (457)

Numbers are presented as mean (SD) or percentage (number of participants).

Table 6: Details of missing data for specific components of the adversity score in Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN). For the main analysis, only complete cases were considered. A sub-analysis after imputing the missing variables using hot deck imputation was also performed.

Instruments included in the Adversity score	**Number of items**	**N available**	**Missing**
Birth information	2	199	0
Household gross income	1	199	0
Child hospitalization	1	199	0
Family Assessment Device	12	136	63
Attachment (scoring 28 min video of parent-child interaction)	1	165	34
Beck Depression Inventory	21	170	29
Edinburgh Postnatal Depression Scale	10	182	17
State-Trait Anxiety Inventory	40	195	4
Pregnancy anxiety	1	172	27
Smoking during pregnancy	1	172	27
Marital strain	9	193	6
Daily Hassles Scale	5	199	0
Physical/sexual abuse	2	199	0
Adversity score	**composite**	**101**	**98**

Figure 3. Block scheme describing sample selection in the ALSPAC cohort.

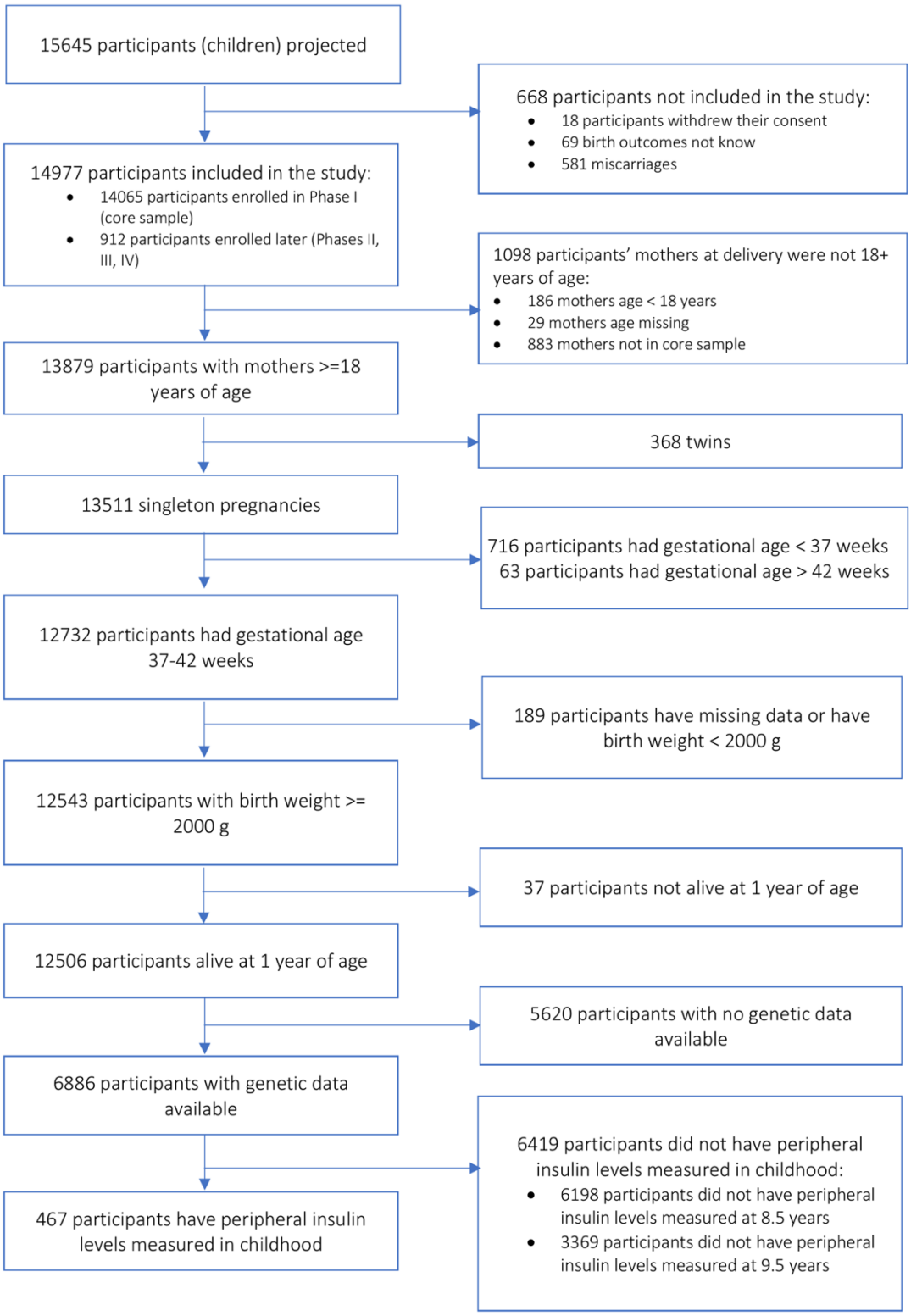


Figure 4. Block scheme describing sample selection in the MAVAN cohort.

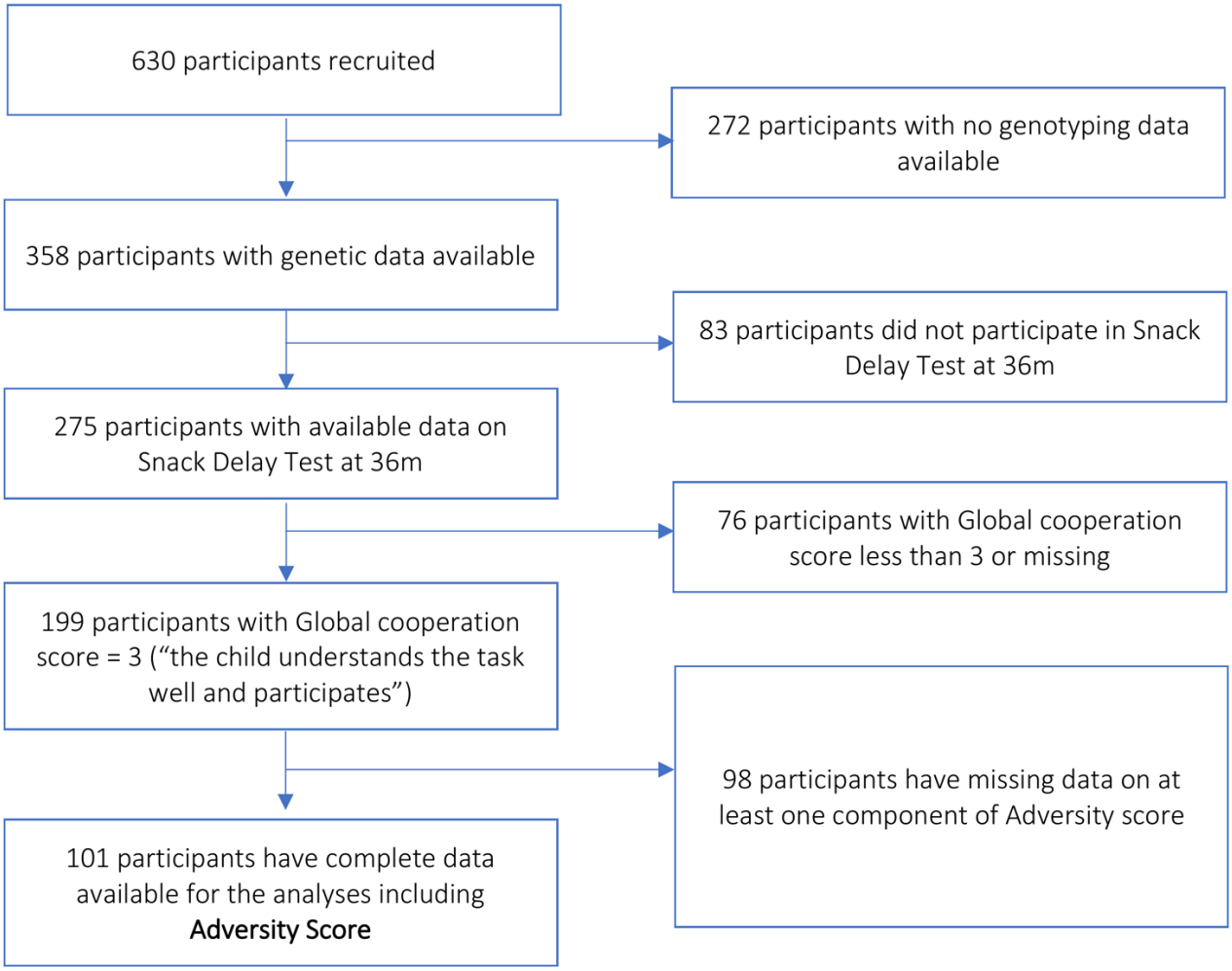


Figure 5. Genome-Wide Association Study (GWAS) for Fasting Insulin from the Meta-Analyses of Glucose and Insulin-related traits Consortium. Each dot represents a single nucleotide polymorphism (SNP), with the x-axis showing genomic location and the y-axis showing the association level of each respective loci to fasting insulin. The gene names for all significant SNPs are displayed in the plot. This plot was obtained from FUMA.

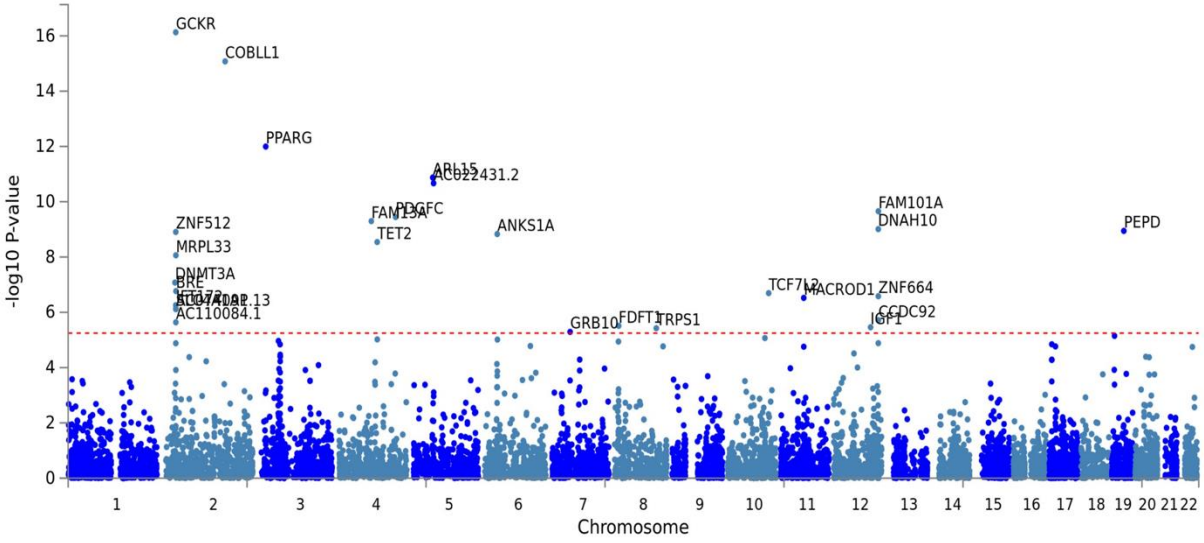


Figure 6. Association between Fasting Insulin PRS and peripheral insulin levels. We calculated polygenic risk scores from the fasting insulin GWAS at 100 different thresholds. Using ALSPAC as a discovery cohort, we identified the PRS threshold with the strongest correlation in predicting peripheral insulin levels in children at age 8.5 years. The strongest (R^2 = 0.039) and most significant (p = 0.071) association was identified to be 0.24 in children at age 8.5 years in the ALSPAC cohort [N = 467; $p\tau_{initial}$ = 0.24 (10,296 SNPs)].

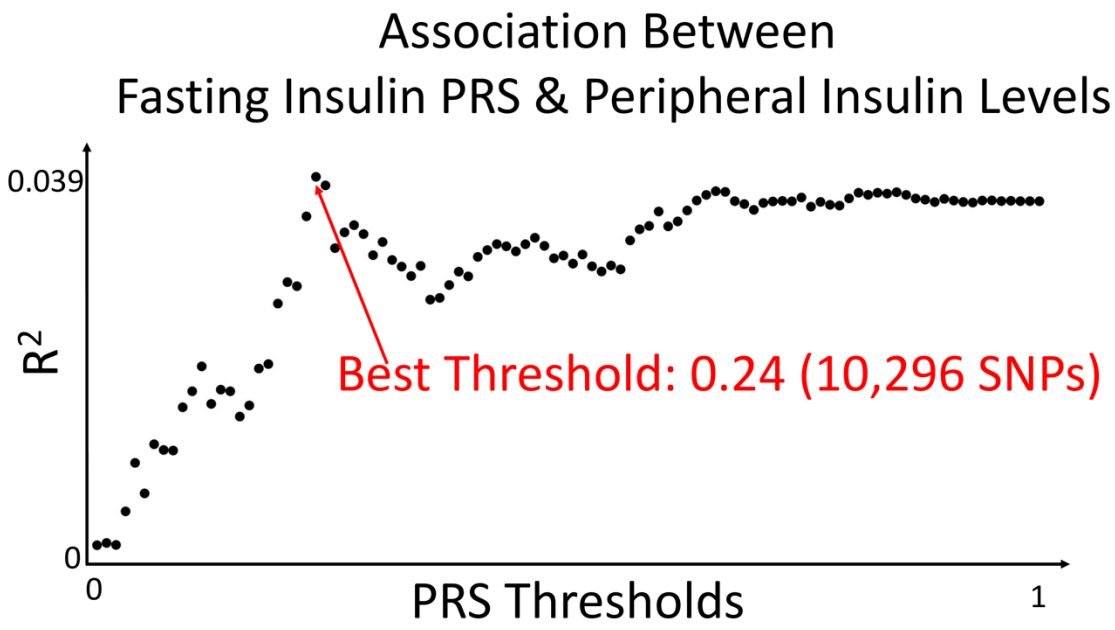


Figure 7. Refined PRS Methodology. This flow chart depicts the refined PRS process. Each color in the gradient on the Fasting Insulin GWAS represents a threshold. In step 1, we used 100 different thresholds between p value 0 and 1 from the Fasting Insulin GWAS and found the threshold that best predicts insulin levels in ALSPAC Children (PRS Threshold 0.24), indicated by the white dotted line on the GWAS. In step 2, we ran a correlation for each SNP within the 0.24 PRS threshold to find which SNPs significantly predicted ($p < 0.05$) the peripheral insulin levels. Lastly, in step 3, using the SNPs that significantly predicted peripheral insulin levels from step 2, we calculated the rPRS in the MAVAN cohort.

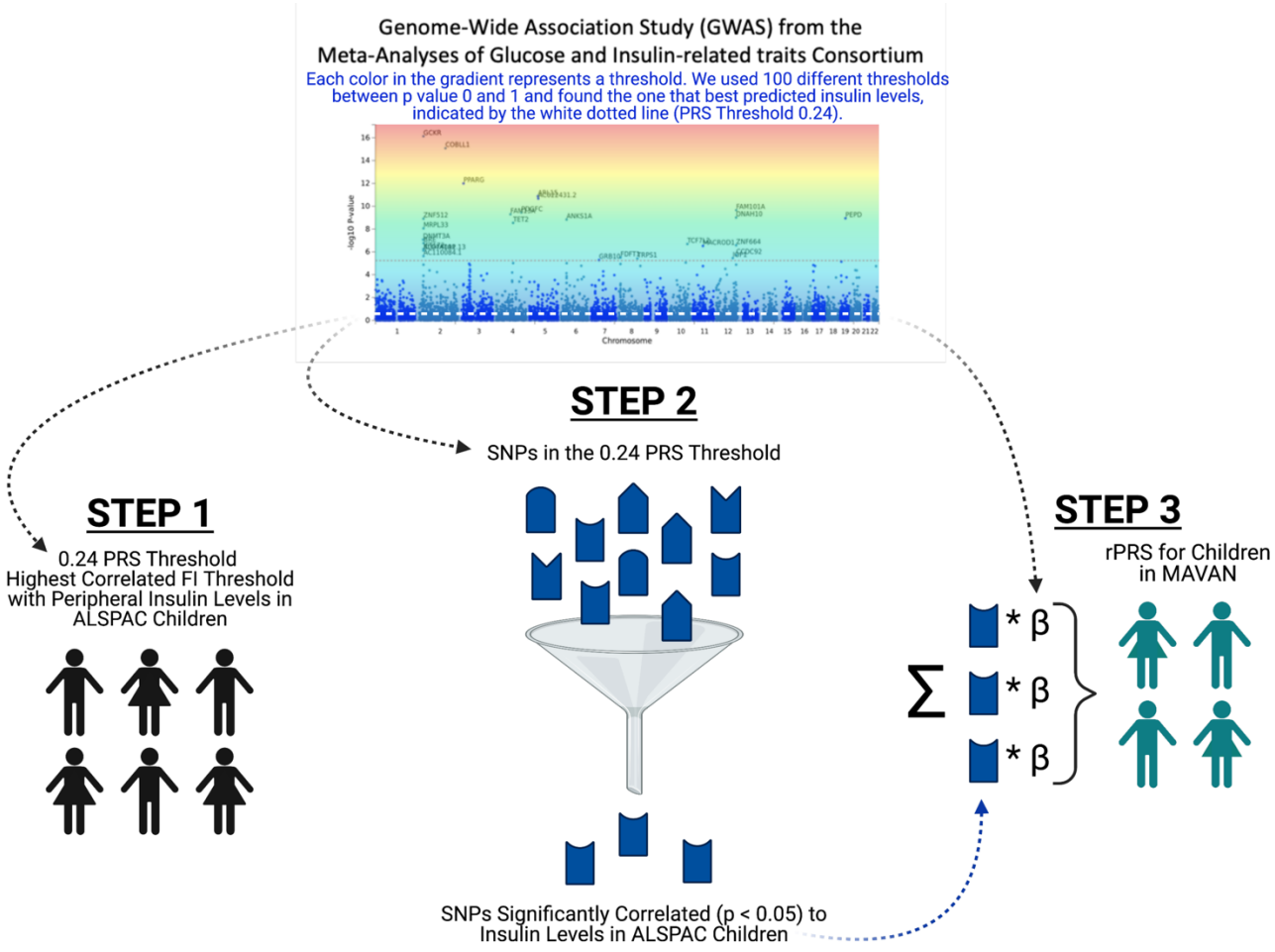


Figure 8. Maternal Adversity, Vulnerability and Neurodevelopment regression analysis. rPRS was calculated for children in MAVAN using the fasting insulin GWAS with significant SNPs identified in ALSPAC through the rPRS methodology. Linear regression analysis showed a significant interaction between fasting insulin PRS and adversity exposure on impulsivity [N = 101, β = -0.329, *p* = 0.024], tested by the Snack Delay Task applied at 36 months: higher adversity is linked to higher impulsivity in children with higher rPRS [rPRS = mean±1 SD, β = -0.551, *p* = 0.009], represented by the solid blue line. The dashed light blue line represents the low rPRS group, where the effect of adversity on impulsivity using the Snack Delay Task was not significant [β = 0.139, *p* = 0.348]. We also analyzed the form of these interactions according to Roisman. The regions of significance, as well as the proportion of interaction (PoI 0.984) and proportion affected (PA 0.782) are consistent with diathesis stress form of interaction.

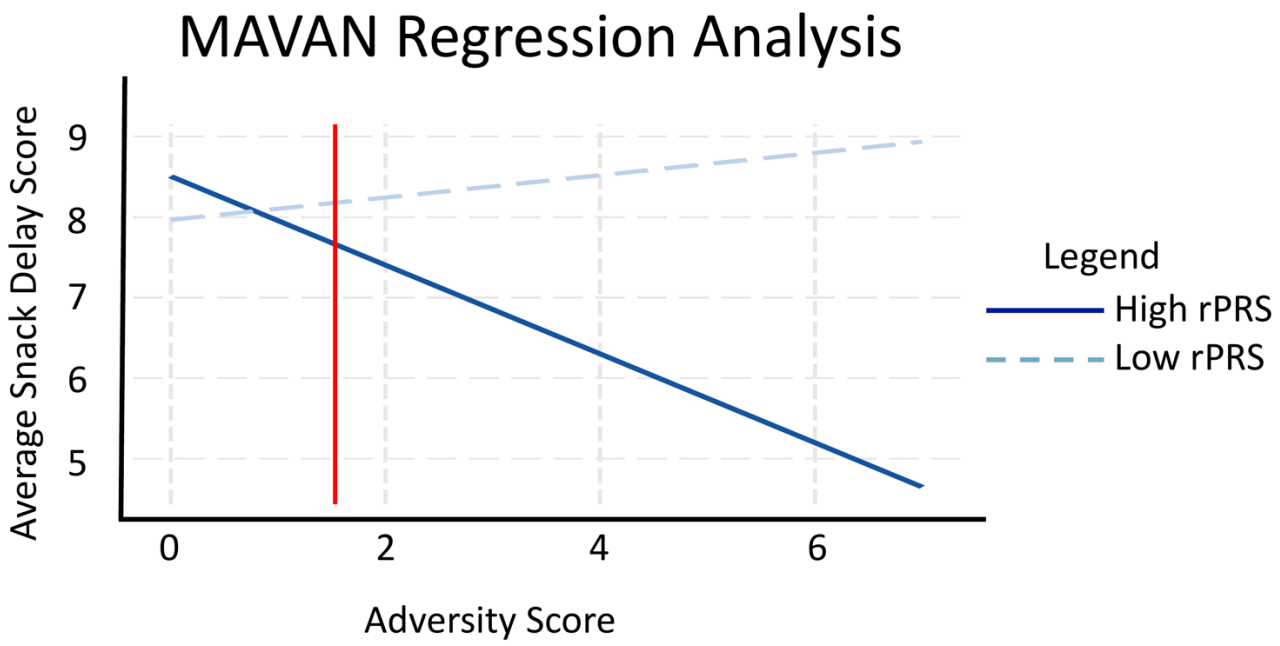


Figure 9. Enrichment analysis through Metacore™. Enrichment analysis of the SNPs that compose the Fasting Insulin rPRS shows that this subset of SNPs is significant for certain nervous system development processes: neurophysiological process of GABA-B receptor signaling at postsynaptic sides of synapses (p = 0.019, FDR = 0.05), neurophysiological process of dopamine D2 receptor signaling in the CNS (p = 0.034, FDR = 0.05), single-stranded RNA binding (p = 0.022, FDR = 0.038), and development involving neurogenesis and synaptogenesis (p = 0.019, FDR = 0.023).

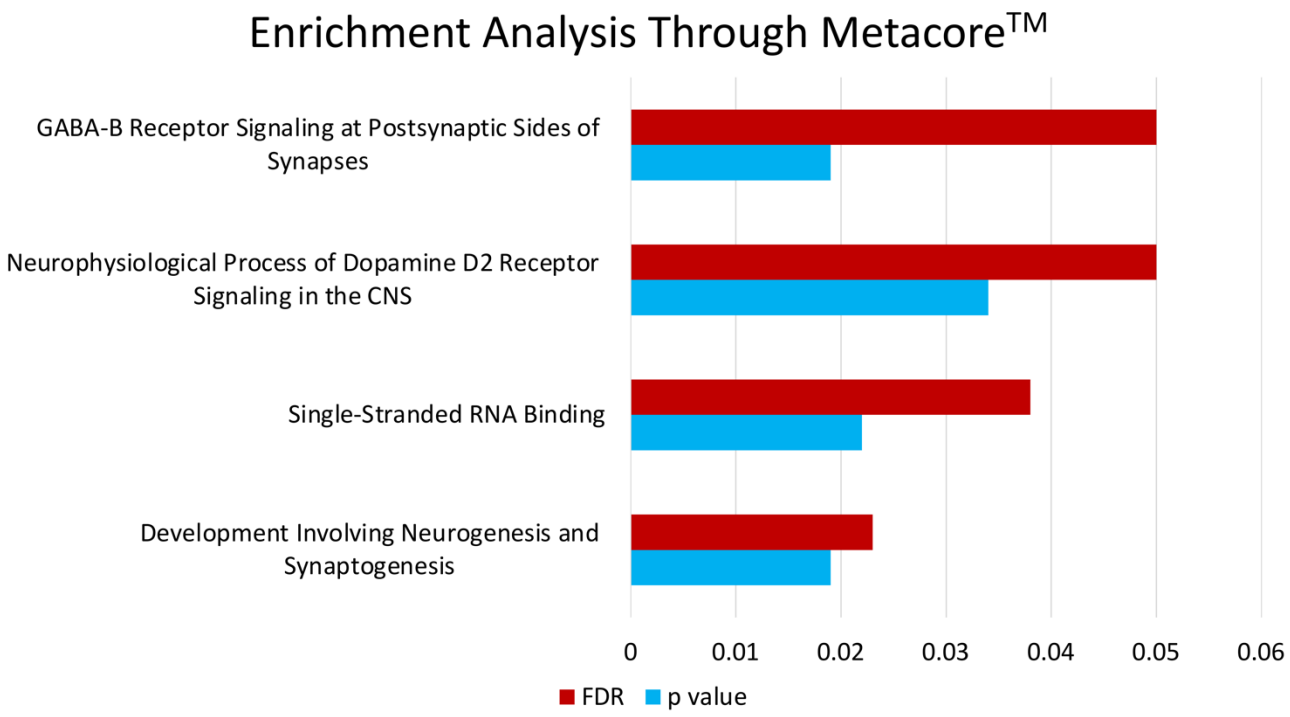


Figure 10. Brain tissue differentially upregulated by fasting insulin genes. Enrichment analysis through FUMA of the genes mapped by the SNPs that compose the Fasting Insulin rPRS that these genes are significantly differentially upregulated in brain specific tissues identified in the figure.

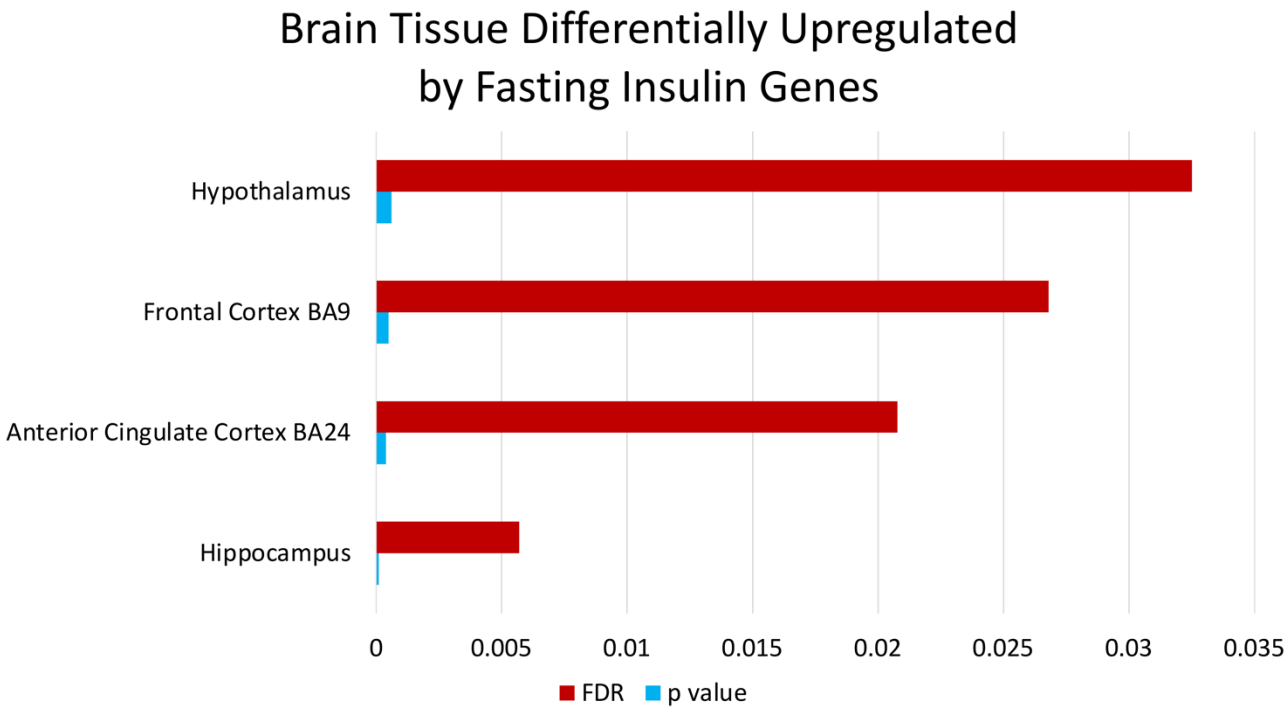
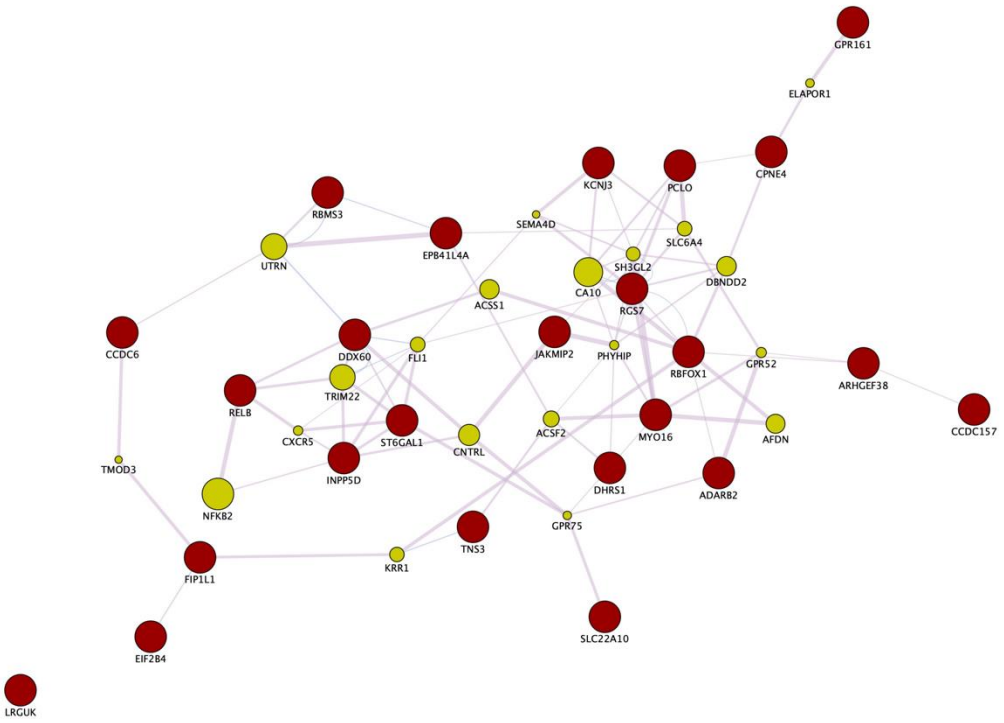


Figure 11. Gene Co-Expression Network. Enrichment analysis through GeneMANIA and Cytoscape of the genes mapped by the SNPs that compose the Fasting Insulin rPRS show that these genes are part of a co-expression network in Homo sapiens. Red circles indicate query genes, whereas green circles indicate related genes added by GeneMANIA. GeneMANIA translates mRNA expression data from Gene Expression Omnibus (GEO) to functional association networks that connect co-expressed genes through the pink lines displayed. The node sizes represent gene scores, indicating the number of paths that start at a given gene node and end up in one of the query genes.



Supplementary Information

Table 7. Adversity Score Calculation Markers in MAVAN. For each individual in the MAVAN cohort, an adversity score is calculated. Each item, except birth size, was binarized using either 15th or 85th percentile as the cut-off. Depending on the scoring system of the item, values below 15th or values higher than 85th percentile were considered as adversity, other values were considered as no adversity. The only item that includes both below 10th as well as above 90th percentiles as adversity is birth size (in other words, both low and high birth size were considered adversity). Presence of each component yields one point and the final postnatal score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

Adversity Cumulative Score
Hospitalizations in the first 6 months of life
Birth Size
Gestational age below or equal to 37 weeks
Maternal mental health (BDI, EPDS, STAI)
Household total gross income
Lack of money score
Disorganized attachment
Poor family function (FAD)
Presence of domestic violence or sexual abuse
Presence of marital strain
Smoking during pregnancy
Pregnancy anxiety

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Chapter IV. Refined fasting insulin polygenic score predicts addiction in adults exposed to childhood adversity

Preface

In our previous study, described in the previous chapter, we were able to show a significant interaction effect of early life adversity and high fasting insulin levels on impulsive behaviors in childhood in the MAVAN cohort [1] in which higher PRS was linked to more impulsivity in individuals exposed to more adversity. The SNPs that constitute the fasting insulin rPRS were also significantly associated with dopamine D2 receptor signaling [1]. This intrigued us as the dopamine system is known to also be associated with addiction related behaviors and directly impacted by impulsivity [66]. Impulsivity has been characterized as a behavioral endophenotype mediating the risk for stimulant dependence, an addictive behavior [41]. Impulsivity in SUD is viewed at contributing to an individual's lack of inhibition towards consuming a harmful substance, but prolonged substance abuse can lead to cortical degeneration which can also increase impulsive behavior [67] creating a cycle between impulsive behavior and addiction behavior. Therefore, for this study, we hypothesized that the genetic score associated with higher fasting insulin, which has been shown to impact childhood impulsivity in children exposed to ELA [1], would also interact with ELA to influence addiction related behaviors in adulthood. We were especially interested in adulthood addiction because we wanted to inspect the long-term impact of ELA on psychiatric outcomes. This provides us with a broader picture of other executive functions that are being affected in adulthood while still being associated with impulsivity.

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Refined fasting insulin polygenic score predicts addiction in adults exposed to childhood adversity

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Resource Availability

SAGE and UKB data can be obtained from the respective cohorts. For code availability and further information, please contact Dr. Patricia Silveira at patricia.silveira@mcgill.ca

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Author Contributions

The manuscript to be submitted was written by Aashita Batra with editing from Irina Pokhvisneva and Dr. Patricia Silveira. Statistical analyses were supervised by Dr. Patricia Silveira and Irina Pokhvisneva and conducted by Guillaume Elgbeili, Sachin Patel, and Aashita Batra. The entire project was planned and supported by Dr. Patricia Silveira.

Declaration of Interests

None.

Abstract

Previously, we showed that the genetic background associated with higher fasting insulin (FI) in children moderates the impact of early adversity on childhood impulsivity, an endophenotype associated with development of addiction behaviors. We hypothesized that the same genetic signal is associated with addiction behaviors in adults exposed to adversity. We found a significant interaction effect between the FI refined PRS (rPRS) and adversity on drug dependence (other than marijuana, cocaine, or opiates) [β = 0.123, p = 0.015] in adults from the SAGE cohort. In the UKB cohort, we found a significant interaction effect between FI rPRS and adversity on addiction to a behavior, such as gambling, or addiction to anything other than alcohol, prescription, over the counter medication, or illicit or recreational drugs [β = 0.051, p = 0.054]. Refined FI rPRS, which associates with adversity-induced childhood impulsivity also associated with adversity induced adulthood addiction.

Introduction

Early life adversity (ELA) augments the risk of adult chronic diseases, such as mental and cardio-metabolic disorders [1-6]. Some prenatal adversities have been linked to changes in structural and functional brain development trajectories [1, 2, 7-10]. Postnatal conditions similarly affect brain areas that are vulnerable as they continue developing until late adolescence. These adversities have been correlated with emotional problems [11], aggressive behaviors [12], changes in brain electrical activity [13, 14], cognitive functions [15], and executive functions (EF) [16]. The mechanisms encompassing EF behaviors, such as attention, impulsivity, and food preferences, involve gene by environment interactions but not everyone exposed to an adversity develops

these alterations. Response to ELA exposure results in individual differences that are predominantly driven by the genetic background.

In our previous work, we developed a model of association between the EF behavior of impulsivity in children and the interaction between biological markers of elevated fasting insulin (FI) levels in children and ELA [17]. While insulin is responsible for acquiring glucose in peripheral tissue, it has a different role as a neuroendocrine hormone as it is not needed for glucose transport into neurons [18]. In the brain, insulin acts as a neuroregulatory peptide [19-21]. Insulin function within the brain influences the development and expression of several EF behaviors [18], such as attention, inhibitory control, and working memory. Since brain insulin levels are not readily measured, genetic markers associated with peripheral insulin levels in children were used to inspect insulin's role in neurodevelopmental behaviors. First, the association between a polygenic risk score (PRS) for high FI [22] using a GWAS conducted in adults and measured peripheral insulin levels in children from the ALSPAC cohort [23, 24] was established. These markers were refined to only include single nucleotide polymorphisms (SNPs) that significantly predicted peripheral insulin levels in children to compose a refined polygenic risk score (rPRS). We showed that there was a significant interaction effect between the rPRS for high FI and ELA exposure on impulsivity in children in the MAVAN cohort [17] in which higher rPRS was linked to more impulsivity in individuals exposed to more adversity. Through enrichment analysis, it was identified that SNPs composing the FI rPRS are associated with nervous system development and dopamine D2 receptor signaling in the Central Nervous System [17]. The dopamine system has been associated with impulsivity in animal and human studies [25, 26]. Animal studies have shown that

striatal dopamine signaling and D2 receptor levels are decreased in addiction and directly impacted by impulsivity [27]. In humans, non-substance addiction has also been linked to reduced D2/D3 receptor availability [28] enforcing the idea that addiction and impulsive-related behaviors are associated with dopamine signaling in the striatum. This finding is interesting because it suggests a potential neurodevelopment pathway to support the relationship between the genetic background linked to insulin, ELA, and dopamine in the context of impulsivity and addiction. Animal work from our lab has previously demonstrated that rats exposed to ELA exhibit a delay in dopamine release in response to palatable food, an effect that is reversed by insulin administration [29] showing that the relationship between ELA and dopamine is moderated by insulin, and that relationship affects behavioral outcomes.

Impulsivity has also been linked to the absence of protracted pruning of the prefrontal cortex (PFC) during teen years which is associated with acquiring control over behavior [30]. It is often argued that the PFC has not completely matured when adolescents engage in risky behavior because when properly matured and functioning, the frontal lobes provide the knowledge from past experience to make behavior decisions [31]. These immature impulse control behaviors have been shown to accompany brain imagining alterations where there is a heightened responsiveness in the accumbens in adolescents and less activation in top-down PFC regions [32]. In fact, some researchers classify behavioral addictions as impulse control disorders [33, 34] because impulsive choices may prone individuals, especially adolescents, to addiction [35]. Impulsivity has been associated with addiction due to several reasons [36]: (1) impulsivity could contribute to the initial use or to the transition from recreational use to abuse [37], (2)

drugs may adversely affect the neurocognitive systems controlling impulses contributing to the continuation of use [38], or (3) impulsivity could help subdue urges which are an essential component for abstinence leading to relapses [39]. The same associations between impulsivity and addiction have been made in adults as well where cortico-cortical connectivity and corticostriatal connectivity were associated with impulsivity in cocaine use disorder [40, 41]. Adults who used multiple substances have shown to score lower on a measure of inhibitory control [42]. Therefore, for this study, we hypothesized that the genetic score associated with higher FI, which has been shown to impact childhood impulsivity in children exposed to ELA [17], would also interact with ELA to influence addiction related behaviors in adulthood. To test this hypothesis, we used two adult cohorts: the Study of Addiction, Genetics and Environment (SAGE) cohort and the UK Biobank (UKB) cohort.

Methods

Participants

We used data from two prospective cohorts: (1) Study of Addiction, Genetics and Environment (SAGE) [55-61] and (2) UK Biobank (UKB) [62] to analyze the gene by environment interaction effects on addiction related outcomes.

Study of Addiction, Genetics and Environment [55-61]*:* The SAGE repository was acquired from dbGaP (https://www.ncbi.nlm.nih.gov/gap, Accession number: phs000092.v1.p). This dataset is a compilation of three studies: the Collaborative Study on the Genetics of Alcoholism, the Family Study of Cocaine Dependence, and the Collaborative Genetic Study of Nicotine Dependence. The dataset contains genotyping and clinical phenotypes associated with substance dependence for adult subjects. There

are a total of 4,121 participants within the cohort but only 2,647 were included in this study, as indicated by *Figure 14.* Access to the SAGE dataset was granted by the NIH Data Access Committee based on the Data Use Certification submitted.

UK Biobank [62]: UKB is a population-based cohort with 502,543 recruited participants between the ages of 37 and 73 from 2006 to 2010 in the UK. This immense dataset contains information on participants' lifestyle and health data at baseline or follow-up, which were collected through questionnaires, physical measurements, and biological samples. All participants provided informed written consent before data collection. This research was conducted using the UK Biobank Resource under Application Number 41975. Approval for the UK Biobank was obtained by the North West Multicentre Research 580 Ethics Committee (REC reference 11/NW/0382; www.ukbiobank.ac.uk/ethics/), the National Information Governance Board for Health and Social Care and the Community Health Index Advisory Group. There are a total of 502,543 participants within the cohort but only 61,340 were included in this study as indicated by *Figure 15.*

Genotyping

In SAGE, the imputation was performed using BEAGLE. After post imputation QC, there was 4,563,702 SNPs available. In UKB, the imputed genotypes were provided in the data repository. For more details on genotyping and quality control please refer to (Bycroft et al., 2018). We also removed variants with minor allele frequency < 0.01%, as well as duplicated and ambiguous SNPs. As a result, there were 74,896,698 variants available in the data set.

Polygenic Risk Scores

In a previous study, we created a refined polygenic risk score (rPRS) that is highly associated with fasting insulin (FI) in children (ALSPAC) and then investigated its association with impulsive behavior when exposed to adversity, as an outcome in an independent cohort of children (MAVAN), thus refining a polygenic risk score (PRS) based on an adult cohort GWAS [22]. Our goal within this study was to inspect whether the genetic markers of higher FI, which are shown to be associated with impulsivity in childhood [17], are associated with adulthood addiction. To examine whether this specific impulsivity associated genetic background mapped to adulthood addiction, it was important to include the same single nucleotide polymorphisms (SNPs) as the ones used in the rPRS of Batra et al. [17]. Using a conventional PRS would only demonstrate an association between a genetic background of adult high FI and adulthood addiction. The rPRS allows us to use a genetic background relevant in childhood while still examining behavior in adulthood. The PRS was calculated using PRSoS pipeline [44] from the FI GWAS[22] at different thresholds and identified the subset of SNPs that best predicted peripheral insulin levels in children from the ALSPAC cohort as displayed by our previous work [17]. The rPRS for FI was calculated at the specific threshold in the individuals from the Study of Addiction, Genetics and Environment (SAGE) cohort and we investigated its interaction effect with adversity on the Diagnostic and Statistical Manual of Mental Disorders (DSM4) substance dependence outcomes available in the SAGE cohort. When we found a significant association between the interaction of FI rRPS and adversity on an addiction behavior in SAGE, we investigated the interaction effect of the rPRS for FI and adversity on addiction behaviors available in the UK Biobank (UKB) cohort. Out of the 57

SNPs that were used to predict childhood impulsivity, only 39 were retained in SAGE but all 57 SNPs were retained in UKB.

Early Life Characterization

Early life adversity exposure was estimated using a cumulative score involving different environmental variables [63] for each individual in the SAGE and UKB cohorts as described by Silveira et al [63]. The adversity score was created by combining several markers of adversity.

The following instruments were included as markers in the SAGE cohort: sexual abuse, physical abuse, sexual trauma, physical trauma, non-assault trauma. Each individual in the cohort was asked to rate abuse/trauma instrument on a scale of 0-4 where 0 indicated that they had never experienced the instrument and 4 indicated that they had extremely experienced the instrument. A marking of 0 was not considered to be adverse but markings 1-4 were categorized as adverse. We also included education category, specifically education less than college was considered adverse, since it can be representative of socio-economic status which has been associated with early life adversity effects [64]. Each adverse instrument was given a score of 1 and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

The following instruments were included as markers in the UKB score: birth size, maternal smoking, felt hated, not felt loved, not have someone to take to the doctor, physical/sexual abuse, and multiple births. Birth size (ID20022) was marked as adverse if it was below 10^{th} percentile or above 90^{th} percentile. The exposure to maternal smoking question (ID1787) is part of a questionnaire related to early life exposures (1 point if the

answer was yes). From an online follow-up questionnaire called Thoughts and Feelings, we obtained information about feeling hated by family member during childhood (1 point if the answer was sometimes, often, or very often; ID20487). The same questionnaire provided information about feeling loved during childhood (1 point if the response was never or rarely; ID20489). Having nobody to take to the doctor when needed was counted as well (1 point if the response was often, half a point was given if the response was rarely had someone; ID20491). Presence of domestic violence during childhood (physical or sexual) was counted as one point (ID20488). Lastly, a questionnaire asked whether an individual was part of a multiple birth (1 point if the response was yes; ID1777). Each adverse instrument was given a score of 1 and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

Behavioral Outcomes

The SAGE cohort used the DSM-IV criteria to determine whether an individual exhibited substance abuse and substance dependence. The Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA-II) tool was used to assess the physical, psychological, and social manifestations of substance dependence [65]. For each type of substance (alcohol, cocaine, marijuana, nicotine, opiates, other drugs than marijuana/cocaine/opiates), dependence was described as a maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three or more of the following occurring at any time in the same 12-month period: (a) tolerance, (b) withdrawal, (c) the substance is often taken in larger amounts or over a longer period than intended, (d) there is a persistent desire or unsuccessful efforts to cut

down or control substance use, (e) a great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects, (f) important social, occupational, or recreational activities are given up or reduced because of substance use, g) the substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance. For this study, we used all DSM4 dependence outcomes, as indicated in *Table 10*.

The UKB cohort applied a questionnaire to assess the addiction behaviors, as indicated in *Table 11*. In the questionnaire, a more general question “Have you been addicted to or dependent on one or more things, including substances (not cigarettes/coffee) or behaviours (such as gambling)?” was included in the analysis of addiction to any substance or behaviours (ID20401). Other questions from this instrument related to more specific outcomes and were also considered in the analysis: 1) “Have you been addicted to alcohol?” was included in the analysis of addiction to alcohol (ID20406); 2) “Have you been addicted to or dependent on prescription or over-the-counter medication?” was used for the analysis of addiction to prescription or over-the-counter medication (ID20503); 3) “Have you been addicted to Illicit or recreational drugs?” was applied for the analysis of addiction to illicit or recreational drugs (ID20456); and 4) “Have you been addicted to a behaviour (such as gambling) or to anything else we have not mentioned?” was used for the analysis of addiction to a behavior (ID20431). If the answer to the first question (ID20401) was no, the participant was not asked the subsequent questions and it was assumed that no addictions were present. To maintain the full sample, those who reported no addictions were added as having no addictions to the

following for questions (ID20406, ID20503, ID20456, ID20431) The score of 1 was assigned to answering yes to a question and a score of 0 was assigned to answering no to a question. All 5 outcomes were used in the analysis of this study.

Statistical analysis

Data analyses were carried out using R [66]. Baseline comparisons between low and high rPRS groups were done in the SAGE and UKB cohort, as indicated in *Tables 1 and 2* respectively. Mean differences of the main confounding variables were assessed using the Student's *t*-test for independent samples if they were continuous variables or the chi-square test if they were categorical variables. Significance levels for all measures were set at $\alpha < 0.05$ in SAGE and $\alpha < 0.1$ for replication analysis in UKB.

We applied logistic regression analysis to test the association between addiction outcomes in SAGE, as indicated in *Table 10*, and the predictors including adversity exposure, FI rPRS, and their interaction term, adjusting for sex, the first three genetic PCs, and income. In UKB, logistic regression analysis was done to test the association between addiction outcomes, as indicated in *Table 11*, and the predictors including adversity exposure, FI rPRS, and their interaction term with the first 40 genetic PCs, age, sex, genotyping array, and assessment center.

Enrichment Analysis

Enrichment analyses were performed for the genes mapped from the SNPs composing the FI rPRS using DisGeNET [43]. DisGeNET contains information on genes associated to diseases based on various publicly available databases: the Comparative Toxicogenomics Database™ (CTD™), UniProt/SwissProt, the Cancer Genome

Interpreter, Orphanet, the Mouse Genome Database (MGD), PsyGeNET, Genomics England, ClinGen, and the Rat Genome Database (RGD).

Results

Baseline characteristics

We performed baseline comparisons between low and high rPRS groups in the SAGE cohort and UKB cohort. The only difference found in the main confounding variable for SAGE is in the variable of income earned per year which was identified as a categorical variable as less than $50,000, as shown in *Table 8*. There were no significant differences between the low and high rPRS groups in the main confounding variables in UKB as shown in *Table 9*.

Interaction between FI rPRS and the adversity score associates with substance use disorders (SUD) in SAGE

We performed a logistic regression analysis to examine the interaction effect between the FI rPRS and adversity exposure on the DSM4 dependence outcomes applied during adulthood, listed in *Table 10* with the results, in individuals from European ancestry in the SAGE cohort. The analyses were adjusted by population stratification PCs, sex, and the categorical variable income earned per year which was significantly different between high and low rPRS groups. A significant interaction effect was observed before false discovery rate (FDR) correction, as highlighted in *Table* 10 and displayed in *Figure 12*, between FI rPRS and adversity exposure on addiction measured by the DSM4 dependence on drugs other than marijuana, cocaine, and opiates on the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA-II) assessment tool [N = 2643, β = 0.123, p = 0.015]. Simple slope analysis at $\pm$1 SD rPRS showed that higher early life

adversity is linked to higher probability of SUD, specifically dependence on drugs other than marijuana, cocaine, and opiates, in adults with higher rPRS [β = 0.707, p < 0.001] and the same trend was observed in adults with lower rPRS [β = 0.487, p < 0.001]. When applying FDR to account for multiple testing, no significant interaction effects were observed.

The main effect of the rPRS on DSM4 dependence on drugs other than marijuana, cocaine, and opiates in SAGE applied at adulthood, adjusted by PCs, sex, and income earned was not significant [N = 2643, β = -0.077, p = 0.241]. However, the main effect of adversity on DSM4 dependence on drugs other than marijuana, cocaine, and opiates in SAGE applied at adulthood, adjusted by PCs, sex, and income earned was significant [N = 2643, β = 0.588, p < 0.001].

Interaction between FI rPRS and the adversity score associates with addiction behaviors in UKB

A significant interaction effect was observed before FDR correction, as highlighted in *Table* 11 and displayed in *Figure 13*, between FI rPRS and adversity exposure on addiction to a behavior, such as gambling, or addiction to anything other than alcohol, prescription, over the counter medication, or illicit or recreational drugs [N = 61298, β = 0.051, p = 0.054]. Simple slope analysis at $\pm$1 SD rPRS showed that higher early life adversity is linked to higher probability of addiction to a behavior, in adults with higher rPRS [β = 0.399 p < 0.001] and the same trend was observed in adults with lower rPRS [β = 0.303, p < 0.001]. No significant interaction effect was observed when adjusting for multiple testing.

The main effect of the rPRS on addiction to a behavior in UKB applied at adulthood, adjusted by PCs, age, sex, genotyping array and assessment center was not significant [N = 61298, β = 0.013, p = 0.675]. However, the main effect of adversity on addiction behaviors in UKB applied at adulthood, adjusted by PCs, age, sex, genotyping array and assessment center was significant [N = 61298, β = 0.352, $p < 0.001$].

Enrichment analysis on FI rPRS

Enrichment analysis performed through DisGeNET[43] of the genes mapped by the SNPs composing the FI rRPS was significantly associated (p = 0.003) with addictive behavior.

Discussion

The purpose of this study was to explore whether the genetic background associated with higher FI, which when interacted with early life adversity predicts impulsivity in childhood [17], also has a significant interaction effect on addiction related behaviors in adulthood. In our previous work, we derived a rPRS consisting of SNPs most associated with peripheral insulin levels in children from a FI GWAS composed of adults. The rPRS and ELA had a significant interaction effect on impulsivity in children in the MAVAN cohort and these SNPs and their mapped genes were significantly correlated with various nervous system development processes [17]. The same rPRS, as shown in this study, displays a significant interaction effect with ELA on SUD in the SAGE cohort and on addiction to a behavior in the UKB cohort.

As described in Batra et al [17] in more detail, the rPRS calculation was done by first calculating the PRS, through the PRSoS tool [44], at one hundred different thresholds in the independent cohort ALSPAC as a training sample. We were then able to identify

the threshold of 0.24 to best predict peripheral insulin levels in children [45]. Then, we refined the rPRS by associating each SNP within the subset obtained from the 0.24 threshold rPRS with peripheral insulin levels in the ALSPAC children to subset for SNPs that significantly associated with the peripheral insulin levels for the rPRS. This refinement ensured that the genetic markers used in the rPRS calculation were the ones that associated with high FI levels in children. Since the GWAS used was constructed in adults, this refinement ensured that the genetic markers used from the GWAS were relevant for the outcome in children. Although the current study inspected behavior outcomes in adults, we still used the SNPs that were most associated with peripheral insulin levels in children because the aim was to inspect whether the SNPs associated with childhood impulsivity would also be associated to a behavior in adulthood. It is important to note that all the SNPs included in rPRS had a p-value in the GWAS that was equal to or less than 0.24, since that was the original PRS threshold, ensuring that all SNPs used have at most a p-value of 0.24 in the association with adult high FI levels. This subset of SNPs therefore contains SNPs associated with high FI levels in adults which are also significantly associated with high FI levels in children making them the ideal subset to use in inspecting the trend from childhood to adulthood EF behavior.

Impulsivity has been characterized as a behavioral endophenotype mediating the risk for substance dependence, an addictive behavior [46]. Impulsivity in substance use is viewed as contributing to an individual's lack of inhibition towards consuming a harmful substance, but prolonged substance abuse can lead to cortical degeneration which can also increase impulsive behavior [31], creating a cycle between impulsive behavior and addiction behavior. Clinical studies have shown that siblings of chronic stimulant abusers

demonstrate increased impulsivity enforcing the notion that impulsivity is an endophenotype for addiction as opposed to a consequence of substance use [46, 47]. Additionally, reduced D2 and D3 receptor availability in the nucleus accumbens has been associated with addiction vulnerability while also exhibiting signs of impulsive behavior [48-50]. Therefore, addiction related behaviors were the ideal behavior to test in adulthood for our study as impulsivity is an endophenotype of addiction.

Our results in SAGE show that although several measures of addiction were assessed, only one demonstrated a significant association with an interaction: DSM4 dependence on drugs other than marijuana, cocaine, and opiates. Similarly, in the UKB, all addiction outcomes were tested but only one resulted in a significant interaction effect: addiction to a behavior, such as gambling, or addiction to anything other than alcohol, prescription, over the counter medication, or illicit or recreational drugs. SUD has been shown to converge on the brain's reward pathways which consists of the dopamine mesolimbic pathway [51]. It is possible that the addiction behaviors that came out as significantly associated with the interaction effect of FI and adversity could be directly linked to impulsive action through a different biological mechanism than addiction behaviors that did not show significant association with the interaction effect in this study. The dopamine pathway is involved in the reward circuitry and in EF but the connection between the two regarding the results found in this study needs to be studied further.

The significant results from the enrichment analysis performed using DisGeNET were of particular interest for our study because the SNPs composing the FI rPRS were associated with SUD in addition to behavioral addiction. Interestingly, impulsive behaviors are also encompassed within the same disease category as addictive behaviors on

DisGeNET, confirming that impulsivity is a genetic endophenotype of addiction. It is intriguing that our refined insulin polygenic score was enriched for addictive behaviors when using DisGeNET, which is in direct agreement to our findings in the two human samples. This also aligns with studies showing that insulin signaling regulates dopamine neurotransmission and by consequence affects addiction and reward related behaviors [52] and our previous findings where we showed that this set of SNPs is enriched for D2 receptor signaling [17]. Together, these findings suggest a relationship between insulin, dopamine, impulsivity, and addiction.

In conclusion, these results affirm that ELA and biological markers associated with higher FI levels are important factors influencing impulsivity in children and addiction behaviors in adults. Our analyses showed that the genetic background associated with high FI levels in childhood moderates the effects of adversity on adulthood substance use and non-substance use addiction behaviors. This research can be highly impactful as it provides insights into the vulnerability to EF disorders which start early on in an individual's life but leave a lasting impact on psychopathology. The biological mechanisms that we discovered to be involved in these processes can inform the development of early interventions and more efficient management of such health outcomes.

Limitations of the Study

There are limitations within our study. The cohorts used in this study were restricted to White/European ancestry because the GWAS used in the analysis was built from data on European ancestry. Unfortunately, this means that this subset of SNPs would not necessarily be relevant for a different ancestry in the same manner because

different ancestries have distinct allele frequencies [53] which could result in differences in the interaction effects between the genetic background and the environment.

A second limitation to this work is that previously, we performed the analysis in children [17], and here we perform the analysis in adults, leaving a gap to be filled by studying the effects in adolescents. Since interventions aid individuals the earlier they are given [54] and addiction is not identified as a disorder as early as childhood, it would be beneficial to understand the development of this association in adolescence to understand when the earliest treatments could be implemented. Additionally, since SUD starts to emerge at a higher frequency in adolescence, it is important to inspect the effects of ELA and insulin signaling in this context in adolescents too. Following which, interventions would then target individuals at an earlier stage and possibly prevent the disorder from following these individuals into adulthood.

Tables and Figures

Table 8: Participants' characteristics in SAGE.

	Total (n = 2647)	Low rPRS (n = 1325)	High rPRS (n = 1322)	*p*
Sex - male	44.5% (1178)	44.3% (587)	44.7% (591)	0.865
Age at interview (years)	38.72 (9.72)	38.8 (9.44)	38.65 (10)	0.691
Education (at least 1 year college)	66.8% (1768)	67.3% (892)	66.3% (876)	0.592
Income/year (<$50,000)	44.2% (1169)	42% (557)	46.3% (612)	**0.030**
DSM4 marijuana dependence	16.5% (435)	16.6% (219)	16.4% (216)	0.916
DSM4 nicotine dependence	45.2% (1157)	46% (590)	44.4% (567)	0.465
DSM4 alcohol dependence	45.3% (1200)	45.8% (607)	44.9% (593)	0.649
DSM4 cocaine dependence	19.4% (513)	19.5% (258)	19.3% (255)	0.929
DSM4 opiate dependence	6.7% (177)	6.7% (88)	6.7% (89)	0.996
DSM4 dependence on drugs other than marijuana, cocaine or opiates	13.4% (354)	13.5% (178)	13.3% (176)	0.961

Numbers are presented as mean (SD) or percentage (number of participants). Comparison between Low/High rPRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

Table 9: Participants' characteristics in UKB

	Total (n = 61340)	Low rPRS (n = 30700)	High rPRS (n = 30640)	*p*
Sex - male	37.6% (23057)	37.7% (11561)	37.5% (11496)	0.730
Birth weight (grams)	3348 (611)	3352 (614)	3343 (609)	0.099
Breastfeeding - yes	71.7% (40471)	71.8% (20240)	71.7% (20231)	0.706
Smoking during pregnancy	28% (17171)	27.8% (8531)	28.2% (8640)	0.262
Age at recruitment (years)	54.86 (7.66)	54.89 (7.67)	54.84 (7.64)	0.362
Townsend Deprivation Index at recruitment	-1.92 (2.69)	-1.91 (2.7)	-1.93 (2.69)	0.572
Body Mass Index	26.67 (4.6)	26.68 (4.61)	26.67 (4.58)	0.792
Ever addicted to any substance or behavior	5.7% (3480)	5.7% (1753)	5.6% (1727)	0.706
Ever addicted to alcohol	2.2% (1355)	2.2% (686)	2.2% (669)	0.691
Ever addicted to prescription or over-the-counter medication	0.9% (536)	0.9% (266)	0.9% (270)	0.878
Ever addicted to illicit or recreational drugs	0.4% (249)	0.4% (126)	0.4% (123)	0.911
Ever addicted to a behavior or miscellaneous	1.3% (822)	1.3% (399)	1.4% (423)	0.405

Numbers are presented as mean (SD) or percentage (number of participants). Comparison between Low/High rPRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

Table 10. Interaction effect between the FI rPRS and adversity exposure on the DSM4 dependence outcomes in SAGE cohort. We performed a logistic regression analysis to examine the interaction effect on the DSM4 dependence outcomes applied during adulthood in individuals from European ancestry in the SAGE cohort, adjusted by population stratification PCs, sex, and the categorical variable income earned per year which was significantly different between high and low rPRS groups. We applied FDR to account for multiple testing. The outcome significantly associated with an interaction before FDR correction is highlighted in the table.

Outcome	Beta	SE	Beta OR	p	FDR-adjusted p-value
DSM4 marijuana dependence	-0.023	0.046	0.977	0.614	0.774
DSM4 nicotine dependence	0.035	0.040	1.035	0.382	0.774
DSM4 alcohol dependence	-0.019	0.042	0.981	0.645	0.774
DSM4 cocaine dependence	0.032	0.049	1.032	0.516	0.774
DSM4 opiate dependence	0.010	0.060	1.010	0.873	0.873
DSM4 dependence on drugs other than marijuana, cocaine or opiates	**0.123**	**0.050**	**1.131**	**0.015**	0.087

Table 11. Interaction effect between the FI rPRS and adversity exposure on addiction outcomes in UKB cohort. We performed one-sided logistic regression analysis to examine the interaction effect on addiction outcomes applied during adulthood in individuals of Caucasian ancestry in the UKB cohort, adjusted by population stratification PCs, age, sex, genotyping array, and assessment center. We applied FDR to account for multiple testing. The outcome significantly associated with an interaction before FDR correction is highlighted in the table.

Outcome	Beta	SE	Beta OR	p	FDR-adjusted p-value
Ever addicted to any substance or behavior	-0.003	0.016	0.997	0.568	0.827
Ever addicted to alcohol	-0.015	0.025	0.985	0.731	0.827
Ever addicted to prescription or over-the-counter medication	0.054	0.038	1.055	0.078	0.195
Ever addicted to illicit or recreational drugs	-0.050	0.053	0.952	0.827	0.827
Ever addicted to a behavior, such as gambling, or addicted to anything other than alcohol, prescription, over the counter medication, or illicit or recreational drugs	**0.051**	**0.032**	**1.052**	**0.054**	0.195

Figure 12. SAGE logistic regression analysis results for dependence on drugs other than marijuana, cocaine, and opiates. rPRS was calculated for adults in SAGE using the fasting insulin GWAS with significant SNPs identified in ALSPAC through the rPRS methodology. Logistic regression analysis showed a significant interaction effect between fasting insulin rPRS and early adversity exposure on the dependence on drugs other than marijuana, cocaine, and opiates [N = 2643, β = 0.123, *p* = 0.015]: higher adversity is linked to higher probability of substance use in adults with higher rPRS [rPRS = mean+1 SD, [β = 0.707, *p* < 0.001]] represented by the solid blue line. Similar association was observed in adults with lower rPRS [rPRS = mean-1 SD, [β = 0.487, *p* < 0.001]] represented by the dashed blue line, though the effect of adversity on the outcome is smaller.

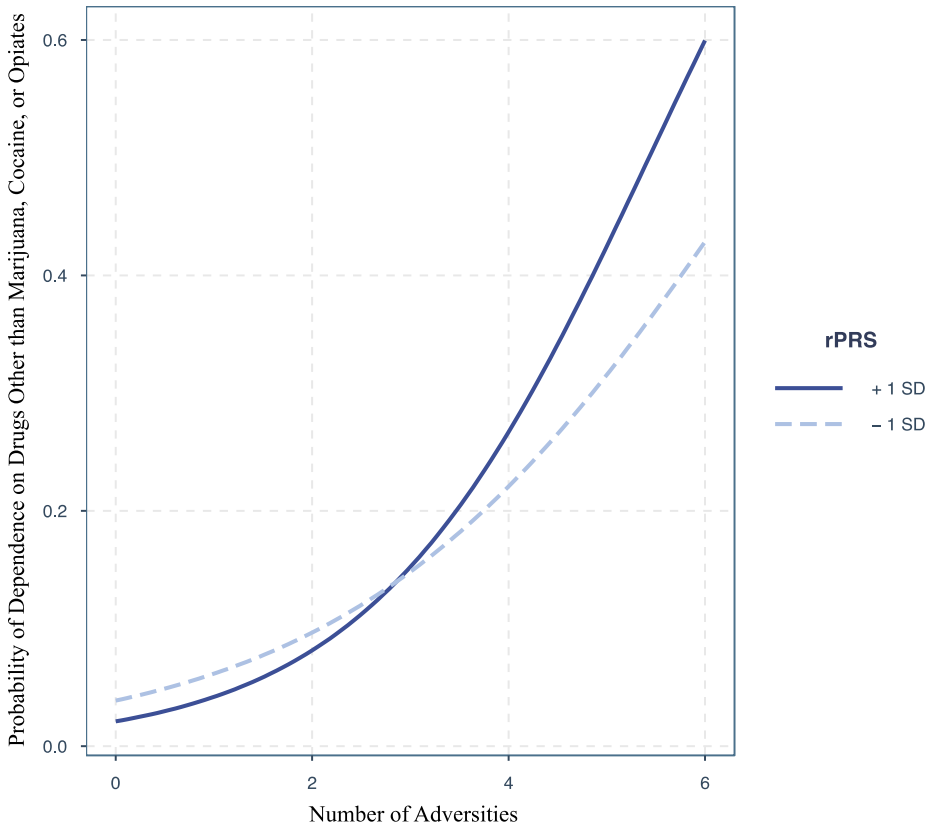


Figure 13. UKB logistic regression analysis results for addiction. rPRS was calculated for adults in UKB using the fasting insulin GWAS with significant SNPs identified in ALSPAC through the rPRS methodology. Logistic regression analysis showed a significant interaction effect between fasting insulin rPRS and adversity exposure on addiction to a behavior, such as gambling, or addiction to anything other than alcohol, prescription, over the counter medication, or illicit or recreational drugs [N = 61298, β = 0.051, *p* = 0.054]: higher adversity is linked to higher probability of addiction in adults with higher rPRS [rPRS = mean+1 SD, [β = 0.399, *p* < 0.001]] represented by the solid blue line. Similar association was observed in adults with lower rPRS [rPRS = mean-1 SD, [β = 0.303, *p* < 0.001]] represented by the dashed blue line, though the effect of adversity on the outcome is smaller.

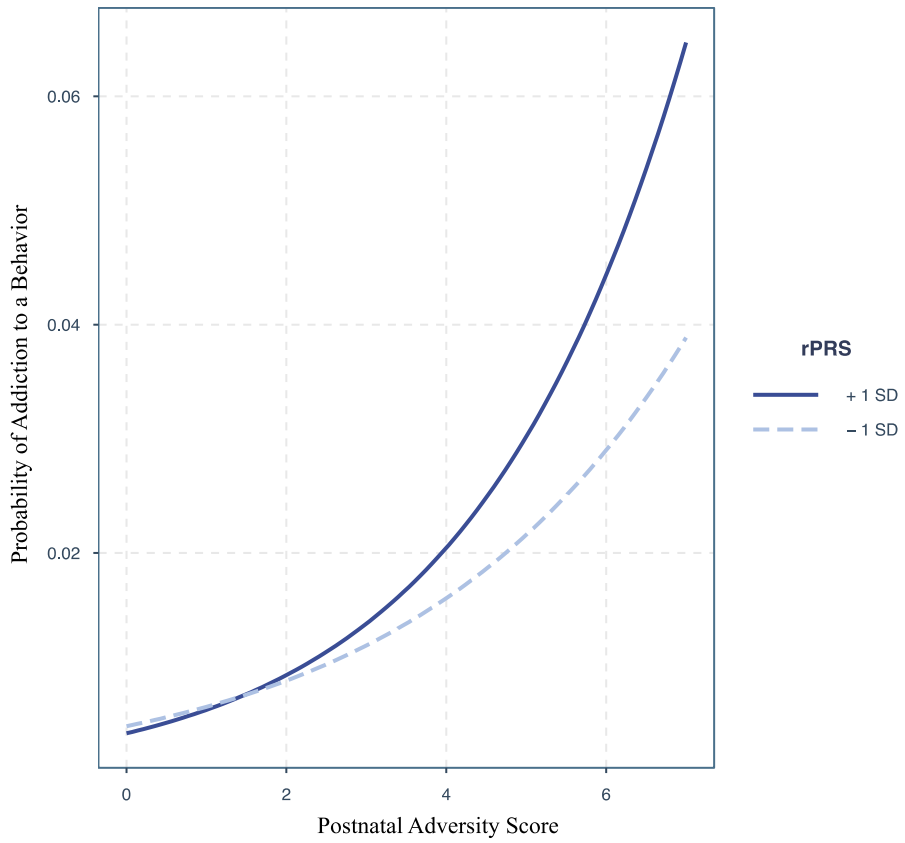


Figure 14. Block scheme describing sample selection in SAGE cohort.

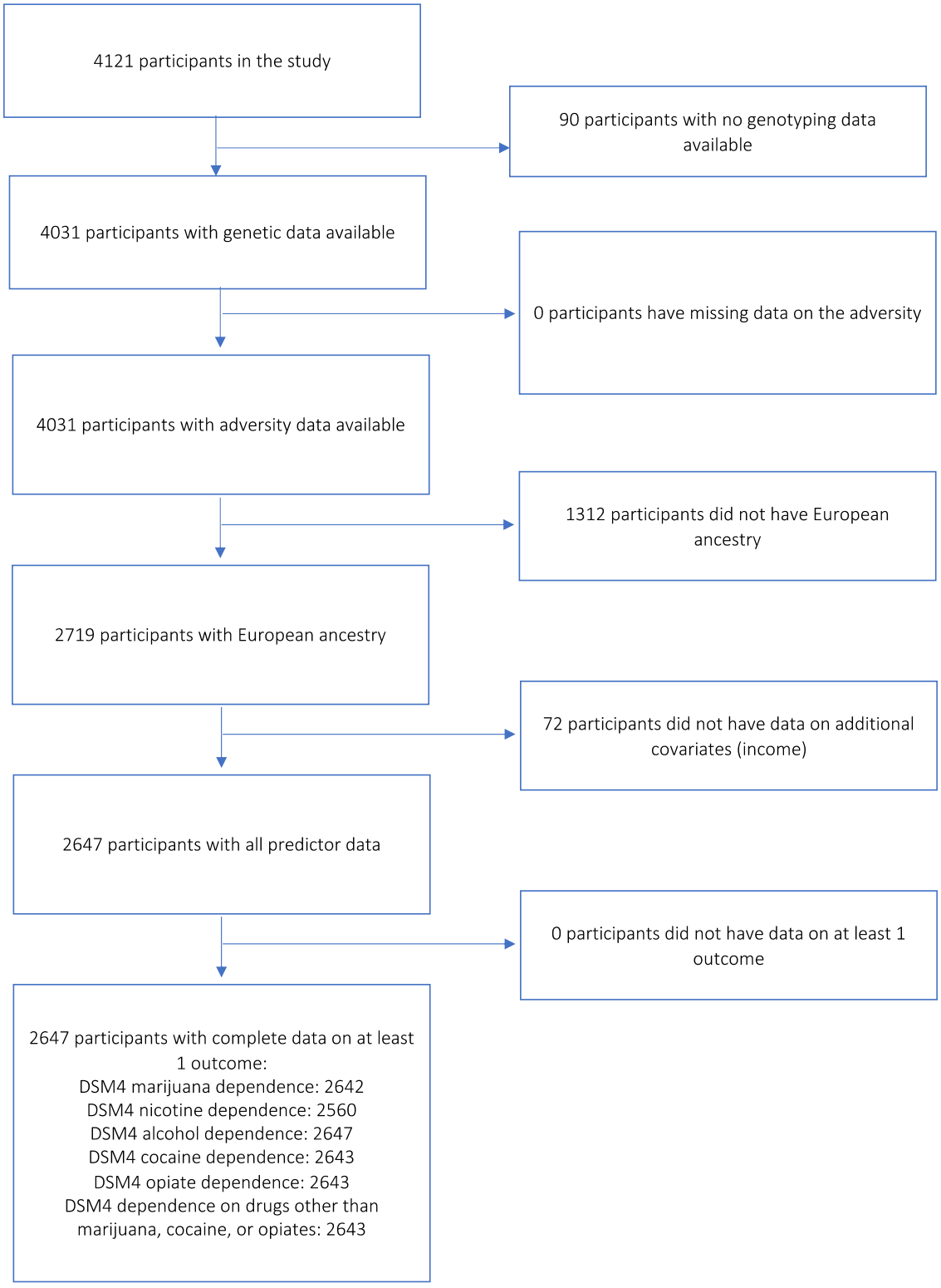
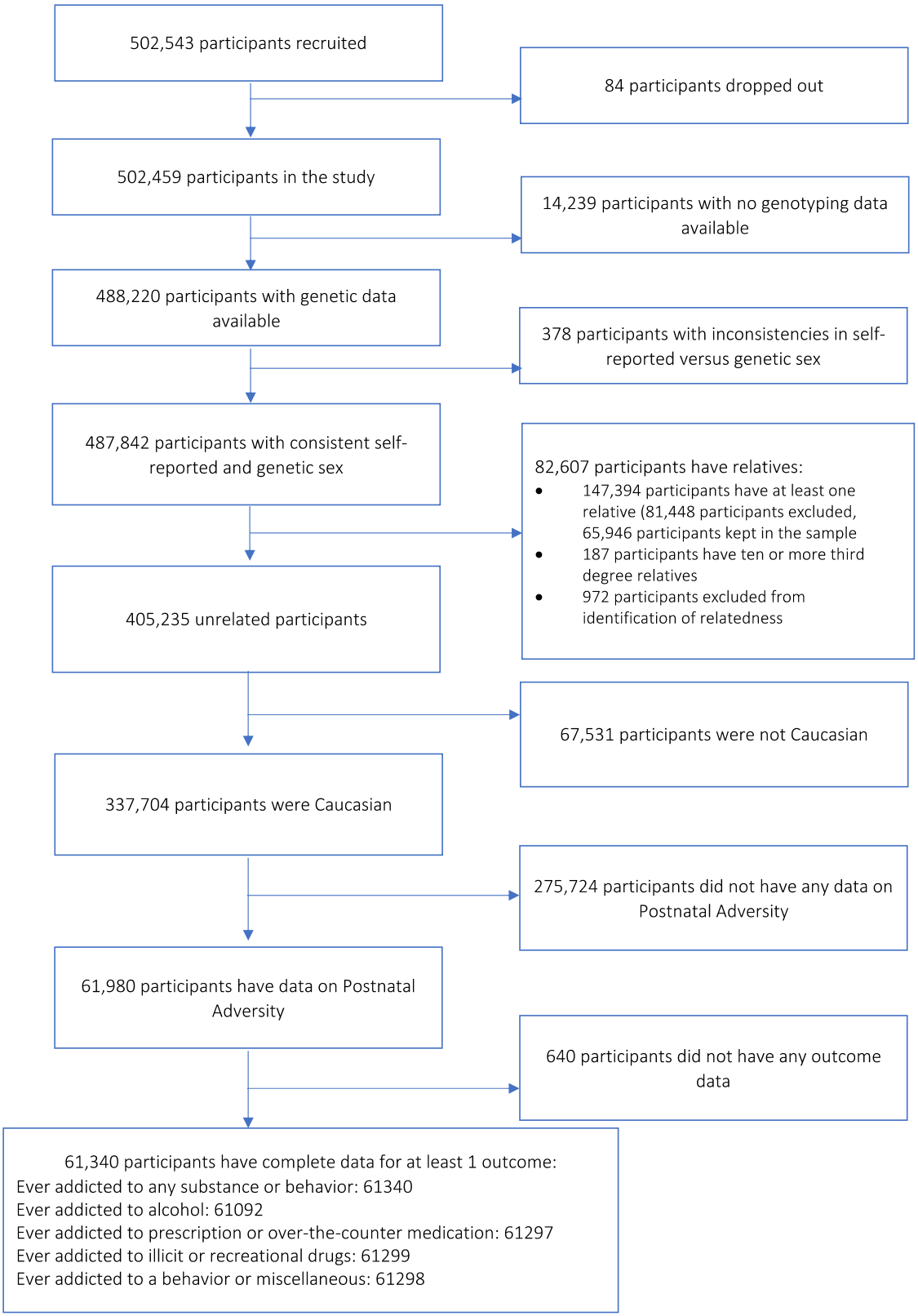


Figure 15. Block scheme describing sample selection in UKB cohort.



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Chapter V. Insulin mediates the effects of early life adversity on executive functioning in a sex-specific manner

Preface

So far, we have been able to show that there is a significant interaction effect of early life adversity and fasting insulin on executive functioning in childhood, through impulsivity, and in adulthood, through addiction. While this informed us that executive function is altered through this interaction in childhood and adulthood, it does leave us to wonder what happens during adolescence. Adolescence is a crucial developmental period affected by early life adversity [68, 69]. Additionally, the prefrontal cortex, the brain area largely responsible for executive functioning, is developing in adolescence until adulthood making it a critical period of development where executive function pathways are being fine-tuned [70]. Therefore, it was important for us to examine the interaction effect of adversity and fasting insulin on executive functioning throughout development instead of just during a particular age period. Additionally, we thought it was important to inspect whether this interaction effect is specific to impulsivity and addiction or does it apply to other executive functions as well. Hence, we decided to inspect the interaction effect on multiple executive function behaviors. Lastly, we thought it was important to further study this effect in a sex-specific manner as the psychiatric disorders typically display sex differences [71]. By studying the interaction effect of early life adversity and fasting insulin on multiple executive functions in a sex-specific manner, we will be one step closer to understanding the underlying mechanisms involved in these behaviors.

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Insulin mediates the effects of early life adversity on executive functioning in a sex-specific manner

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Abstract

Objective: Considering the high co-morbidity between altered metabolism and executive function (EF) problems, we hypothesized that the genetic background associated with altered fasting insulin (FI) and EF could be shared. Early life adversity (ELA) is associated with altered insulin signaling and altered EF behaviors in a sex-specific manner and could be involved in this relationship.

Methods: Conjunctional false discovery rate (ConjFDR) was used to identify the shared genetic architecture between FI and the EFs impulsivity and ADHD. We identified the PRS threshold from FI GWAS that best predicted insulin levels in male and female ALSPAC children [N_{males}=1,901, $N_{females}$=1,834; $p_{t\text{-}intial\text{-}males}$= 0.05 (11,121 SNPs), $p_{t\text{-}intial\text{-}females}$= 0.15 (27,202 SNPs)], further refining it to only include SNPs significantly associated with insulin levels in children [$N_{SNP\text{-}males}$= 635 SNPs, $N_{SNP\text{-}females}$ = 1,449 SNPs]. PheWAS was run to identify EF associated with the interaction between the refined PRS (rPRS) and early adversity. To inquire a direct causal relationship between FI and impulsivity in the presence of adversity, we applied mendelian randomization (MR).

Results: ConjFDR showed no shared SNPs significantly associated with sex specific FI GWAS and sex-specific impulsivity or ADHD GWASs. PheWAS showed impulsivity and attention-related outcomes to be highlighted in interaction models between FI rPRS and early adversity. Two-sample MR suggests a causal association between higher fasting insulin levels and impulsive behavior in females exposed to adversity ($p < 0.001$).

Conclusion: This solidifies that the relationship between high FI and EF is not direct but rather interacting with early life adversity exposure, especially in females.

Introduction

Exposure to early life adversity (ELA) is a common developmental risk factor associated with both metabolic and psychiatric phenotypes. ELA leads to alterations in responsivity to stress and alters insulin sensitivity at different ages. Childhood adversity as well as adult stress exposures have been linked to diabetes and altered glucose regulation while chronic stress in adulthood has been associated with glucose intolerance and metabolic syndrome [1]. All these findings emphasize that although a myriad of systems is affected by ELA, insulin regulation and function is one of them, deserving to be further inspected.

Insulin is a principal hormone in respect to metabolism regulation in animals while also being a neuroregulatory peptide in several parts of the central nervous system including the prefrontal cortex (PFC) [2]. Within the PFC, insulin modulates the development and expression of different executive function (EF) behaviors [3], such as attention, inhibitory control, and working memory. Insulin reduces activity in the prefrontal areas that control behaviors such as inhibitory control of eating [4]. In Alzheimer's patients, insulin dysregulation has been linked to learning deficits and memory formation impairments [5].

A recent study shows that metabolic syndrome and IR were associated with poorer executive performance in women [6] suggesting that insulin could be related with EF in a sex-specific manner. Co-morbidity has been established between metabolic and psychiatric diseases and interestingly, among adults with ADHD, there is a 18.7% higher prevalence of type 2 diabetes in males rather than females [7] suggesting that these sex-specific associations need to be further studied.

We have previously reported [8] that a polygenic risk score associated with higher FI interacts with ELA to predict the development of inhibitory control in children at 36 months, reinforcing the idea that insulin may moderate the effects of ELA on EF. However, as the effects of ELA are often sex-specific [9, 10], a better understanding of sex-specific relationships between ELA, insulin, and the development of EF is needed. Our hypotheses for the current study are 1) the genetic backgrounds associated with high FI and EF share polygenic architecture; 2) if (1) is rejected, models including the interaction term between the genetic background associated with high FI and childhood adversity exposure could show association with EF over the life-course; 3) if (2) shows significance results, then there could be a causal relationship between fasting insulin levels and impulsivity in the presence of adversity, which we will inspect through mendelian randomization (MR).

Methods

Investigating the shared genetic background between higher fasting insulin and altered executive function phenotypes (impulsivity and ADHD)

GWASs

Sex specific FI GWAS: We obtained complete FI GWAS results in the form of summary statistics p-value from the Meta-Analyses of Glucose and Insulin-related traits Consortium [11]. Please see more details in the Supplement.

Sex specific ADHD GWAS: ADHD GWAS was obtained from sex-specific meta-analyses of case-control ADHD by the Psychiatric Genomics Consortium and the Lundbeck Foundation Initiative for Integrative Psychiatric Research [12]. Please see more details in the Supplement.

Sex specific Impulsivity GWAS: As this GWAS was not available in the literature, we have performed a sex specific GWAS for impulsivity using the UK Biobank data. Please see more details in the Supplement.

Conditional/conjunctional FDR

Analyses applied here used false discovery rate (FDR) methods previously published and established by Andreassen et al [13]. To evaluate the cross-phenotype polygenic architecture, we produced conditional quantile-quantile (Q-Q) plots, conditioning FI on impulsivity or ADHD and vice versa. To identify shared loci between FI and impulsivity or ADHD, we applied the conditional FDR (condFDR) and conjunctional FDR (conjFDR) methods [13]. A conservative FDR level of 0.01 per pairwise comparison was set for condFDR/conjFDR, corresponding to 1 false positive per 100 reported associations. More details can be found in the Supplement, as well as in the original and subsequent publications [13].

Investigating the interactive effects between polygenic scores for higher fasting insulin and childhood adversity on altered executive function phenotypes in multiple cohorts in a sex-specific manner

Participants

We used data from five prospective birth cohorts: 1) Avon Longitudinal Study of Parents and Children (ALSPAC) [14, 15]; 2) Maternal Adversity, Vulnerability, and Neurodevelopment (MAVAN) [16]; 3) Growing Up in Singapore Towards healthy Outcomes (GUSTO) [17]; 4) Adolescent Brain Cognitive Development ^{SM} Study (ABCD®) [18]; and 5) UK Biobank (UKB) [19] to analyze the gene by environment interaction effects on EF outcomes. Details about recruitment, ethical approval, inclusion criteria for this

study and genotyping information on the cohorts can be found in supplementary materials.

The Avon Longitudinal Study of Parents and Children (ALSPAC) [20]: For the purpose of our analysis, we included children of 8.5 and 9.5 years old (individuals with peripheral insulin levels data), whose mothers had a pregnancy duration between 37 and 42 weeks, a maternal age at delivery greater than 18 years, a child birthweight greater than 2 kg, child alive at 1 year of age, and we only included singleton pregnancies in the analysis. There were 1,901 males and 1,834 females with complete data available for the analyses.

Maternal Adversity, Vulnerability, and Neurodevelopment (MAVAN) Project [16]: There were 161 subjects with complete data on the predictors used within this study available for the analyses after the exclusion criteria was applied, as described in *supplementary Figure 23*.

Growing Up in Singapore Towards healthy Outcomes (GUSTO) [17] prospective cohort: Study sample was selected based on data availability for each analysis. There were 466 subjects with complete data on the predictors used within this study available for the analyses, as described in *supplementary Figure 23*.

Adolescent Brain Cognitive Development[SM] Study *(ABCD®) [18]*: There were 7,655 subjects with complete data on the predictors used within this study available for the analyses, as described in *supplementary Figure 23*.

UK Biobank (UKB) [19]: There are a total of 502,543 participants within the cohort. There were 71,036 subjects with complete data on the predictors used within this study available for the analyses, as described in *supplementary Figure 23*.

Refined Polygenic Risk Scores (rPRS)

In this study, we sought to identify the high FI PRS threshold that best predicted FI levels in children from the ALSPAC cohort in a sex-specific manner (using a sex-specific FI GWAS). For that, we used the refined-PRS (rPRS) method previously described in our previous study [8]. Subsequently, the rPRS was applied to inspect the interaction effect between the rPRS for higher FI and ELA on EF behaviors in four separate cohorts to assess the effect in four different age groups, separately for males and females.

ALSPAC: The FI PRSs were calculated using the FI GWASs separately for males and females (N_{males} = 47,806, $N_{females}$ = 50,404) from the Meta-Analyses of Glucose and Insulin-related traits Consortium PRSs were calculated at 100 different p-value thresholds for each individual in the ALSPAC cohort as a sum of the risk alleles count weighted by the effect size described in the GWAS for each SNP; we then utilized Generalized Estimating Equations (GEE) analysis to identify the threshold of PRS at which the model predicting peripheral insulin levels in children at age 8.5-9.5 years had the best fit to the data, separately in males and females. To further refine the PRS, a process explained in Batra et al [8] was applied, we ran a GEE analysis for each SNP within the identified PRS threshold to find which SNPs were significantly associated with the peripheral insulin levels separately for males and females [$N_{SNP\ males}$= 635 SNPs, $N_{SNP\ females}$ = 1,449 SNPs]. Please see more details in the Supplement.

MAVAN, GUSTO, ABCD, and UKB: The SNPs we discovered to associate with peripheral insulin levels in the ALSPAC cohort were used to construct a rPRS in these four cohorts. Because the SNPs were selected in a refinement process of a PRS that was

created through conventional means, we therefore refer to this PRS as the refined PRS. The rPRS was calculated similarly to the PRS scores in ALSPAC, as a weighted sum of 635 SNPs for males and 1,449 SNPs for females.

Early Life Adversity

To inspect the interaction effect between the PRS and early life adversity, we calculated adversity exposure using a cumulative score involving different environmental variables for each individual in the cohorts as described by Silveira et al. [21]. Details on the components of the scores for each cohort can be found in supplementary materials.

Interaction PheWAS

We performed a linear regression (for continuous outcomes) and logistic regression (for binary outcomes) analyses for each PheWAS to investigate the interaction effect between the rPRS for FI and postnatal adversity exposure on each EF behavior separately in males and females in MAVAN, GUSTO, ABCD, and UKB, adjusted by population stratification PCs, age and other covariates if they were found to be significantly different between low/high PRS groups (as presented in *Tables 12-15*). We also used GEE if an outcome was measured several times. Details on the EF behaviors analyzed for each cohort can be found in supplementary materials.

Investigating the causal relationship between high fasting insulin and altered executive functions according to early adversity exposure in a sex-specific manner

Two-Sample Mendelian Randomization Analyses

Two-sample MR analyses were performed using R and the TwoSampleMR package [22]. Since we were interested in inspecting the interaction effect between FI and adversity exposure on impulsivity, we ran two analyses separately in males and

females: 1) two-sample MR between the FI GWAS and an impulsivity GWAS constructed with data from individuals exposed to adversity; 2) two-sample MR between the FI GWAS and an impulsivity GWAS constructed with data from individuals that were not exposed to adversity. Please see more details in the Supplement.

Results

Investigating the shared genetic background between higher fasting insulin and altered executive function phenotypes (impulsivity and ADHD)

The conditional Q-Q plots did not show enrichment for FI given impulsivity or ADHD as evident in *supplementary Figure 24*. The blue lines in *supplementary Figure* are drawn using the FI GWAS including all the SNPs regardless of their association with impulsivity or ADHD. An increasingly leftward deflection from the dashed line of no association was not observed when SNPs with stronger association with impulsivity and ADHD were plotted. To provide a list of shared loci between FI and impulsivity or ADHD, we performed conjFDR analyses where we found no SNPs in common between the genetic datasets.

Summary statistics for the sex-specific impulsivity GWAS generated in this study can be found at https://github.com/SilveiraLab/Sex-Specific_Impulsivity_GWAS. A brief description of the findings from this GWAS can be found in the Supplement (*Figures 17, 18, 19, 20, 21, and 22*).

Investigating the interactive effects between polygenic risk scores for higher fasting insulin and childhood adversity on altered executive function phenotypes in multiple cohorts in a sex-specific manner

Baseline comparisons between low and high PRS groups were performed in all four cohorts. In the MAVAN cohort, the only main confounding variable that was significantly different between low and high PRS was birth weight in males ($p = 0.034$), as shown in *Table 12.* In the GUSTO cohort, ethnicity in females ($p = 0.016$) was significantly different between the low and high PRS as shown in *Table 13*. In the ABCD cohort, household income in females ($p < 0.001$), was significantly different between low and high PRS as shown in *Table 14*. In the UKB cohort, there were no significantly different confounding variables between low and high PRS as shown in *Table 15*.

PheWAS Analysis

We performed linear regression and logistic regression analyses to investigate the interaction effect between the rPRS for FI and postnatal adversity exposure on each EF behavior separately in males and females in MAVAN, GUSTO, ABCD, and UKB, adjusted by population stratification PCs, age, and other covariates if they were found to be significantly different between low/high PRS groups which are shown in *Tables 12 through 15*. The outcomes significantly associated with the interaction, by nominal p-values, in each cohort are highlighted in *Figure 16*. None of the outcomes were significantly associated after adjusting for multiple testing.

Investigating the interactive effects between polygenic scores for higher fasting insulin and childhood adversity on altered executive function phenotypes in multiple cohorts in a sex-specific manner

Mendelian randomization analyses allowed testing of potential causal association between FI and impulsivity. Before running two sample MR, we used the MR Egger intercept test to inspect for horizontal pleiotropy. The MR Egger intercept test did not

reject the hypothesis of the presence of horizontal pleiotropy in males exposed to adversity (intercept = -0.00008; standard error = 0.00002; $p < 0.001$), in males not exposed to adversity (intercept = 0.00009; standard error = 0.00001; $p < 0.001$), and in females not exposed to adversity (intercept = 0.00004; standard error = 0.00001; $p = 0.001$). MR-PRESSO was used to identify outliers in those three instances and once those outliers were removed, MR Egger was performed again. The test could not significantly deny the presence of horizontal pleiotropy in males exposed to adversity (intercept = -0.0001; standard error = 0.00002; $p < 0.001$), in males not exposed to adversity (intercept = 0.00008; standard error = 0.00001; $p < 0.001$), and in females not exposed to adversity (intercept = 0.00003; standard error = 0.00001; $p = 0.017$).

However, the MR Egger intercept test suggested that there was no horizontal pleiotropy in females exposed to adversity (intercept = -0.000006; standard error = 0.00002; $p = 0.766$). Therefore, two-sample MR analysis was only used to inspect the potential causal association between FI and impulsivity in females exposed to adversity. All MR methods resulted in a significant association between the exposure FI and the outcome impulsivity in females exposed to adversity [IVW $p < 0.001$, IVW $\beta = -0.028$, IVW 95% CI: -0.032 to -0.023; Simple Median $p < 0.001$, Simple Median $\beta = -0.035$, Simple Median 95% CI: -0.042 to -0.029; Weighted Median $p < 0.001$, Weighted Median $\beta = -0.022$, Weighted Median 95% CI: -0.029 to -0.015].

Discussion

The purpose of this study was to explore whether there is a moderating effect of FI in the association between ELA and altered EF, throughout development, in a sex-

specific manner, and whether there is a causal relationship between FI and altered EF according to the exposure of adversity.

CondFDR did not show a shared genetic architecture between FI and impulsivity, an EF previously identified to be associated with the interaction between high FI levels and ELA in [8], or between FI and ADHD, a disorder in which impulsivity is a symptom [23]. A previous study from our lab has shown that a mesocorticolimbic-specific expression based PRS of insulin receptor (IR-ePRS) was associated with childhood impulsivity in males [24]. Based on these findings, we expected that insulin and impulsivity would share genetic architecture, however our study could not confirm this neither in males nor females. The lack of shared genetic architecture suggested that there could be a factor interacting with FI, such as ELA, to result in alterations in EF behaviors. These findings led us to inspect the interaction effect between FI and ELA on EF behaviors including a broad range of outcomes.

We demonstrated that the calculation of a rPRS, consisting of SNPs most highly associated with peripheral insulin levels in children and representing the risk for high FI levels early in life, can be derived from the GWAS of FI in adults [8]. Insulin resistance has been shown to be associated with childhood social relationship problems [25], and these could also affect EF development as observed in our results. Since conduct problems often occur with ADHD [26] and heritability has been established in molecular genetic studies between both traits [27], it made sense that we observed a significant interaction effects on conduct problems and rule breaking behavior in GUSTO females. While in MAVAN, GUSTO, and ABCD, attention and impulsivity traits were being highlighted through this interaction effect using different measures, in the UKB, the

significant interaction effect was seen on psychiatric outcomes such as addiction to alcohol in males and depression in females. The significant outcomes in the UKB show a trend from childhood to adulthood because impulsivity and attention related problems map to addiction [28] and depression [29] in adulthood. Additionally, internalizing problems are significantly associated with symptoms of anxiety and depression [30], confirming results that we saw as early as adolescence in ABCD females.

Although none of these interaction effects were significant after adjusting for multiple testing, the trend is present throughout development, in both males and females in childhood as evident through MAVAN and GUSTO. But the association changes in adolescents to only being significant in females in the ABCD cohort. This could be explained by males exhibiting higher insulin sensitivity at this age serving as a metabolic corrector [31]. When examined in adults, both analyses in males and females showed significant interaction between FI and ELA on psychiatric outcomes, thus reaffirming the trend of insulin functioning's association with cognitive functions [32] in childhood, which has been shown in literature and in our studies.

The gene ontology enrichment analyses, done through MetaCore^{TM}, showed that the SNPs composing the FI rPRS are associated with nervous system development. Some of the enriched processes should be highlighted, such as dopamine D2 receptor transactivation in males as the dopamine system has been linked to impulsive behavior in animal models and human studies [33, 34]. This finding suggests a potential neurodevelopment pathway to support the relationship between the genetic background linked to insulin, ELA, and dopamine. In previous animal models from our lab, it has been shown that animals that have experienced ELA have a delay in dopamine release in

response to palatable food, but insulin administration reverts this delayed effect [35]. These studies show that the relationship between ELA and dopamine is moderated by insulin. Enrichment analyses in FUMA revealed that the genes, to which the SNPs composing the FI rPRS map to, were significantly enriched for cognition in both males and females for several GWASs for ADHD in both males and females and for impulsivity in females, aligning with our executive function results from the interaction analysis.

MR is a powerful tool as it uses the Mendel law as its basis to assess the potential causal association between two traits through a naturally occurring randomized clinical trial [36]. In our study, MR showed significant associations between FI and impulsivity in females exposed to adversity. In all other cases, the Egger test in addition to the MR-PRESSO test failed to significantly deny the presence of horizontal pleiotropy meaning that there may be causal association between FI and impulsivity in those cases, but MR could not be used to determine its presence. These results are consistent with previous studies from our lab where ELA has affected specifically female behavior. Barbieri et al [37] showed that women born with severe intrauterine growth (IUGR) show a preference to carbohydrates in adulthood compared to those born with normal birth weight, without effects in men [37]. IUGR has been associated with metabolic stress which can result in altered appetite regulation. Appetite regulation involves the EF inhibition of impulsive behaviors [38] explaining the results from Barbieri et al in the context of our study. A similar effect was observed in another study done by our lab where IUGR girls demonstrated increased impulsivity in the Snack delay task, while there were no effects of IUGR in boys [39]. Rodent studies done on the topic also show that ELA leads to learning impairments in female rats and prepubertal stress leads to compulsive behavior

in female rats, but this effect was not observed with male rats [40]. And while we cannot exclude that this causal relationship might exist in males as well upon the examination through further studies, we can conclude that there is a causal association between insulin and impulsivity in the context of adversity in females.

In conclusion, our present findings provide support for the moderating effects of FI on the long-term impact of ELA on altered EF such as impulsivity and attention throughout development in males and females. Additionally, high FI has a causal association with increased impulsivity in females exposed to adversity. This research can be highly impactful as it can provide further insights into the mechanisms involved in insulin moderating effects of ELA on EF disorders, as well as the development of potential interventions.

Tables and Figures

Table 12. Participants' characteristics in MAVAN. Numbers are presented as mean (SD) or percentage (number of participants). Comparison between Low/High PRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

Sample descriptive	Males				Females			
	Total (n = 80)	**Low PRS (n = 40)**	**High PRS (n = 40)**	***p***	**Total (n = 81)**	**Low PRS (n = 40)**	**High PRS (n = 41)**	***p***
Age at delivery (years)	30.58 (4.31)	30.54 (3.95)	30.61 (4.7)	0.942	30.88 (4.82)	31.14 (4.74)	30.62 (4.93)	0.625
Birth weight (grams)	3449 (459)	3341 (373)	3558 (514)	**0.034**	3233 (429)	3169 (406)	3294 (446)	0.191
Breastfeeding at 3 months- yes	76.9% (60)	79.5% (31)	74.4% (29)	0.788	68.4% (54)	68.4% (26)	68.3% (28)	1
Smoking during pregnancy	12.5% (10)	12.5% (5)	12.5% (5)	1	11.1% (9)	5% (2)	17.1% (7)	0.169
Household income < $30000	0.14 (0.34)	0.08 (0.27)	0.19 (0.4)	0.155	0.13 (0.34)	0.08 (0.27)	0.18 (0.39)	0.18
Gestational age at birth (weeks)	39.35 (1.21)	39.27 (1.2)	39.42 (1.24)	0.583	39.12 (1.18)	38.92 (1.14)	39.32 (1.19)	0.134

Table 13. Participants' characteristics in GUSTO. Numbers are presented as mean (SD) or percentage (number of participants). Comparison between Low/High PRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

Sample descriptive	**Males**				**Females**			
	Total (n = 249)	**Low PRS (n = 124)**	**High PRS (n = 125)**	***p***	**Total (n = 217)**	**Low PRS (n = 108)**	**High PRS (n = 109)**	***p***
Age at delivery (years)	31.16 (4.86)	31.21 (4.7)	31.11 (5.03)	0.880	31.1 (5.31)	30.81 (4.8)	31.38 (5.77)	0.434
Birth weight (grams)	3149 (427)	3126 (439)	3172 (415)	0.398	3081 (427)	3065 (421)	3098 (433)	0.570
Breastfeeding at 3 months- yes	61.6% (146)	60.2% (71)	63% (75)	0.750	55.2% (117)	59.8% (64)	50.5% (53)	0.219
Smoking during pregnancy	2% (5)	3.2% (4)	0.8% (1)	0.361	2.8% (6)	2.8% (3)	2.8% (3)	1
Household income < SG$2000	0.13 (0.34)	0.1 (0.31)	0.16 (0.37)	0.201	0.16 (0.36)	0.15 (0.36)	0.17 (0.37)	0.732
Ethnicity Chinese Indian Malay	53.8% (134) 15.3% (38) 30.9% (77)	54% (67) 14.5% (18) 31.5% (39)	53.6% (67) 16% (20) 30.4% (38)	0.944	58.5% (127) 13.8% (30) 27.6% (60)	60.2% (65) 7.4% (8) 32.4% (35)	56.9% (62) 20.2% (22) 22.9% (25)	**0.016**
Gestational age at birth (weeks)	38.77 (1.28)	38.7 (1.3)	38.84 (1.25)	0.400	38.96 (1.21)	38.89 (1.19)	39.03 (1.22)	0.395

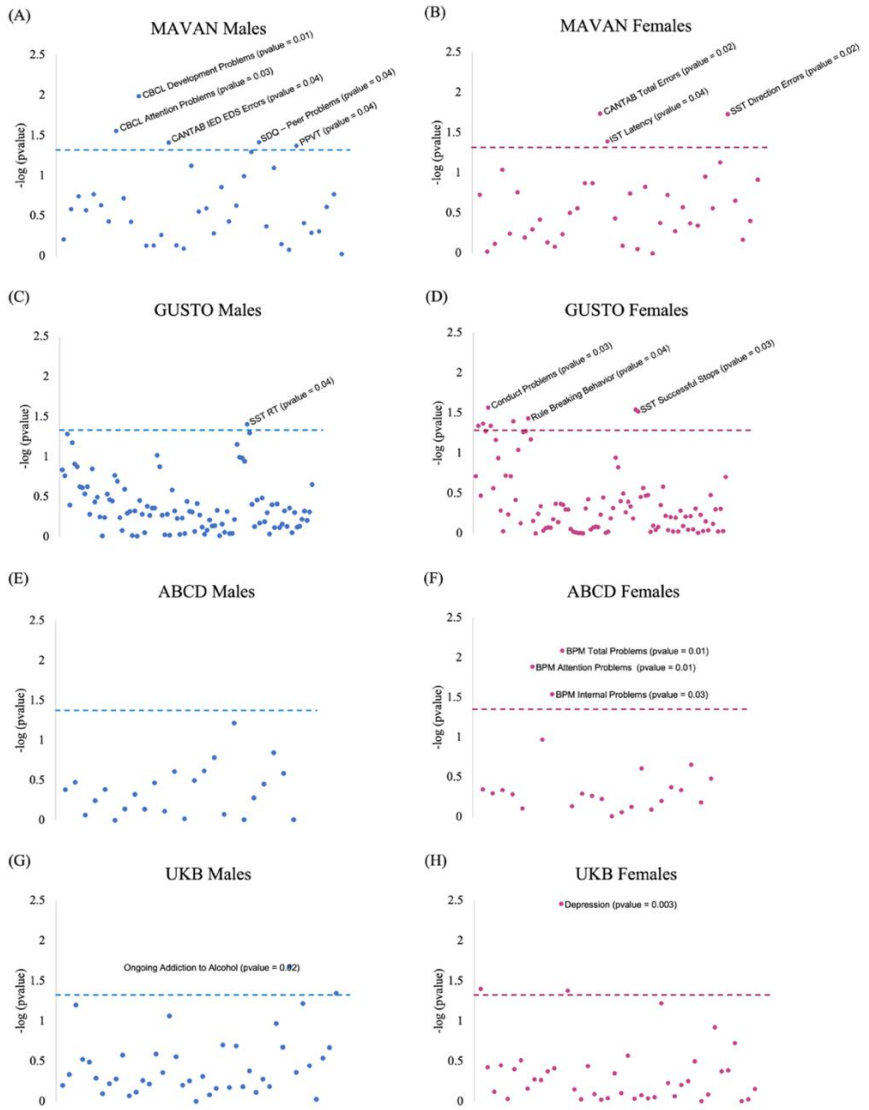
Table 14. Participants' characteristics in ABCD. Numbers are presented as mean (SD) or percentage (number of participants). Comparison between Low/High PRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

Sample descriptive	**Males**				**Females**			
	Total (n = 4012)	**Low PRS (n = 1988)**	**High PRS (n = 2024)**	***p***	**Total (n = 3643)**	**Low PRS (n = 1782)**	**High PRS (n = 1861)**	***p***
Age at recruitment (months)	119.08 (7.55)	118.92 (7.59)	119.24 (7.51)	0.19	118.9 (7.46)	118.86 (7.33)	118.93 (7.58)	0.757
Birth weight (grams)	3270 (676)	3260 (672)	3279 (679)	0.375	3131 (644)	3145 (635)	3118 (652)	0.22
Breastfeeding at 3 months- yes	69.7% (2770)	69.7% (1375)	69.7% (1395)	1	69.1% (2519)	68.5% (1220)	69.8% (1299)	0.402
Smoking during pregnancy	13.1% (527)	13.4% (266)	12.9% (261)	0.683	12.6% (459)	13.5% (241)	11.7% (218)	0.111
Household income < $25000	0.12 (0.32)	0.12 (0.32)	0.11 (0.32)	0.516	0.12 (0.32)	0.14 (0.35)	0.09 (0.29)	**<0.001**
Gestational age at birth (weeks)	39.04 (2.2)	39.04 (2.2)	39.04 (2.2)	0.985	39.08 (2.15)	39.15 (2.04)	39.01 (2.24)	0.052

Table 15. Participants' characteristics in UKB. Numbers are presented as mean (SD) or percentage (number of participants). Comparison between Low/High PRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

Sample descriptive	**Males**				**Females**			
	Total (n = 26527)	**Low PRS (n = 13275)**	**High PRS (n = 13252)**	***p***	**Total (n = 44509)**	**Low PRS (n = 22133)**	**High PRS (n = 22376)**	***p***
Age at recruitment (years)	54.96 (7.84)	54.92 (7.86)	55 (7.82)	0.396	54.4 (7.61)	54.91 (7.88)	55 (7.81)	0.714
Birth weight (grams)	3461 (635)	3462 (636)	3461 (634)	0.812	3274 (589)	3464 (637)	3459 (634)	0.181
Breastfeeding - yes	74.1% (17757)	74% (8846)	74.3% (8911)	0.574	70.7% (29313)	74.1% (8726)	74.2% (9031)	0.131
Smoking during pregnancy	28.5% (7547)	28.7% (3805)	28.2% (3742)	0.45	27.4% (12183)	27.8% (3627)	29.1% (3920)	0.398
Townsend Deprivation Index at recruitment	-1.83 (2.79)	-1.79 (2.81)	-1.86 (2.78)	0.059	-1.72 (2.81)	-1.81 (2.82)	-1.85 (2.77)	0.111
Body Mass Index	27.34 (4.07)	27.3 (4.05)	27.39 (4.1)	0.071	26.24 (4.89)	27.28 (4.1)	27.41 (4.05)	0.152

Figure 16. Executive functions PheWAS Results. Linear/logistic regression analyses were done to investigate the interaction effect between the rPRS for FI and postnatal adversity exposure on executive function behaviors in MAVAN (A) males and (B) females, GUSTO (C) males and (D) females, ABCD (E) males and (F) females, and UKB (G) males and (H) females, adjusted by covariates described in the methods section. In all cohorts, there were no significant interaction effects found on executive functions when considering the adjusted FDR p-values. The results highlight outcomes that were significant before the FDR correction was applied.



Supplementary Information

Methods

Investigating the shared genetic background between higher fasting insulin and altered executive function phenotypes (impulsivity and ADHD)

GWASs

Sex specific FI GWAS: We obtained complete FI GWAS results in the form of summary statistics p-value from the Meta-Analyses of Glucose and Insulin-related traits Consortium [11]. These GWAS meta-analysis results are provided for 47,806 men and 50,404 women. All participants were of European ancestry, without diabetes and mostly adults while data from a total of 8,222 adolescents were included in the meta-analyses. The additive genetic effect of each SNP was estimated using a linear regression model adjusting for age, study site, and genetic principal components. More information on this dataset can be found in Lagou et al [11].

Sex specific Impulsivity GWAS: As this GWAS was not available in the literature, we have performed a sex specific GWAS for impulsivity using the UK Biobank data. Genotyping data in the UKB cohort was available for 487,409 subjects. We excluded participants who withdrew their consent, with inconsistencies in genetic and reported sex, as well as outliers for heterozygosity. Also, we restricted our analysis only to the participants who identified themselves as “Caucasians” (ID 22006) to ensure the GWAS was comparable to the sex specific FI GWAS. We also removed variants with minor allele frequency < 0.01, an imputation accuracy info score < 0.1 as well as duplicated and ambiguous SNPs, resulting in 7,351,435 variants in the data set. The impulsivity phenotype was defined using mean time to correctly identify matches (ID 20023),

obtained at initial assessment visit (2006-2010) during a reaction time test based on the card-game “Snap”. This variable measures the combined processing and reaction speed of a participant. There were 128,277 male and 146,607 female unrelated subjects with the impulsivity phenotype and genotype data available and included in the GWAS, as displayed in *Figures 17 and 18* respectively. Additionally, *Figures 19 and 20* show Q-Q plots of the observed P-values on those expected using all the variants analyzed in UK Biobank for males and females respectively. We applied linear regression analysis using SNPTEST v2.5.4 to assess the effect of each SNP on impulsivity, adjusting for age, genotyping array, and 40 genetic principal components.

As this is an original GWAS, a small description of the findings is provided. Gene- and region-based analyses of the significant genes ($P < 2.6*10^{-6}$) were conducted using MAGMA (Multi-marker Analysis of GenoMic Annotation) available on FUMA GWAS (Functional Mapping and Annotation of Genome-Wide Association Studies) [41]. For gene-set pathway analysis we used the results obtained from the gene-based analysis considering SNPs at 10^{-5} as the threshold to conduct a further gene-set pathway analysis to test for gene enrichment using FUMA GWAS (Functional Mapping and Annotation of Genome-Wide Association Studies), Gene2func, gene set analysis, GO molecular functions.

Sex specific ADHD GWAS: ADHD GWAS was obtained from sex-specific meta-analyses of case-control ADHD by the Psychiatric Genomics Consortium and the Lundbeck Foundation Initiative for Integrative Psychiatric Research [12]. The GWAS included only subjects of European ancestry, 32,102 males (N=14,154 cases & 17,948 controls) and 21,191 females (N=4,945 cases & 16,246 controls), and SNPs with MAF >

0.01 and info-score > 0.8. We excluded duplicated and ambiguous SNPs, which resulted in 5,768,802 SNPs in male-specific GWAS and 5,748,208 SNPs in female-specific GWAS.

Conditional/conjunctional FDR

Analyses applied here used false discovery rate (FDR) methods previously published and established by Andreassen et al [13]. To evaluate the cross-phenotype polygenic architecture, we produced conditional quantile-quantile (Q-Q) plots, conditioning FI on impulsivity or ADHD and vice versa. Q-Q plots show the quantiles of the observed p values on the y axis and the theoretical quantiles under no association on the x axis. If there is no association, then the Q-Q plot falls on a straight null line but if there is any systematic association, the plot would deflect from the null line. Each Q-Q plot contains SNP p values of trait 1 conditional on different strength of association with trait 2 allowing us to determine if conditioning on a second trait indicates to a stronger association in the first trait of interest which would suggest a shared polygenic architecture between the traits.

To identify shared loci between FI and impulsivity or ADHD, we applied the conditional FDR (condFDR) and conjunctional FDR (conjFDR) methods [42, 43]. The condFDR method employs genetic association summary statistics from a trait of interest (FI) together with those of a conditional trait (impulsivity or ADHD) to estimate the posterior probability that a SNP has no association with the primary trait, given that the p values for that SNP in both the primary and the conditional traits are lower than the observed p values. This method identifies loci associated with the primary trait by using the associations present with the conditional traits. The conjFDR statistic is defined as

the maximum of the 2 mutual condFDR values and is a conservative estimate of the posterior probability that a SNP has no association with either trait, given that the p values for that SNP in both the primary and conditional traits are lower than the observed p values. Using this method of the conjFDR identifies loci which are associated with both traits. A conservative FDR level of 0.01 per pairwise comparison was set for condFDR/conjFDR, corresponding to 1 false positive per 100 reported associations. More details can be found in the original and subsequent publications [13, 42-44].

Investigating the interactive effects between polygenic scores for higher fasting insulin and childhood adversity on altered executive function phenotypes in multiple cohorts in a sex-specific manner

Participants

We used data from five prospective birth cohorts: 1) Avon Longitudinal Study of Parents and Children (ALSPAC) [14, 15, 20]; 2) Maternal Adversity, Vulnerability, and Neurodevelopment (MAVAN) [16]; 3) Growing Up in Singapore Towards healthy Outcomes (GUSTO) [17]; 4) Adolescent Brain Cognitive Development ^{SM} Study (ABCD®); and 5) UK Biobank (UKB) [19] to analyze the gene by environment interaction effects on EF outcomes.

The Avon Longitudinal Study of Parents and Children (ALSPAC) [20]: The ALSPAC cohort included pregnant women from the county of Avon, UK [14, 15] (N = 14,541) with expected delivery dates between April 1991 and December 1992. Additional recruitment (N = 906) was done later during phases, bringing the total sample size to 15,447. Participants provided informed written consent to participate in the study. Consent for biological samples had been collected in accordance with the Human Tissue

Act (2004). Ethics approval for the study was obtained from the ALSPAC Ethics and Law Committee and the local research ethics committees (a full list of the ethics committees that approved different aspects of the ALSPAC studies is available at http://www.bristol.ac.uk/alspac/researchers/research-ethics/). Data were collected during clinic visits or with postal questionnaires. Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool at http://www.bristol.ac.uk/alspac/researchers/our-data/. For the purpose of our analysis, we included children of 8.5 and 9.5 years old (individuals with peripheral insulin levels data), whose mothers had a pregnancy duration between 37 and 42 weeks, a maternal age at delivery greater than 18 years, a child birthweight greater than 2 kg, child alive at 1 year of age, and we only included singleton pregnancies in the analysis. There were 1,901 males and 1,834 females with complete data available for the analyses.

Maternal Adversity, Vulnerability, and Neurodevelopment (MAVAN) Project [16]: MAVAN is a birth cohort which follows children from birth up to 6 years of age in Montreal (Quebec) and Hamilton (Ontario), Canada, and has 629 recruited participants [16]. Mothers aged 18 years or above, with singleton pregnancies, and fluent in French or English were included in the study. Approval for the MAVAN project was obtained by the ethics committees and university affiliates (McGill University and Université de Montréal, the Royal Victoria Hospital, Jewish General Hospital, Centre hospitalier de l'Université de Montréal and Hôpital Maisonneuve-Rosemount) and St. Joseph's Hospital and McMaster

University, Hamilton, Ontario, Canada. Informed consent was obtained from all participants. There were 161 subjects with complete data on the predictors used within this study available for the analyses after the exclusion criteria was applied, as described in *supplementary Figure 23*.

Growing Up in Singapore Towards healthy Outcomes (GUSTO) [17] prospective cohort: The GUSTO study recruited pregnant women of at least 18 years in age, who were attending their first trimester antenatal ultrasound scan between June 2009 and September 2010 at one of Singapore's two major public maternity units: 1) National University Hospital and 2) KK Women's and Children's Hospital. Initially, the main GUSTO cohort recruited 1,450 mothers. By delivery, from 1216 participants, we excluded twins, subjects without genotyping data available, and participants without complete data for the analysis. Sociodemographic characteristics were collected using standardized self-report questionnaires. Study sample was selected based on data availability for each analysis. There were 466 subjects with complete data on the predictors used within this study available for the analyses, as described in *supplementary Figure 23*.

Adolescent Brain Cognitive ***Development*** [SM] Study *(ABCD®)*: This is a large-scale study tracking 9 years old and 10 years old individuals recruited from 21 research sites across the United States. ABCD® Data Release 2.0 which includes 4 waves of data: baseline (N = 11 875), 6-month follow-up (N = 8 623), 12-month follow-up (N = 4 951), and 18-month follow-up (N = 1 919). These data were accessed from the National Institutes of Mental Health Data Archive. There were 7,655 subjects with complete data on the predictors used within this study available for the analyses, as described in *supplementary Figure 23*.

UK Biobank (UKB) [19]: UKB is a population-based cohort with 502,543 recruited participants between the ages of 37 and 73 from 2006 to 2010 in the UK. This dataset contains information on participants' lifestyle and health data at baseline or follow-up, which were collected through questionnaires, physical measurements, and biological samples. All participants provided informed written consent before data collection. There are a total of 502,543 participants within the cohort. There were 71,036 subjects with complete data on the predictors used within this study available for the analyses, as described in *supplementary Figure 23*.

Genotyping

Processing of the genotyping data was done using PLINK 1.9 [45].

ALSPAC: Children in ALSPAC cohort were genotyped using the Illumina HumanHap550 quad chip genotyping platform by the Wellcome Trust Sanger Institute, Cambridge, UK and the Laboratory Corporation of America, Burlington, NC, US [46]. Standard quality control (QC) procedure was applied: participants with inconsistent self-reported and genotyped sex, minimal or excessive heterozygosity, high levels of individual missingness (>3%) and insufficient sample replication (IBD < 0.8) were excluded. Also, SNPs with call rate < 95%, MAF < 1%, or not in Hardy-Weinberg Equilibrium (HWE) ($p < 5 \times 10^{-7}$) were removed. Following the QC, the genotyping data was imputed using Impute v3 and Haplotype Reference Consortium (HRC) imputation reference panel (release 1.1), which resulted in 38,898,739 SNPs available for analysis. The population structure of ALSPAC cohort was described using principal component analysis [47, 48], which was conducted on the genotyped autosomal SNPs with MAF > 5% with the following pruning parameters for linkage disequilibrium: 100-SNP sliding

window, an increment of 5 SNPs, and variance inflation factor (VIF) threshold of 1.01. To account for population stratification, the first ten principal components were included in the analysis.

MAVAN: Genome-wide platforms (the Infinium PsychArray v1 or the PsychChip v1.1/v1.2, Illumina, Inc.) were used to genotype 229,456 autosomal SNPs of buccal epithelial cells of children in MAVAN, according to manufacturer's guidelines. SNPs with a low call rate (<95%), low *p*-values on HWE exact test ($p < 1 \times 10^{-30}$), and a MAF < 5% were removed. Afterwards, imputation using the Sanger Imputation Service (McCarthy et al., 2016) and HRC as the reference panel (release 1.1) was performed and SNPs with an info score > 0.8 were retained for the analysis, resulting in 16,249,769 autosomal SNPs. Similar to the ALSPAC cohort, the population structure of the MAVAN cohort was evaluated using principal component analysis of all autosomal SNPs that passed the quality control (MAF > 5%) and not in high linkage disequilibrium ($r^2 > 0.2$) across 50-SNP region and an increment of 5 SNPs [48]. Based on the inspection of the scree plot, the first three principal components (PCs) were the most informative of population structure and were included in all subsequent analyses.

GUSTO: Genomic DNA was extracted from the frozen umbilical cord specimens for each child and genotyped via Illumina OmniExpress arrays and Ilumina Exome arrays following the manufacturer's instructions. Similar to the MAVAN cohort, SNPs with a call rate < 95% or a MAF <5% or which deviated from HWE (p < 1e-40) were removed. Samples with call rates < 95% were removed. The Sanger Imputation Service was used to perform genome-wide imputation using 1000G as the reference, which resulted in 4,869,008 SNPs with an info score > 0.8. To assess population structure, we

performed principal component analysis on a pruned data set, like the MAVAN cohort. Based on the inspection of the scree plot, the first three principal components (PCs) were considered as the most informative of population structure in GUSTO cohort and were included in all analyses.

ABCD: The ABCD genetic data on 517,724 genotyped SNPs for 10627 subjects was obtained from ABCD data repository and subjected to the QC procedure, which was carried out using PLINK 1.9 [49]. We removed SNPs with a low call rate (<95%), minor allele frequency less than 5% or with low p-values on HWE exact test ($p < 1e\text{-}40$). Also, subjects with a missing call rate > 5% or relatedness issues were excluded. In total, 493,811 autosomal SNPs for 10,329 subjects passed the QC. Then, we imputed the data using the Sanger Imputation Service [50] and the HRC as the reference panel (release 1.1) resulting in 10,027,736 SNPs with an info score > 0.8. The population structure was evaluated using principal component analysis in a similar procedure as for the MAVAN cohort. First, from a set of 10,329 subjects we retained 8,873 unrelated subjects by keeping only one subject per family. Then we applied PCA of all autosomal SNPs that passed the quality control (MAF > 5%) and not in high linkage disequilibrium ($r2 > 0.2$) across 50-SNP region and an increment of 5 SNPs to generate the PCs, then projected the results on the related subjects' subset to obtain the PCs estimates for the remaining subjects. The first ten genetic principal components were included in all subsequent analyses.

UKB: Blood samples from UK Biobank were genotyped at the Affymetrix Research Services Laboratory in Santa Clara, California, USA. Genotyping was conducted using a bespoke BiLEVE Axiom array for 50,000 participants and the remaining 450,000

participants were genotyped using the Affymetrix UK Biobank Axiom array. The two SNP arrays are very similar with over 95% common marker content. Axiom Array plates were processed on the Affymetrix GeneTitan® Multi-Channel (MC) Instrument. Genotypes were then called from the resulting intensities in batches of ~4,700 samples (~4,800 including the controls) using the Affymetrix Power Tools software and the Affymetrix Best Practices Workflow. Individuals with the same genotype at any given SNP will cluster together in a two-dimensional intensity space (one dimension for each targeted allele). For the interim data release, Affymetrix performed further rounds of genotype calling using algorithms customized for the UK Biobank project. These algorithms targeted very rare SNPs with 6 or fewer minor alleles in a batch, and a subset of SNPs for which the generic calling algorithm did not perform optimally. After genotype calling, Affymetrix performed quality control in each batch separately, to exclude SNPs with poor cluster properties. If a SNP did not meet the Affymetrix prescribed QC thresholds in a given batch, it was set to missing in all individuals from that batch. HWE was performed for each batch. Affymetrix also checked sample quality (such as DNA concentration) and genotype calls were provided only for samples with sufficient DNA metrics. For SNP-based QC metrics, only individuals with similar ancestry and the population structure were characterized by computing principal components using only UK Biobank individuals. The array also includes coding variants across a range of minor allele frequencies (MAFs), including rare markers (< 1% MAF); and markers that provide good genome-wide coverage for imputation in European populations in the common (> 5%) and low frequency (1–5%) MAF ranges. More information about the genotyping protocol, QC, and imputation could be found in [51]. The population structure of the UK Biobank cohort was

evaluated using fastPCA algorithm for principal component analysis [52]. To account for population stratification, the first forty principal components were included in the UK Biobank analysis.

Refined Polygenic Risk Scores (rPRS)

A polygenic risk score (PRS) is a sum of the genetic effects of many variants, weighted by an estimated effect of association between alleles and the phenotype of interest described in a GWAS summary statistics. Classically, PRS can be calculated at any p-value thresholds from the GWAS. In this study, we sought to identify the high FI PRS threshold that best predicted FI levels in children from the ALSPAC cohort in a sex-specific manner (using a sex-specific FI GWAS). For that, we used the refined-PRS (rPRS) method previously described in our previous study [53]. Subsequently, the rPRS was applied in this study to inspect the interaction effect between the rPRS for higher FI and ELA on EF behaviors in four separate cohorts to assess the effect in four different age groups, separately for males and females.

ALSPAC: The FI PRSs were calculated using the FI GWASs separately for males and females (N_{males} = 47,806, $N_{females}$ = 50,404) from the Meta-Analyses of Glucose and Insulin-related traits Consortium [11]. Before the PRS calculations, the lists of SNPs from the GWASs were subjected to LD clumping with r^2 of 0.2 and ALSPAC cohort as a reference dataset. PRSs were calculated at 100 different p-value thresholds for each individual in the ALSPAC cohort as a sum of the risk alleles count weighted by the effect size described in the GWAS for each SNP [54, 55]. Using ALSPAC as a discovery cohort, we utilized Generalized Estimating Equations (GEE) analysis, using R package geepack [56-58], which allows to incorporate several measurements from the same participants to

identify the PRS threshold at which the model had the best fit to the data predicting peripheral insulin levels in children at age 8.5-9.5 years, separately in males and females. The best fit model was identified to be with a PRS at $p_{t\text{-}intial\text{-}males}$= 0.05 (11,121 SNPs; N_{males}=1,901) in males and $p_{t\text{-}intial\text{-}females}$=0.15 (27,202 SNPs; $N_{females}$=1,834) in females. To further refine the PRS, a process explained in Batra et al [53] was applied. Precisely, we ran a GEE analysis for each SNP within the identified PRS threshold to find which SNPs were significantly associated with the peripheral insulin levels separately for males and females [$N_{SNP\ males}$= 635 SNPs, $N_{SNP\ females}$ = 1,449 SNPs].

MAVAN, GUSTO, ABCD, and UKB: The SNPs we discovered to associate with peripheral insulin levels in the ALSPAC cohort were used to construct a rPRS in these four cohorts. Because the SNPs were selected in a refinement process of a PRS that was created through conventional means, we therefore refer to this PRS as the refined PRS. The rPRS was calculated similarly to the PRS scores in ALSPAC, as a weighted sum of 635 SNPs for males and 1,449 SNPs for females.

Early Life Adversity

MAVAN: The postnatal adversity score was created by combining the following markers: 1) birth size percentile below 10^{th} percentile or above 90^{th} percentile; 2) gestational age below or equal to 37 weeks; 3) smoking during pregnancy; 4) household total gross income below $30,000; 5) lack of money; 6) presence of domestic violence or sexual abuse during pregnancy; 7) marital strain; 8) pregnancy anxiety; 9) hospitalization in the first 6 months of life; 10) disorganized attachment; 11) poor family function; 12) maternal mental health through the presence of either BDI, EPDS, or STAI. For every item with a continuous score, we used either the 15^{th} or the 85^{th} percentile as the cut-off

to add a point to the adversity score. Presence of each component yields one point, and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

GUSTO: The postnatal adversity score was created by combining the following markers: 1) birth size percentile below 10th percentile or above 90th percentile; 2) gestational age below or equal to 37 weeks; 3) smoking during pregnancy; 4) household monthly income below $2000; 5) poor family function; 6) hospitalization in the first 6 months of life; 7) maternal mental health through the presence of either BDI, EPDS, or STAI. For every item with a continuous score, we used either the 15th or the 85th percentile as the cut-off to add a point to the adversity score. Presence of each component yields one point and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

ABCD: The postnatal adversity score was created by combining the following markers: 1) birth size below 10th percentile or above 90th percentile; 2) gestational age below or equal to 37 weeks; 3) smoking during pregnancy; 4) being in an incubator after birth; 5) household total gross income below $25,000; 6) presence of verbal, physical, or sexual abuse; 7) youth report of caregiver acceptance; 8) FES conflict; 9) caregiver's mental health. Individuals received 1 point for every item they indicated experiencing and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

UKB: The postnatal adversity score was created by combining the following markers: 1) birth size was below 10th percentile or above 90th percentile (ID20022); 2) maternal smoking (ID1787; 1 point if the answer was yes); 3) feeling hated by family

member during childhood (1 point if the answer was sometimes, often, or very often; ID20487); 4) feeling loved during childhood (1 point if the response was never or rarely; ID20489); 5) not having someone to take to the doctor when needed (1 point if the response was often, half a point was given if the response was rarely had someone; ID20491); 6) presence of domestic violence during childhood (physical or sexual) was also counted as one point (ID20488); and 7) whether an individual was part of a multiple birth (1 point if the response was yes; ID1777). Each adverse instrument was given a score of 1 and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

Executive Function Behavioral Outcomes

To inspect the interaction effect of the PRS and early life adversity on EF, we used all the EF behaviors with available data for each cohort. The MAVAN cohort outcomes consisted of EF behavior data from the Bayley Scales of Infant Development II [59], Child Behavior Checklist (CBCL) [60], School Readiness Test Battery [61], Dominique Questionnaire [62], Strengths and Difficulties Questionnaire (SDQ) [63], Cambridge Neuropsychological Test Automated Battery (CANTAB) Intra and Extradimentional shift (IED), and Information Sampling Task (IST) [64]. The GUSTO cohort outcomes consisted of EF behaviors data from Bayley Scales of Infant Development II [59], Child Behavior Checklist (CBCL) [60], Stop Signal Task (SST) [65], Snack Delay Task [39], Sticker Delay Task [66]. The ABCD cohort outcomes consisted of EF behaviors data from the Child Behavior Checklist [60], Brief Problem Monitor (BPM) assessment [67], and UPPS-P Impulsive Behavior Scale [68]. The ABCD Study data repository grows and changes over time. The ABCD Study data used in this report came

from https://dx.doi.org/10.15154/1503209. The UKB cohort analysis consisted of psychiatric outcomes related to EF outcomes.

Enrichment Analysis

Enrichment analyses for gene ontologies were performed using MetaCore™ (Clarivate Analytics) on the SNPs that compose the FI rPRS for males and females separately. Furthermore, gene-based enrichment analyses were performed in FUMA (https://fuma.ctglab.nl/) [69-71] after mapping the SNPs composing the FI rPRS to genes with the biomaRt package in R [72, 73].

Investigating the causal relationship between high fasting insulin and altered executive functions according to early adversity exposure in a sex-specific manner

Two-Sample Mendelian Randomization Analyses

Two-sample MR analyses were performed using R [72, 73] and the TwoSampleMR package [22]. Exposure and outcome GWAS summary statistics were harmonized as described within the package. To assess horizontal pleiotropy (i.e., an association of the genetic instrument with the outcome independent of the exposure), we used the MR-Egger estimation for genetic instruments. When the pleiotropy assumption was not met, we used a different statistical method robust to pleiotropy: MR pleiotropy residual sum and outlier (MR-PRESSO). MR-PRESSO is able to identify outliers with potential horizontal pleiotropy when using multiple genetic variants as an instrument and provides a corrected estimate after removing these outliers [74]. We first performed fixed-effects meta-analysis of genetic instruments using inverse-variance weighting (IVW) [75] and standard errors were computed with the Wald estimator and delta weighting to account for uncertainty in genetic association with the exposure. To assess the

robustness of our findings, the simple median MR and weighted median MR approach were performed. Since we were interested in inspecting the interaction effect between FI and adversity exposure on impulsivity, we ran two analyses separately in males and females: 1) two-sample MR between the FI GWAS and an impulsivity GWAS constructed with data from individuals exposed to adversity; 2) two-sample MR between the FI GWAS and an impulsivity GWAS constructed with data from individuals that were not exposed to adversity.

Results

Sex-specific impulsivity GWAS: Gene-based enrichment analysis

SNPs with $P<10^{-5}$ were selected to describe female and male GWASs. 14 genes were significantly associated with impulsivity in males and 5 in females (*Figures 21 and 22*). 20 genes overlapped between the male and female impulsivity GWASs (out of 101 total genes in males and 114 genes in females) indicating a small degree of shared polygenic architecture between the sexes. Only the female genes exhibited significant association with cellular components, most of which were related to the nervous system: dendritic tree, somatodendritic compartment, neuron projection, synapse, neuron to neuron synapse, and postsynaptic specialization. *ATG13*, which exhibits significant pleiotropy with Alzheimer's disease GWAS and fasting glucose GWAS in addition to reaction time, is enriched in both males and females. Several of the genes enriched in the males GWAS were significantly associated with autism spectrum disorder or schizophrenia GWAS. *MYT1L* and *TSSC1*, genes that are significantly associated with non-planning and motor impulsivity, were enriched only in females.

Investigating the interactive effects between polygenic scores for higher fasting insulin and childhood adversity on altered executive function phenotypes in multiple cohorts in a sex-specific manner

Enrichment Analysis

Enrichment analyses (MetaCore™) show that the subset of SNPs composing the FI rPRS are significant for several nervous system development processes in a sex-specific manner, as shown in *supplementary Table 16*. Additionally, enrichment analysis (FUMA, https://fuma.ctglab.nl/) of the genes mapped by the SNPs that compose the FI rPRS showed that these genes were significantly associated with certain nervous system development process and GWASs in a sex-specific manner, as shown in *supplementary Table 16.*

Supplemental Figures and Tables

Figure 17. Manhattan plot of all the variants analyzed in the UK Biobank for impulsivity in males (N = 128,277).

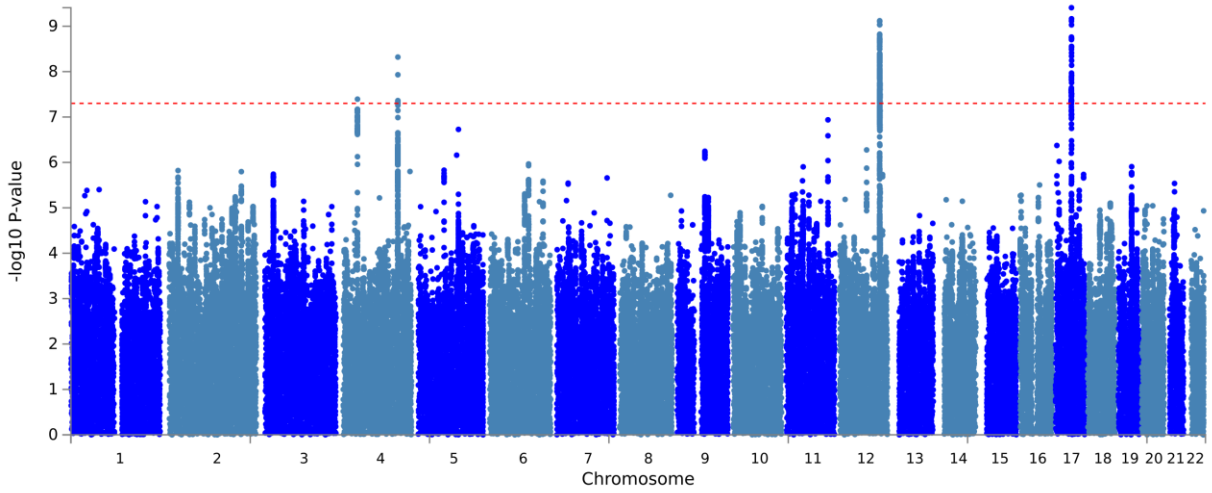


Figure 18. Manhattan plot of all the variants analyzed in the UK Biobank for impulsivity in females (N = 146,607).

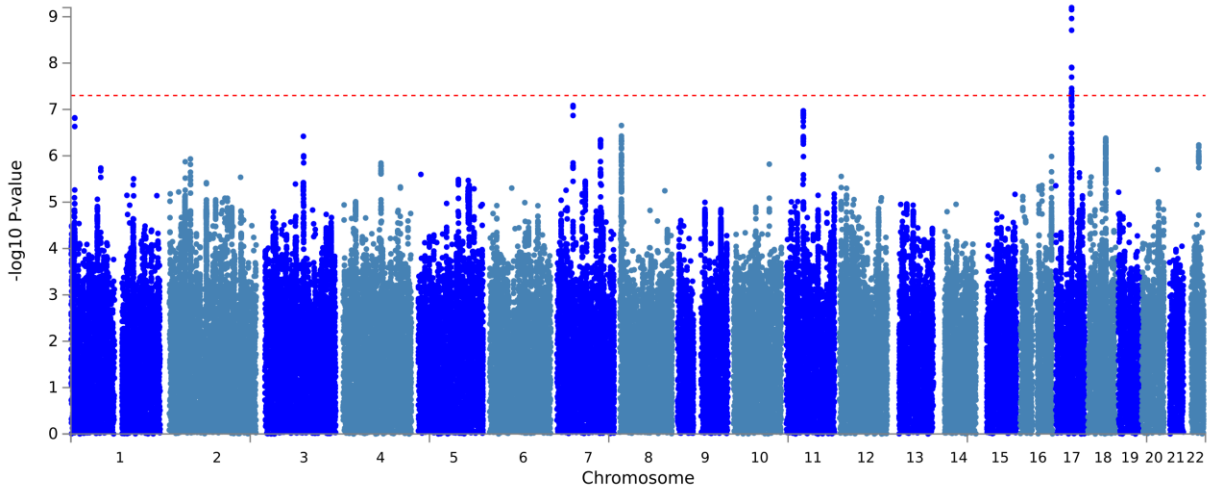


Figure 19. Q-Q plots of the observed P-values on those expected using all the variants analyzed in UK Biobank for impulsivity in males (N = 128,277).

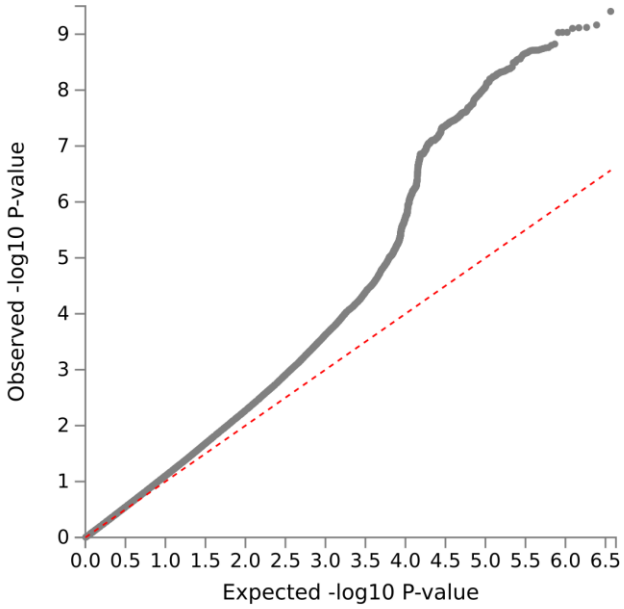


Figure 20. Q-Q plots of the observed P-values on those expected using all the variants analyzed in UK Biobank impulsivity in females (N = 146,607).

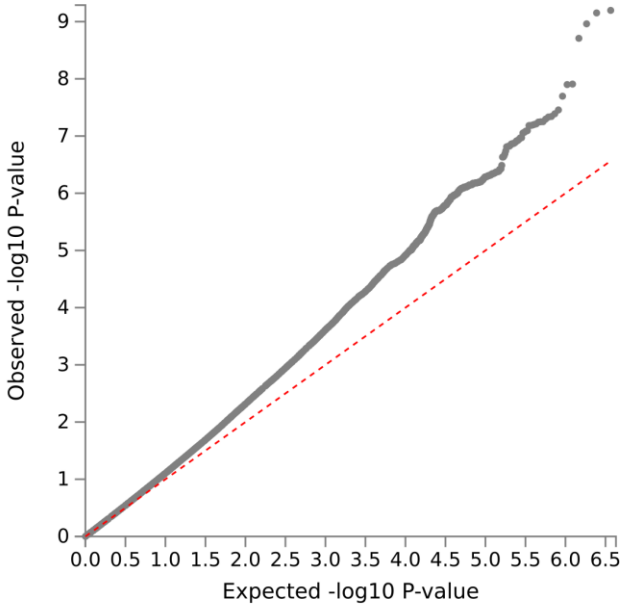


Figure 21. Manhattan plot of the adjusted –log10 P-values of each gene for an association with impulsivity in the UK Biobank cohort in males (N=128,277). The dotted horizontal line represents the gene-based threshold for significance (was defined at P = 0.05/19080 = 2.621e-6, where 19080 is a number of mapped protein coding genes).

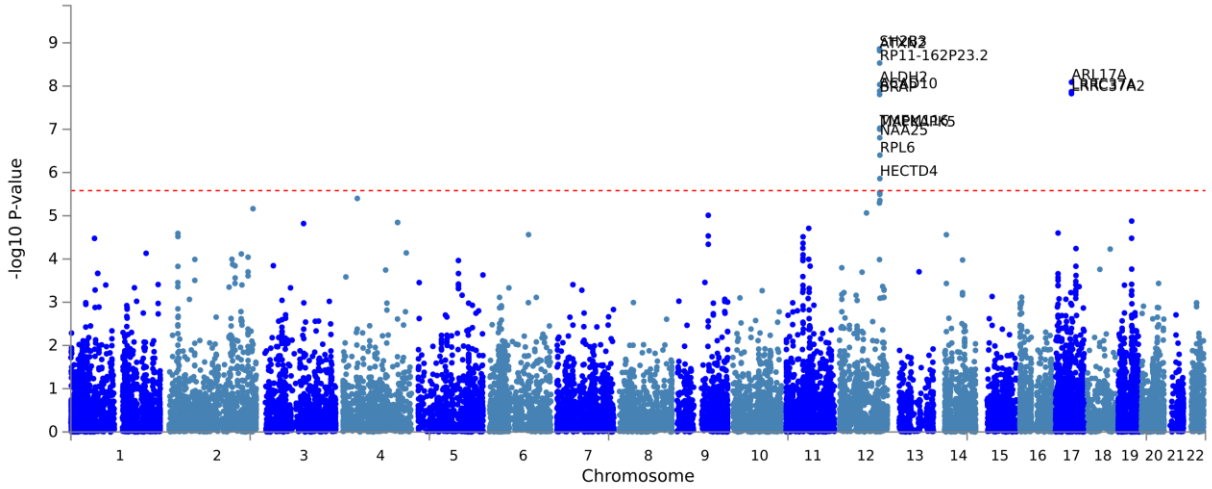


Figure 22. Manhattan plot of the adjusted –log10 P-values of each gene for an association with impulsivity in the UK Biobank cohort in males (N= 146,607). The dotted horizontal line represents the gene-based threshold for significance (was defined at P = 0.05/19080 = 2.621e-6, where 19080 is a number of mapped protein coding genes).

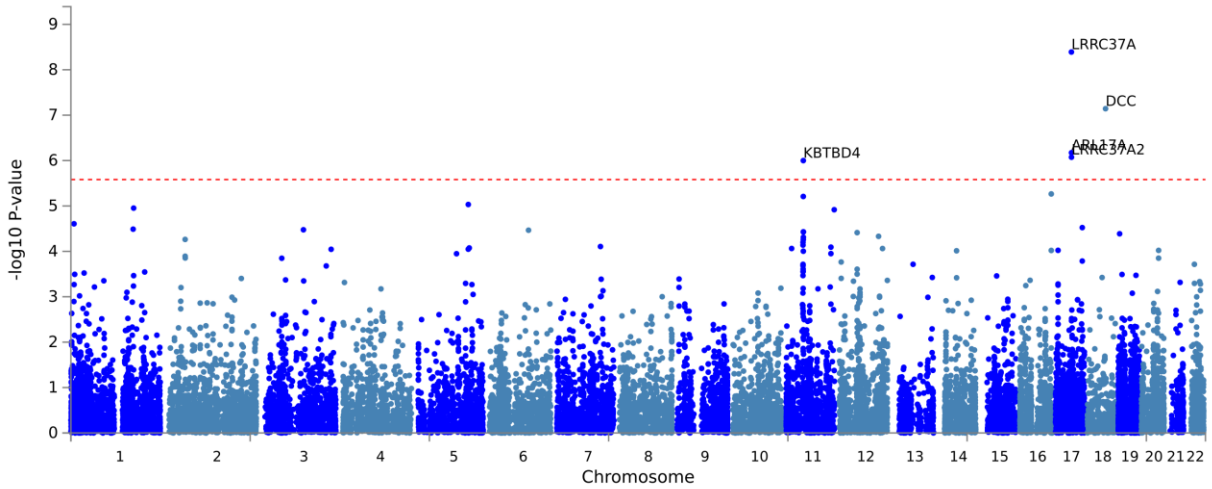


Figure 23. Block scheme describing sample selection in (A) MAVAN, (B) GUSTO, (C) ABCD, and (D) UKB.

Figure 24. Conditional Q-Q plots (A) fasting insulin conditioned on impulsivity (males), (B) fasting insulin conditioned on impulsivity (females), (C) impulsivity conditioned on fasting insulin (males), (D) impulsivity conditioned on fasting insulin (females), (E) fasting insulin conditioned on ADHD (males), (F) fasting insulin conditioned on ADHD (females), (G) ADHD conditioned on fasting insulin (males), and (H) ADHD conditioned on fasting insulin (females). The plots, (A) and (E) for males and (B) and (F) for females, display the nominal -log10p values of the single SNP association statistics versus their empirical distribution in fasting insulin and below the standard GWAS threshold as a function of significance of association with impulsivity/ADHD at the level of $p < 0.1$, $p < 0.01$, and $p < 0.001$. The plots, (C) and (G) for males and (D) and (H) for females, display the nominal -log10p values of the single SNP association statistics versus their empirical distribution in impulsivity/ADHD and below the standard GWAS threshold as a function of significance of association with fasting insulin at the level of $p < 0.1$, $p < 0.01$, and $p < 0.001$. The blue line indicates all the SNPs. The dashed line indicates the null hypothesis. The blue line indicates all the SNPs. The dashed line indicates the null hypothesis.

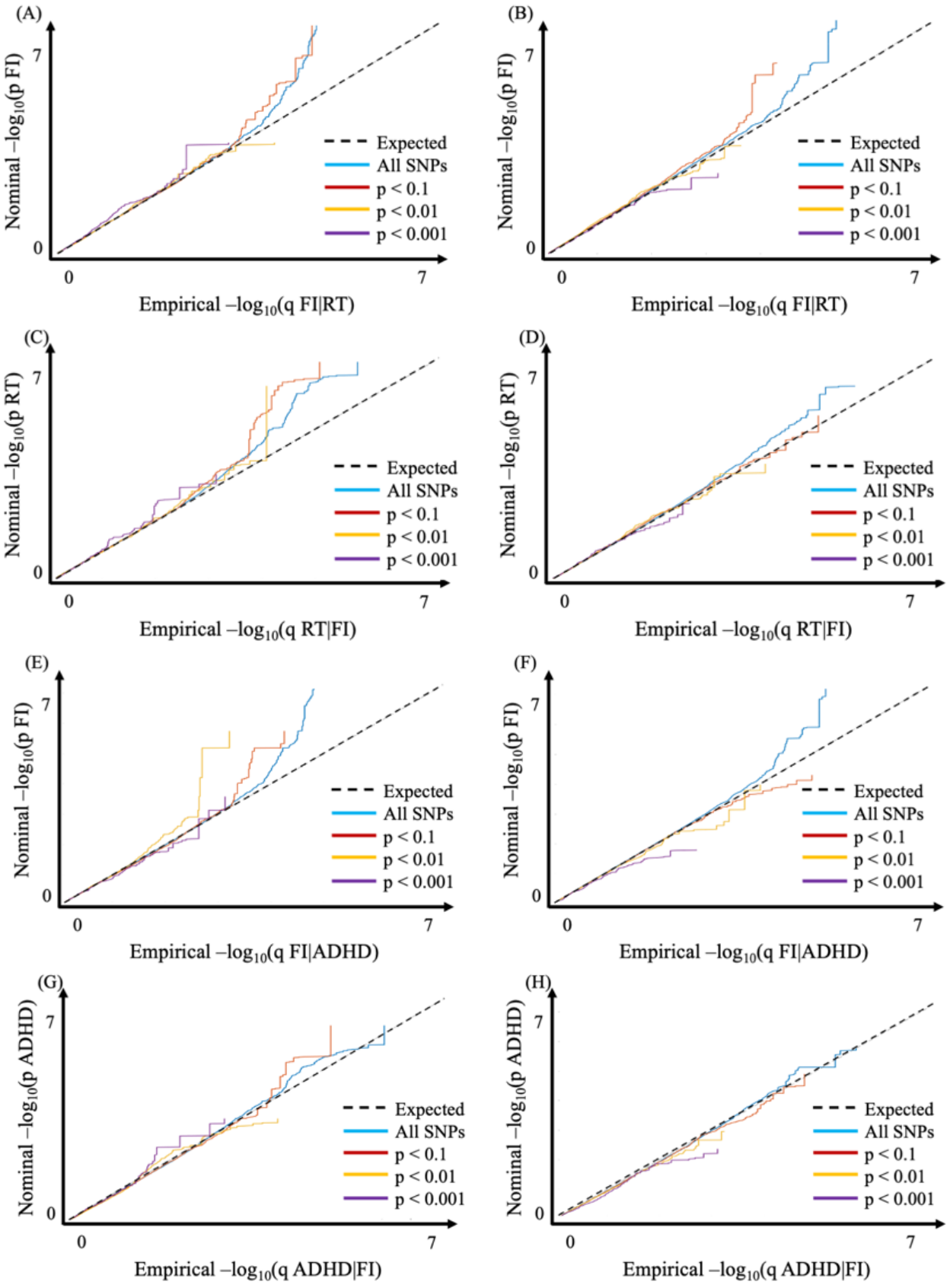

(A)
Nominal −log10(p FI)
Empirical −log10(q FI|RT)
(B)
Nominal −log10(p FI)
Empirical −log10(q FI|RT)
(C)
Nominal −log10(p RT)
Empirical −log10(q RT|FI)
(D)
Nominal −log10(p RT)
Empirical −log10(q RT|FI)
(E)
Nominal −log10(p FI)
Empirical −log10(q FI|ADHD)
(F)
Nominal −log10(p FI)
Empirical −log10(q FI|ADHD)
(G)
Nominal −log10(p ADHD)
Empirical −log10(q ADHD|FI)
(H)
Nominal −log10(p ADHD)
Empirical −log10(q ADHD|FI)
0
7
Expected
All SNPs
p < 0.1
p < 0.01
p < 0.001

Table 16. Enrichment analyses, through MetaCore™ and FUMA, show that the subset of SNPs and genes composed from the males and females FI rPRS are significant for several nervous system development processes and GWASs.

Metacore Results from SNPs composing the FI rPRS		
Processes	**Males FDR adjusted p-value**	**Females FDR adjusted p-value**
Nervous system disease	p < 0.001	p < 0.001
Nervous system development	p < 0.001	p < 0.001
Psychiatry and psychology diseases	p < 0.001	
Mood disorders	p < 0.001	
Neurogenesis axonal guidance		p = 0.007
HTR2A signaling in the nervous system	p = 0.033	p = 0.019
Regulation of intrinsic membrane properties and excitability of cortical pyramidal neurons		p = 0.0275
Neurophysiological process dopamine D2 receptor transactivation of PDGFR in the central nervous system	p = 0.041	
FUMA Results from genes mapped from the FI rPRS		
GWAS Enrichment	**Males FDR adjusted p-value**	**Females FDR adjusted p-value**
Cognitive ability	p < 0.001	
ADHD	p = 0.0002	p < 0.001
Cognitive performance		p < 0.001
Food addiction		p = 0.0048
Impulsivity		p = 0.018
Processes	**Males FDR adjusted p-value**	**Females FDR adjusted p-value**
Biological process cognition	p = 0.0308	p = 0.00228
Nervous system disease	p < 0.001	p < 0.001
Nervous system development	p < 0.001	p < 0.001
Psychiatry and psychology diseases	p < 0.001	
Mood disorders	p < 0.001	
Neurogenesis axonal guidance		p = 0.007
HTR2A signaling in the nervous system	p = 0.033	p = 0.019
Regulation of intrinsic membrane properties and excitability of cortical pyramidal neurons		p = 0.0275
Neurophysiological process dopamine D2 receptor transactivation of PDGFR in the central nervous system	p = 0.041	

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Chapter VI. Relationship between insulin and netrin-1/dcc guidance cue pathway regulation in the prefrontal cortex of rodents exposed to prenatal adversity

Preface

Our findings thus far have informed us that early life adversity and insulin signaling impact executive functioning throughout development. While we were able to establish these associations using human data and even show a causal association between the genetic background of fasting insulin and the genetic background of impulsivity in females exposed to adversity, the mechanism involved still needs to be researched. Based on our findings that the SNPs associated with high fasting insulin are enriched for the dopamine pathway, we chose the mesocorticolimbic dopamine pathway to inspect the mechanism in rodents. Literature has also shown that dopamine is involved in several executive function behaviors while the effect of early life adversity on this pathway and its role in insulin signaling is yet to be fully established. Previous work from our lab has shown that early life adversity, through an intrauterine growth restriction model in rodents, impacts dopamine signaling in the brain and alters behavior. The same model also shows alterations in insulin signaling in the brain. Therefore, with this next and last study, we wanted to inspect how insulin impacts the development of the mesocorticolimbic dopamine pathway to get one step closer to understanding the mechanism involved within this pathway which leads to altered executive functioning behavior. Since the netrin-1/dcc guidance cue pathway is involved in regulating the mesocorticolimbic pathway, within this study, we chose to inspect the effects of early life adversity and insulin on the netrin-1 guidance cue system.

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Relationship Between Insulin and Netrin-1/DCC Guidance Cue Pathway Regulation in the Prefrontal Cortex of Rodents Exposed to Prenatal Adversity

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Conflicts of Interest

None

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals sprague dawley and has been approved by the institutional committees (Research Ethics Committee of Hospital de Clínicas de Porto Alegre (GPPG/HCPA); McGill University; Douglas Mental Health University Institute AUP DOUG-8018).

Abstract

Fetal restriction (FR) alters insulin sensitivity, but it's unknown how the metabolic profile associated with restriction affects development of the dopamine (DA) system and DA-related behaviors. The Netrin-1/DCC guidance cue system participates in maturation of the mesocorticolimbic DA circuitry. We aimed to identify if FR modifies Netrin-1/DCC receptor protein expression in the prefrontal cortex (PFC) at birth and mRNA in adulthood in rodent males. We used cultured HEK293 cells to assess if levels of miR-218, microRNA regulator of DCC, are sensitive to insulin. To assess this, pregnant dams were subjected to a 50% FR diet from gestational day 10 until birth. Medial PFC (mPFC) DCC/Netrin-1 protein expression was measured at P0 at baseline and *Dcc*/*Netrin*-1 mRNA levels were quantified in adult 15 min after a saline/insulin injection. miR-218 levels in HEK-293 cells were measured in response to insulin exposure. At P0, Netrin-1 levels are downregulated in FR animals in comparison to controls. In adult rodents, insulin administration results in an increase in *Dcc* mRNA levels in control but not FR rats. In HEK293 cells, there is a positive correlation between insulin concentration and miR-218 levels. Since miR-218 is a *Dcc* gene expression regulator and our in-vitro results show that insulin regulates miR-218 levels, we suggest that FR-induced changes in insulin sensitivity could be affecting *Dcc* expression via miR-218, impacting DA system maturation and organization. As fetal adversity is linked to non-adaptive behaviors later in life, this may contribute to early identification of vulnerability to chronic diseases associated with fetal adversity.

Keywords: prenatal adversity, intrauterine growth restriction, food restriction, insulin, axonal guidance cues

Introduction

Reduced growth in fetal life has been strongly linked to the development of impaired glucose tolerance and type II diabetes [1], mainly due to altered growth and development of the pancreas [2]. Insulin secretion relative to insulin sensitivity is highly impaired in these individuals throughout their lifetime as a consequence of poor growth before birth [3-8]. Many models have shown that reduced growth due to poor nutrition increases the risk for adult chronic diseases. One of the most important models being that of caloric restriction [1, 9, 10] because it re-capitulates many features seen in intrauterine growth restriction (IUGR) cases in humans such as catch up growth [11], hyperinsulinemia [12], type II diabetes [13], and altered behaviors such as increased preference for palatable foods [9] and impulsivity [14]. Impulsivity includes two dimensions: action and choice [15]. Impulsive action refers to the inability in the inhibition of response such as responding without thinking [16] while impulsive choices involve a decision-making process and are expressed by the tendency to prefer immediate gains over long-term ones [17, 18]. Both action and choice impulsivity involve proper function of the mesocorticolimbic dopamine (DA) system [19].

It is yet to be fully established how IUGR-induced changes in insulin alter impulsivity and whether regulators of mesocorticolimbic DA development are involved. Tyrosine hydroxylase (TH) is the rate-limiting enzyme which converts L-tyrosine to L-3,4-dihydroxyphenylalanine, which ultimately turns into DA [20]. In response to sweet food intake, adult fetal growth restricted (FR) animals, meaning offspring of female rodents that were subjected to caloric restriction during pregnancy, show an increase in the expression of TH in the prefrontal cortex (PFC) in comparison to controls (referred to as AdLib in the

manuscript as the dams were fed ad libitum diet) [9, 21]. Since DA in the PFC is involved in attention and impulsivity-related behaviors in rats, increased TH expression in FR animals could be associated to the animals' reward-associated value of sweet food. This association with sweet food is especially relevant in FR animals [22], as they show an increased preference for palatable foods [9] that could lead to persistent changes in nutritional imbalance, eventually contributing to chronic metabolic diseases in adulthood [23]. When presented with palatable food, such as Froot Loops®, FR adult rats take less time to start eating the pellets in comparison to AdLib animals although both groups consumed the same amount of pellets over a 30 minute period, which could be a result of increased impulsivity [24]. DA signaling seems to be involved in these behavioral changes in FR animals, as evident by Alves et al. where the mean change in DA signaling in PFC was measured in controls and FR animals in response to standard chow and Froot Loops® consumption [24]. They observed that Froot Loops® consumption caused a blunted increase in DA concentration in FR animals when compared to AdLib animals [24]. Another study showed that FR animals exhibit higher peripheral insulin levels than AdLib animals [25] suggesting that IUGR modifies the modulation of mesolimbic DA neurons by insulin beyond the well-known alteration in peripheral glucose metabolism [24]. These studies showing lower TH levels and higher DA levels in control animals in response to palatable food when compared to FR animals could be suggesting that there is a slower signal to convert TH to DA in FR animals, but future studies will need to confirm this. In controls, the PFC DA levels in response to Froot Loops® consumption were greater than the levels in response to standard chow, while FR animals did not show a difference in DA levels in response to chow and Froot Loops®. The weakened response

to sweet food seen in the PFC of IUGR animals may result from decreased activation of striatal areas, leading to a lower valuation of the reward magnitude and therefore, favoring impulsive choices for immediate small rewards [21]. Within the PFC, there seems to be a positive correlation between impulsive choice and expression of D1 receptors [19] since micro-infusion of D1 receptor antagonist in the area increased impulsive choice. Altered expression of D2 receptors, which are found to be increased in the PFC of IUGR animals at 160 days of life, is also linked with impulsive choice [26]. Together, these findings indicate that fetal adversity alters brain sensitivity to insulin and modulates DA-related behavioral responses to palatable foods [24, 25]. Additionally, in rodents exposed to IUGR, there is systemic hyperglycemia/hyperinsulinemia [27] and alterations in the expression of genes or proteins associated with DA signaling in the PFC, including TH, pTH, D1 and D2 [9, 21, 25, 28]. These metabolic and neurochemical effects are accompanied by changes in cognitive flexibility, sensitivity to reward, and poor inhibitory control [9, 21, 24, 28, 29]. The behavioral and metabolic phenotype observed in animals exposed to FR recapitulates the profile detected in humans born after exposure to prenatal adversity [30-50] with striking accuracy. In humans, differences in DA function associate with the increased palatable food consumption in IUGR individuals [51]. Furthermore, Silveira et al. discovered, in two independent cohorts, that catch up growth, an insulin-dependent process, influences decision making and inhibitory control specifically in IUGR children tested in tasks that did not involve the choice for food rewards [14]. Given the involvement of the mesocorticolimbic DA pathway in impulsive behaviors, we are interested in investigating the molecules involved in the maturation of

this pathway and characterize the effect of early life adversity in this developmental process by using the FR animal model.

The guidance cue Netrin-1 participates in the developmental organization of neural networks by either attracting or repelling extending axons and dendrites [2, 10, 52-56] and is highly expressed in terminal fields of mesocorticolimbic dopamine neurons including the PFC [57]. Responses to Netrin-1 can be modulated by regulating the availability of DCC (deleted in colorectal cancer) and UNC5 (uncoordinated) receptors [3, 58-61]. DCC receptors in DA axons promote target recognition events in the NAcc, preventing them from continuing to grow to the PFC, [62-66] and thereby organizing PFC local circuits [57, 67, 68] and inhibitory control in adulthood [67, 68]. DCC receptors are also expressed by PFC local neurons across the lifespan and are involved in susceptibility/resilience to chronic social defeat stress [69]. Changes in DCC expression in DA and cortical neurons induced by exposure to drugs of abuse or to stress, respectively, have been shown to be mediated by the microRNA-218 (miR-218) [69, 70]. miR-218 can be detected in blood with circulating levels matching its expression in brain [71, 72]. Both chronic caloric restriction and chronic high fat diet exposure in adulthood increases the expression of miR-218 in the hypothalamus [73]**.** However, the possible persistent effect of dietary manipulations during fetal development, as well as the effect of differences in insulin sensitivity on the expression of miR-218 and its guidance cue receptor targets in the PFC remain unknown.

Based on these findings, we aimed to investigate if exposure to prenatal adversity alters guidance cue systems known to be intimately involved in dopamine, PFC, cognitive development, and in vulnerability to chronic stress. Using the FR model of IUGR in male

rats, we measured changes in the Netrin-1/DCC guidance cue pathway in the PFC at birth and in adulthood in response to early life adversity and whether insulin sensitivity plated a role in the pathway. Additionally, we assessed if insulin affects microRNA levels, which are shown to impact the Netrin-1/DCC guidance cue pathway, suggesting their potential role in the mechanism involved in DA-related behaviors.

Methods

Primiparous Sprague Dawley rats of approximately 70-80 days were maintained in a controlled environment: standard dark/light cycle of 12 hours each, ambient temperature of the colony housing and testing rooms was 22 ± 1 °C, relative humidity level was approximately 20-50%, cage cleaning once a week, and food and water provided *ad libitum*. Estrous cycle was determined daily by vaginal smearing and females were time-mated. Gestation was confirmed at day 1 by visualizing the presence of sperm cells on the vaginal smear. On gestational day 10, dams were randomly allocated into one of the following dietary groups: control group (AdLib), which received an *ad libitum* diet of standard laboratory chow, or a 50% food restricted group (FR), based on the IUGR model described by Desai et al. [74], which received 50% of the *ad libitum*-fed dams' intake (determined by daily quantification of normal intake in a separate cohort of pregnant Sprague Dawley rats). These diets were provided from day 10 of pregnancy until the pups were born.

Within 24 hours of birth, pups from each litter were individually weighed and standardized to a maximum of 8 pups per litter, with 4 females and 4 males. Cross-fostering was performed, and all litters were adopted by AdLib dams. Only male offspring were used in this study and only one animal per litter was used in each experiment.

Tissues were collected in two separate age groups. The first group's tissue was collected on the day the animals were born. The second group's pups were weaned on postnatal day (PND) 21, separated into groups of two same-sex (same litter/group) individuals per cage, and kept in a controlled environment as previously described. Except for the cage cleaning once a week, animals were left undisturbed from PND 21 until tissue collection which occurred at approximately PND 100.

Tissue Collection

Tissue collection for protein level analysis took place at two different ages: PND 0 and PND 100. For the PND 0 group, the animals were decapitated, the brains were quickly removed and a series of 0.5 mm brain punches from the PFC were dissected from brain coronal sections corresponding to plates 7-9 of the Brain Rat Atlas [75]. Tissue was stored at -80°C until further analysis. For the PND 100 group, the animals received either a peripheral saline injection or insulin injection. Fifteen minutes after the injection, the animals were decapitated, the brains were quickly removed, the PFC was dissected through 1.0 mm thick coronal slices with the aid of an atlas [76], and the tissue was stored at -80°C until further analysis.

Western Blot

Total protein fractions were isolated using the mirVana™ PARIS RNA and Native Protein Purification Kit Protocol (Cat#AM1556, Thermo Scientific, Toronto, ON, Canada). Briefly, protein samples (20 µg) were separated on a 10% SDS-PAGE and transferred to a PVDF membrane that was incubated overnight at 4°C with antibodies against DCC (1:1000, Cat#554223, BD Pharmingen), Netrin-1 (1:1000, cat# ab126729, Abcam), and alpha-tubulin (1:2000, Cat #2144S, Cell Signaling). We obtained the optical

density for each Netrin-1, DCC, and tubulin band, and the fold change was calculated by normalizing to tubulin's optical density.

Tissue RNA extraction and quantitative real time PCR

Total RNA and microRNA fraction were isolated from the frozen tissue using the Norgen Biotek Corp RNA/DNA/Protein Purification Plus Kit (Cat#47700, Norgen Biotek Corp, Thorold, ON, Canada) as per manufacture's instruction. All RNA samples were determined to have 260/280 and 260/230 values ≥ 1.8, using the Nanodrop 1000 system (Thermo Scientific, Toronto, ON, Canada). Reverse transcription for *Dcc*, *Netrin-1* (*Ntn-1)* and *Glyceraldehyde-3-phosphatedehydrogenase* (GAPDH) mRNA were performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Real time PCR, using TaqMan assay (Applied Biosystems, Foster City, CA) was carried out with an Applied Biosystems 7900HT RT PCR system. Data for *Dcc* and *Ntn-1* mRNA expression were analyzed by using the Relative Quantification standard curve method and *Gapdh* was used as reference gene. In all cases, the real-time PCR was run in technical triplicates.

HEK293 In-Vitro Analysis

HEK293 cells were cultured in 12-well plates with DMEM medium with 10% fetal bovine serum (FBS). Once the cells reached 80% of confluency, they were starved in serum-free medium for 16-18 hours. After that period two different treatments were applied: (1) exposure to different concentrations of insulin (50, 100 or 200 nM) or basal medium to measure total micro-RNA levels after 6 hours and (2) exposure to 100 mM of insulin or basal medium to measure total micro-RNA levels at different time points (10, 30, 60 or 360 minutes). In both cases, a control, undisturbed cell culture was kept (UNT).

Cell cultures were harvested by trypsinization (0.125 trypsin/1% EDTA in PBS) and homogenized using the miRNeasy Mini Kit (Qiagen, Toronto, ON, Canada). Total RNA and microRNA fraction were isolated according to the manufacturer's instructions. All RNA samples were determined to have 260/280 and 260/230 values ≥ 1.8, using the Nanodrop 1000 system (Thermo Fisher Scientific, Toronto, ON, Canada). Reverse transcription for miR-218 was performed using the TaqMan MicroRNA Reverse Transcription Kit and TaqMan probes (Applied Biosystems, QC, Canada). Real-time PCR was run in technical triplicates with an Applied Biosystems QuantStudio RT PCR system (Applied Biosystems, QC, Canada). The small nucleolar RNA (snoRNA) RNU6B was used as an endogenous control of miRNA measures. Expression levels of miR-218 were calculated using the Relative Quantitation standard curve method and normalized by RNU6B. The expression of miR-218 was relativized to UNT group and presented as a fold change.

Statistical Analysis

Student's *t*-test was used for the Western Blot analysis for PND 0 animal results. All variables were expressed as mean ± standard error of the mean (SEM). One-way ANOVA was used to analyze the Western Blot analysis for PND 100 animal results and the in-vitro results from HEK293 cultures followed by Tukey post hoc test when appropriate. Results were considered significant when $p \leq 0.05$. Data were analyzed using the RStudio software [77].

Results

PND 0 Measures

Protein levels of Netrin-1 and DCC receptors in the PFC were measured in AdLib and FR animals of age PND 0 (AdLib weight = 7.55 ± 0.21, FR weight = 6.84 ± 0.11, Student's t-test, n = 8/group, t(14) = 3.039; p = 0.009) as shown in *Figure 25*. Netrin-1 protein levels were found to be downregulated in the PFC of FR animals when compared to AdLib animals (W(8) = 51; p = 0.0499). DCC protein expression was not significantly different in the PFC of FR animals when compared to AdLib animals (t(14) = -1.25; p = 0.231).

To confirm our findings, we replicated these results by using additional tissue from new AdLib animals of age PND 0 (AdLib weight = 7.46 ± 0.195, FR weight = 6.84 ± 0.11, Student's t-test, n = 8/group, t(14) = 2.786; p = 0.015). Netrin-1 protein levels were found to be downregulated using one-sided test in the PFC of FR animals when compared to AdLib animals (t(14) = 1.714; p = 0.0543). DCC protein levels were not significantly different in the PFC of FR animals when compared to AdLib animals (t(14) = 1.478; p = 0.162).

PND 100 Measures

Levels of *Ntn-1* and *Dcc* mRNA in the PFC were measured 15 minutes after an intraperitoneal saline or insulin injection after a 4 hour fast (1 IU/kg) in AdLib and FR animals of age PND 100 as shown in *Figure 26*. We measured these transcripts 15 minutes after the injection because previous studies in our lab showed changes in insulin signaling pathway within this time frame and IUGR model [25]. There were no significant differences in *Ntn-1* mRNA levels between the four groups: AdLib Saline, AdLib Insulin, FR Saline, and FR Insulin (F(3,33) = 0.066, p = 0.977). There was a significant difference in *Dcc* levels across the four groups (F(3,33) = 4.407, p = 0.0103). A Tukey post hoc test

revealed that there is a significant *upregulation* of *Dcc* mRNA in AdLib animals which received the insulin injection when comparing to AdLib animals which were administered a saline injection ($p = 0.008$). We also found a significant *downregulation* of *Dcc* mRNA levels in FR animals which received a saline injection when compared to the AdLib animals treated with insulin ($p = 0.032$).

miR-218 Measures in HEK293 Cultures

miR-218 expression was measured in response to four different insulin concentrations, as shown in *Figure 27*, in addition to the control group (UNT): 0 nM, 50 nM, 100 nM, 200 nM. There was a significant difference in the fold change of miR-218 between the five groups ($F(4,10) = 4.394$, $p = 0.0262$). A Tukey post hoc test revealed that there is a significant *upregulation* in miR-218 in response to 100 nM of insulin ($p = 0.018$) when comparing to the control group.

miR-218 expression was measured over a time curve when exposed to 100 nM of insulin, as shown in *Figure 28*, at five different time points in addition to the control group (UNT): 0 minutes, 10 minutes, 30 minutes, 60 minutes, and 360 minutes. There was a significant difference in miR-218 expression across the six groups ($F(5,12) = 27.26$, $p < 0.001$). A Tukey post hoc test revealed that there is a significant *upregulation* in miR-218 at 10 minutes ($p = 0.004$), 30 minutes ($p < 0.001$), 60 minutes ($p < 0.001$), and 360 minutes ($p < 0.001$) when comparing to the control group; *upregulation* at 10 minutes ($p = 0.022$), 30 minutes ($p < 0.001$), 60 minutes ($p = 0.001$), and 360 minutes ($p = 0.001$) when comparing to 0 minutes; and *upregulation* at 60 minutes ($p = 0.043$) when comparing to 10 minutes.

Discussion

The food restriction model that we used in this study was based on the successful IUGR model described by Desai et al. [27]. This model was established as a useful approach able to mimic many of the adverse outcomes frequently observed in IUGR in humans, including decreased plasma leptin levels, increased food intake, obesity, increased percentage of body fat, insulin resistance, and catch-up growth[78]. More recently, this model has been used to investigate alterations in feeding behavior that in some way influence food choices and consumption [27]. In this context, interesting changes were found in attentional skills, in physical activity, in reward sensitivity (indicated by a decreased conditioning for palatable food in a place preference test), in hedonic responses to sweet food, and in impulsive behavior. Therefore, this model was best suited for our study where we investigated whether environmental insults early in life, modeled through metabolic prenatal adversity, affect the Netrin-1/DCC guidance cue system which is known to be involved in DA and PFC maturation and function [65, 70, 79, 80]. We showed that Netrin-1 protein levels are downregulated in the PFC of FR animals in comparison to AdLib animals at age PND 0, indicating that metabolic prenatal adversity affects developmental guidance cue systems as early as birth. In the matured cortex, at PND 100, we do not see changes in *Ntn-1* mRNA transcript expression at baseline (AdLib Saline group and FR Saline group) suggesting that the changes observed at birth do not persist into adulthood. FR does not alter baseline DCC protein expression in the PFC at birth or in mRNA transcript levels in adulthood. However, at PND 100, AdLib animals showed *Dcc* mRNA upregulation in response to an insulin challenge when compared to AdLib animals who received saline, but this response is absent in FR animals. This lack of regulation of *Dcc* by insulin in FR rodents is in line with our previous work showing that

FR animals have different sensitivity to insulin [25] and suggest that exposure to fetal growth restriction prevents insulin-induced regulation of *Dcc* expression in the PFC in adulthood. It is important to note that protein levels were measured in PND 0 animals while mRNA levels were measured in PND 100 animals but the positive correlation between mRNA and protein expression levels [81] allows us to compare these transcripts between these ages.

The expression of the Netrin-1/DCC system in mesocorticolimbic DA systems, including the PFC, decreases dramatically from early postnatal life to adulthood [71, 82, 83]. This shift in expression levels coincides with the distinct role that Netrin-1 and DCC play across postnatal development – from axonal pathfinding and synaptic pruning/refinement in early postnatal life to synaptic plasticity in adulthood [79, 84]. It is likely that the early changes in Netrin-1 expression observed in FR animals impact the core organization of developing PFC neuronal networks, including DA axonal growth, while the altered insulin-response in DCC receptor expression in adulthood involve reorganization of matured local synaptic connectivity. In the PFC, Netrin-1 has been shown to be highly expressed by pyramidal and GABA interneurons, while DCC receptors are expressed by pyramidal neurons as well as non-DA axons [57, 83]. It remains to be determined whether changes in guidance cue expression induced by FR or by insulin administration in adulthood localize to specific PFC neuronal populations and if they are predominately occurring at the pre- or postsynaptic level.

miR-218 levels in the brain have been known to be affected by altered metabolism as shown by Sangiao-Alvarellos et. al. [73] where caloric restriction and high fat diet in adulthood resulted in an increase in miR-218 expression in the hypothalamus in

comparison to the control group. Since miR-218 regulates *Dcc* mRNA and DCC protein expression [70], it could be involved in the effect of metabolic prenatal adversity on this guidance cue system. To assess this hypothesis, we did in-vitro analysis involving miR-218 in HEK-292 cells. We showed that there is a time-dependent and dose-dependent increase in miR-218 expression in HEK-293 cells. Whether and how insulin-induced regulation of miR-218 may be involved in the changes in DCC expression observed in the PFC in adulthood remains to be established. Indeed, previous studies have shown that miR-218 can stimulate the transcription of certain transcripts [85] and cumulative evidence indicates that miRNAs can upregulate genes and protein expression in specific cell types and under particular conditions [86]. Since miR-218 is a regulator of *Dcc* gene expression and our in-vitro results show that insulin regulates miR-218 levels, we suggest that FR-induced changes in insulin sensitivity could be affecting *Dcc* expression via miR-218.

In conclusion, our work explores how insulin mediates the effects of prenatal adversity, modeled through FR, on the Netrin-1/DCC guidance cue system which is known to be involved in PFC maturation. Previous animal work from our lab has shown that animals exposed to prenatal adversity showed a pronounced aversion to delayed rewards in addition to an increase in PFC D2 levels [24]. Our current findings may partially explain the mechanism involved in this relationship. This guidance cue pathway is affected by metabolic prenatal adversity through changes in insulin, as shown by our results, which could ultimately reorganize pre- and postsynaptic dopamine networks and impact executive functions in adulthood. As the FR model parallels many of the alterations

observed in IUGR in humans, the current findings have the potential to inform future studies to explore these mechanisms in human populations.

Figures

Figure 25. PFC Netrin-1 and DCC expression in PND0 males. Levels of Netrin-1 and DCC receptors in the PFC were measured in AdLib and FR animals of age PND 0. *(a)* Netrin-1 protein levels were found to be downregulated in the PFC of FR animals when compared to AdLib animals (W(8) = 51; p = 0.0499). *(b)* DCC protein expression was not significantly different in the PFC of FR animals when compared to AdLib animals (t(14) = -1.25; p = 0.231).

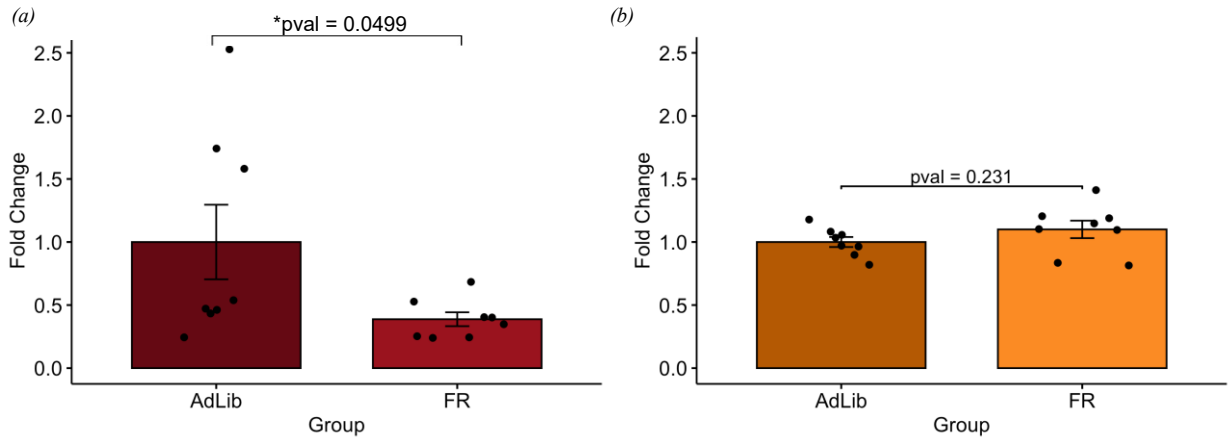


Figure 26. PFC *Netrin-1* and *Dcc* mRNA expression in PND 100 males. Levels of *Netrin-1* and *Dcc* in the PFC were measured 15 minutes after a peripheral saline or insulin injection in AdLib and FR animals. *(a)* There were no significant differences in *Netrin-1* mRNA levels across the four groups: AdLib Saline, AdLib Insulin, FR Saline, and FR Insulin ($F(3,33) = 0.066$, $p = 0.98$). *(b)* There was a significant difference in *Dcc* mRNA levels over groups ($F(3,33) = 4.41$, $p = 0.0103$). There is a significant upregulation of *Dcc* mRNA in AdLib animals treated with an insulin injection when comparing to AdLib animals administered a saline injection ($p = 0.008$) and a significant downregulation in *Dcc* mRNA levels in FR animals treated with the saline injection when compared to the AdLib animals given the insulin injection ($p = 0.032$).

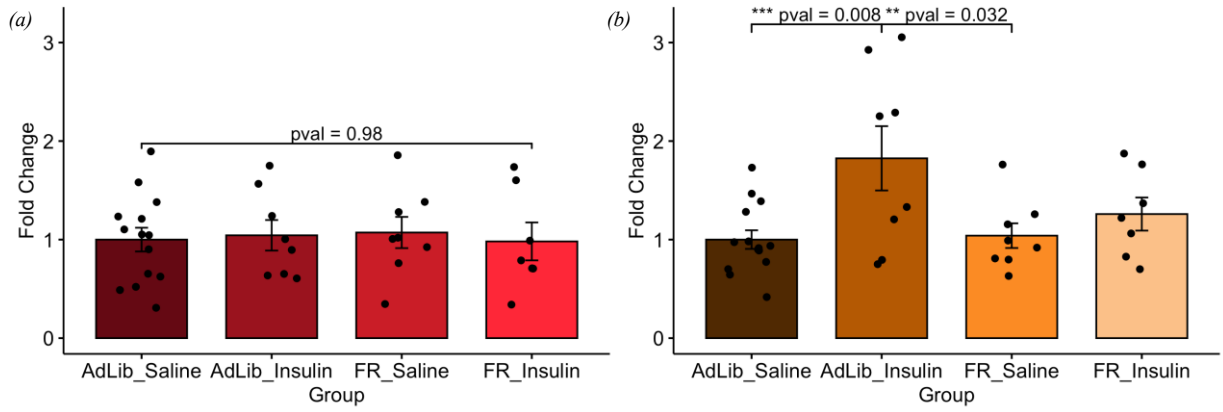


Figure 27. Insulin regulates miR-218 expression in a concentration-dependent manner. miR-218 expression was measured in response to four different insulin concentrations in addition to the control group (UNT): 0 nM, 50 nM, 100 nM, 200 nM. There was a significant difference in miR-218 expression across groups ($F(4,10) = 4.394$, $p = 0.0262$). miR-218 levels were upregulated at 100 nM of insulin when compared to the control group.

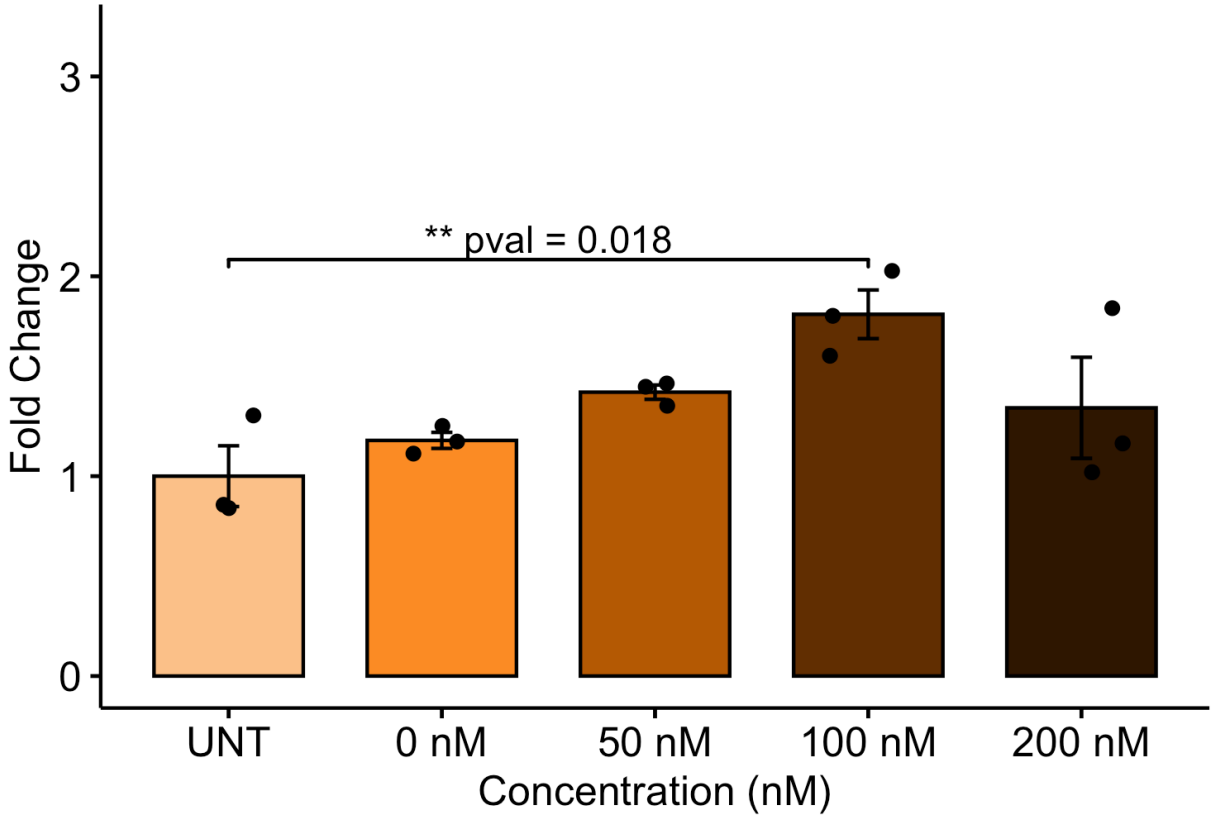
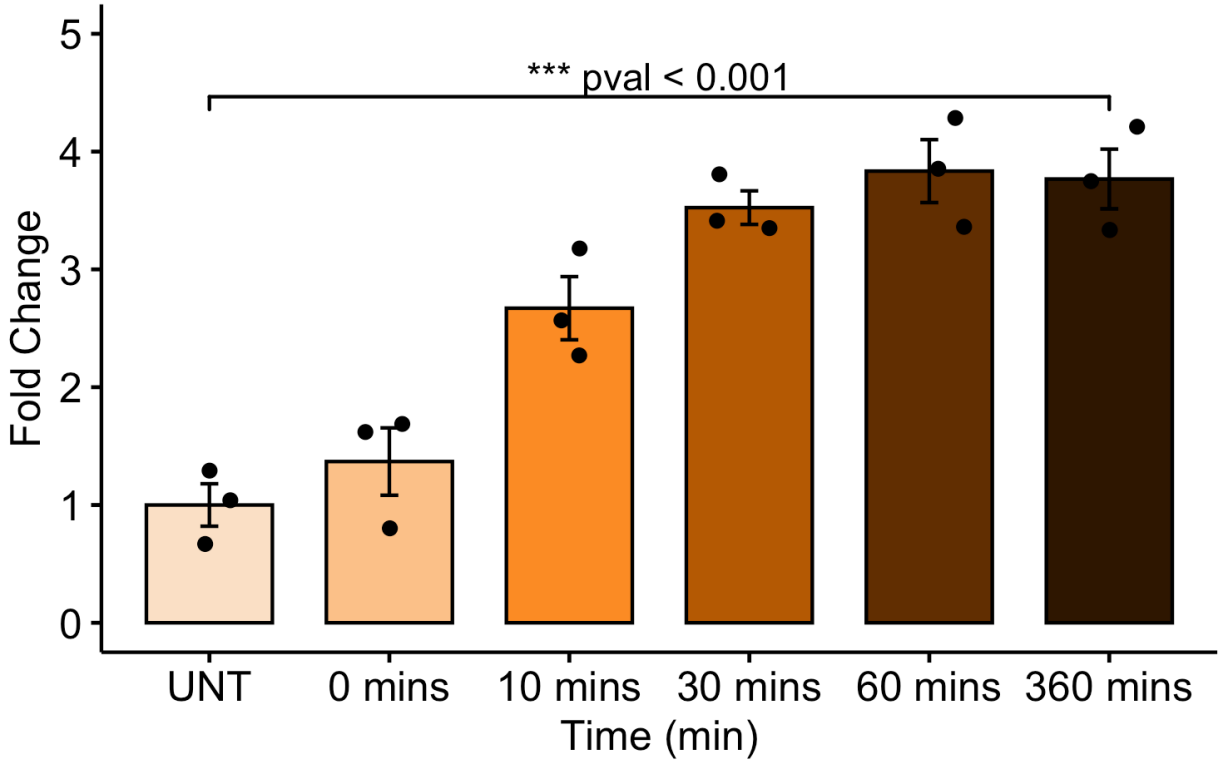


Figure 28. Effects of insulin signaling on miR-218 levels over time. Fold change in miR-218 were measured over a time curve at five different time points in addition to the control group (UNT): 0 minutes, 10 minutes, 30 minutes, 60 minutes, and 360 minutes. There was a significant difference in miR-218 expression across groups ($F(5,12) = 27.26$, $p < 0.001$). miR-218 levels were upregulated at 10 minutes, 30 minutes, 60 minutes, and 360 minutes in comparison to the control group.



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Chapter VII. Discussion

Through this thesis work, our objective was to investigate how variations in insulin function modulate the effects of early life adversity on executive functions. By using genome wide association studies (GWAS), we were able to investigate the role of fasting insulin-related single nucleotide polymorphisms (SNP) within the interaction effect of early life adversity and fasting insulin on executive functioning. GWASs have been used for about a decade to inspect the genetic marker associations with diseases or traits, making them a reliable and tested method [72]. It is especially helpful to identify the genetics behind a trait that is considered complex, meaning that it is influenced by many genes, which may interact in additive or non-additive ways [73]. Fasting insulin is one such trait, affected by several genetic markers working together to result in a higher risk for high fasting insulin levels, where the loci have been well-replicated implying true associations [74]. Most of the disease-associated markers are parts of the noncoding regions of the human genome, meaning that they effect the function through transcription, splicing, or mRNA stability. This is important to consider when studying the mechanisms behind the associations found through GWASs. Through regular GWAS associations, we found a list of SNPs that were associated with fasting insulin levels. But we took an alternative approach in our study, the refined polygenic risk score (rPRS), which allowed us to identify a subset of SNPs that were most associated with fasting insulin levels in children. This method was essential for our studies as we wanted to investigate the effects of early life adversity and fasting insulin in childhood. Since there are no fasting insulin GWASs in children, the rPRS was a technique to use the SNPs relevant in children. What is particularly interesting is that these SNPs are still valid in adults and by using the rPRS

method, we get a way of identifying SNPs that would be relevant at both age groups. Since most GWASs available are either in adults or use meta-analysis encompassing results from various ages, this new method can be used by anyone who would like to inspect the effects of genetic markers in a specific age group. It is especially relevant to do when it comes to metabolic hormones since they are involved in child growth and can vary depending on the age of an individual [75]. By using the rPRS approach, any study can be applied to the age group needed while using the stable genetic background instead of varying and oscillating levels of hormones.

An interesting aspect of this thesis work is that we were able to use sex-specific and non sex-specific FI GWASs. This gave as an opportunity to start the research work by looking at the effects of FI, interacting with ELA, in a broad perspective, on a specific executive function. While literature had established that insulin impacts executive functioning, it was important to keep the study broad, inspecting several executive functioning skills, while we inspect the interaction effect of fasting insulin and ELA to understand which ones are specifically impacted by the interaction instead of just insulin signaling. Once we had established that fasting insulin and adversity are working together in association with the impulsivity executive function outcome in childhood and addiction outcome, which consists of executive function behaviors, we could further investigate whether there was a sex-specific impact of fasting insulin. Even more so, we were able to investigate executive functions in a broad manner to identify all executive functions associated with the interaction effect of fasting insulin and early life adversity, as shown in the study described in chapter V.

While individual associations between insulin and executive functioning, adversity and executive functioning, and adversity and insulin had been established previously in the literature, we were specifically interested in investigating the interaction between insulin and adversity on executive functioning. In other words, we postulated that insulin would mediate the effects of adversity on executive function, likely in a sex-specific manner. Instead of doing moderation-mediation models, we decided to use a more novel and causality-related approach called Mendelian randomization.

It is widely accepted that diseases are not solely caused by genetics or the environment, but rather by an interaction between the two [76]. By investigating the interaction, we are securing a more accurate description of how the disease is occurring because it provides a correct estimate of the proportion of the disease that is explained by genes, the environment, and their joint effect. The success of studies looking into gene by environment interaction depends on the genetic data and environment data that is reliable and processed to be replicated in multiple studies. High-throughput genotyping technologies such as whole-genome SNP scans provide with quality information on the genetic background [77], which is why we used the FI GWASs in our studies. The environment component was assessed through self-reported information through interviews and questionnaire. We believe our method of calculation the early adversity score encompasses all aspects of an adverse environment to provide us with clear information on the impact it will have within the study. The calculation of the prenatal adversity score, used in the study described in chapter V, and the postnatal adversity score, used in the studies described in chapters III, IV, and V, has been established through previous work done in lab [78]. The cumulative score contained different

environmental variables and instruments, allowing us to analyze the presence of adversity in general and encompassing all types of adversity rather than focusing on one particular adversity. This method gave us the opportunity to inspect the extreme role of the environment, in terms of early adversity prenatally and postnatally, within the context of the study. By encompassing several aspects of an adverse environment, we are also able to ensure that our results apply to many environmental exposures, whether that be malnutrition, abuse, socioeconomic status, or maternal mental illnesses. We are, therefore, able to strongly suggest that insulin modulates the effect of different adverse environments, prenatal or postnatal, on executive functions related to impulsivity [1], attention (chapter V), and addiction (chapter IV).

One inherent problem that occurs in the gene by environment model is the use of multiple genes, multiple exposures, and multiple interactions, which could lead to type 1 errors, or false positives. While we use statistical approaches to control for false positives, as described in the individual chapters for each study, the reproducibility of this specific gene by environment interaction across multiple studies and multiple cohorts informs us of the reliability of the results we found. We investigated our hypothesis in five separate cohorts: MAVAN (ages 3 to 6 years), GUSTO (ages 2 to 7 years), ABCD (ages 10 and 11 years), SAGE (adults), and UKB (adults). In every cohort, we were able to establish that there is a significant interaction effect of early life adversity and fasting insulin on executive functioning. Moreover, we were able to also reproduce the effect in different age groups using the multiple cohorts, exhibiting that this interaction effect has long lasting impact on the psychological health of a being. And while executive functioning implicates the prefrontal cortex's functioning, the gene by environment interaction starts

impacting the behaviors while the prefrontal cortex is developing. While this is informative for identification of disease, it can also be used to create interventions early on in childhood and in adolescence to help individuals pre-disposed to altered executive function behaviors.

This thesis focused on executive functions because these are essential in long-term cognitive and social developmental outcomes. Executive functions refer to a set of neurocognitive skills that are concerned with goal-directed problem solving [79], encompassing working memory, inhibitory control, and set shifting/flexibility. While executive functions heavily depend on the neural networks in the PFC which continue to develop into early adulthood, there are major advances in EF which occur during preschool life [79]. Therefore, it was important for us to examine whether adversity and fasting insulin are working together to influence changes in EF as early as 2 years. With our results, we see that the interaction between childhood adversity and fasting insulin impacts EF early in childhood and has long term impact, as shown through our second and third studies where we inspect addiction in adulthood and EF throughout development. Because inhibitory control is a focus of executive functions, we started the first study with the specific hypothesis of testing impulsive behavior as function of the interaction between fasting insulin and childhood adversity. Once we were able to show that there is a significant association with impulsivity, we wanted to further inspect EF. Because impulsivity can have various definitions and be measured in a variety of ways, some researchers consider it to be a separate trait from EF rather than a part of it. One thing that EF and impulsivity have in common is the role they play in addiction [80]. Impulsivity observed in addicted individuals could be a result of dysfunction of several EF

[80] making addiction a logical outcome to inspect in our second study. When we were able to show that the genetic markers of fasting insulin which associated with childhood impulsivity in the context of adversity were also the same genetic markers of fasting insulin which associated with addiction in adulthood in the presence of adversity, it strongly suggests that fasting insulin moderates the impact of early life adversity in the development of EF, and these relationships have a long-term impact on the risk for developing addiction-related psychopathology. But to confirm that this effect was not isolated to impulsivity or addiction, we also did a PheWAS analysis investigating the interaction effect of childhood adversity exposure and the genetic background linked to fasting insulin on multiple EF, in males and females. One outcome of interest that showed up in association with the interaction effect in every age group, in addition to impulsivity, was attention or ADHD. This is interesting because impulsivity and attention share traits to the point that there is also a trait called attention deficit impulsivity, which is defined as the diminished ability to persist in engagement of relevant rather than irrelevant stimuli [80]. Even more interesting is that addicted individuals often have attention deficit impulsivity and therefore, it seems like EF encompasses these individual traits and we have shown that each one is individually affected by the interaction between early life adversity and high fasting insulin throughout development. Learning of factors that result in altered EF is especially relevant in medicine as early interventions can help improve EF skills. It has been shown that diverse activities such as computerized training, noncomputerized games, aerobatics, martial arts, yoga, mindfulness, and school curricula can aid in improving EF development in children as early as 4 years old and in pre-teens [81].

While GWASs provide associations between a genetic marker and a trait, it is not possible to determine whether a causal effect exists between the identified genetic marker and the associated trait from the GWAS. Mendelian randomization (MR) analysis brings us one step closer to identifying whether a causal relationship exists between an exposure and the trait of interest. However, there are limitations to MR that should be discussed. One of the limitations is that the GWAS used within the MR analysis are often a case-control design, meaning that there was a selection of cases and controls specific to the design of the GWAS conducted, introducing biases and inclusion/exclusion of criteria from several population, leading to population stratification [82]. This can be a problem because the differences seen in allele frequencies between the cases and controls could be because of systematic differences in ancestry rather than association of genes with disease [83]. Therefore, it is important to test within the same ancestry groups when it comes to two sample MR. While the majority of the individuals in the FI GWAS and the impulsivity GWAS used in our study consist of European ancestry, it is important to remember that: 1) the results would need to be further validated in a separate European ancestry cohort to confirm its reproducibility and 2) the results are only relevant for the specific ancestry the analysis was performed in. It is also important to note that because MR only uses GWASs, and in this case, both GWASs were constructed from data gathered in adults, that the results we found may also be only relevant in adults. GWASs would need to be created at different ages to test MR at different ages to assess whether the association holds throughout development.

When discussing MR, it is important to note that because MR studies are comparable to randomized clinical trials, they are often used to infer causality, but true

causality can only be proven with further biological testing. MR increases the likelihood of identifying the genetic background of traits that are acquired together, implying the function of both traits to result in a biological change. In our study, we established a significant MR association between fasting insulin and impulsivity in females exposed to adversity. This tells us that the risk for high fasting insulin levels is accompanied by the risk of impulsive behavior in females exposed to adversity. To assess whether insulin signaling causes alterations in impulsive behavior in females exposed to adversity, it would be important to conduct further studies in rodents and inspect the mechanism. But MR provides us with more confidence than the initial GWAS did of the association between FI and impulsivity.

Traditional MR analysis has a caveat that because of the complexity of LD structure in a genome, an association between a genetic variant and a disease identified in a GWAS could exist because of causality but also because of LD with another causal marker, or pleiotropy of the variant [84]. Two-sample MR tackles this problem by using summary level data from the GWAS and accounting for LD as part of the analysis. The pleiotropy is also tested as part of this analysis and by using the Egger test, we were able to successfully deny the presence of horizontal pleiotropy in females exposed to adversity. Therefore, in our case, with the assumptions satisfied, we can be confident in the results we found in females exposed to adversity.

We need to make note of the unique approach we took with two-sample MR method to inspect the interaction effect between fasting insulin and early life adversity. To our knowledge, so far, two-sample MR have only been able to examine main effects between a genetic exposure and a genetic outcome. Since we were interested in

investigating the causal association between the interaction of early life adversity with fasting insulin and impulsivity, we tested two-sample MR using our exposure GWAS of fasting insulin and we constructed two impulsivity GWASs: first a GWAS in which individuals exhibited impulsivity and were exposed to adversity and in the second GWAS, individual exhibited impulsivity and were not exposed to adversity. As mentioned earlier, gene by environment interactions provide a more detailed picture of the biological occurrences leading to the phenotype of interest. This approach, validated and peer-reviewed, opens the possibility of further investigating gene by environment interactions with the possibility of establishing causal associations.

To explore mechanisms, we decided to use rodents for the last study so we could inspect whether insulin affects the guidance cue molecules that are involved in the development of the dopamine (DA) pathway in a rodent model of prenatal adversity. While our study does not establish the causality exhibited by the associations we found in the human studies, it is the first step to bring us closer to understanding the underlying mechanism involved in this relationship. Through our study, we were able to show that the netrin-1/DCC guidance cue pathway is affected by prenatal adversity, modeled through FR, which has previously been linked to enhanced insulin sensitivity, at birth. We were also able to show that insulin modulates the guidance cues expression in adulthood in rodents exposed to prenatal adversity, implying the long-term impact of insulin and early adversity. Prenatal adversity programs biological changes in individuals impacting diseases in the long term. The changes seen in the netrin-1/DCC guidance cue pathway in response to prenatal adversity in adulthood display the lasting effects of programming in this animal model. Previous studies indicating that insulin sensitivity is affected in

adulthood because of prenatal adversity combined with the responses to insulin we showed within the guidance cue pathway indicate that insulin sensitivity is impacting the development of this pathway in throughout development. As described in earlier chapters, the netrin-1/DCC guidance cue pathway is involved in the development of the DA pathway in the PFC which is linked to EF. A different study from our lab on the corticolimbic-specific DCC gene co-expression network, in the PFC and nucleus accumbens, was associated with impulsivity in children [85] solidifying our hypothesis. Furthermore, other recent human studies showing that altered levels of DCC gene expression was related to numerous neuropsychiatric conditions of development characterized by deficits in PFC function and impulse control [59, 61]. Although the prenatal adversity modeled within this project is metabolic in nature causing caloric deficiency and weight loss in the pregnant dam and offspring brings to question whether other adversities would result in the same developmental abnormalities. While extreme adversity, of any kind, can trigger stress signals to the body to direct the available resources to the organs needed for survival, we showed that a metabolic adversity results in alterations due to the lack of nutrients. It will be interesting to investigate whether other adversities result in the same changes in insulin signaling and sensitivity as the metabolic one tested in our rodent study. Therefore, our results in the last study, are on track to understanding the mechanism involved linking early life adversity, insulin signaling, and executive functions such as impulsivity. Future studies will need to done to establish this mechanism: 1) measure insulin's action on its receptor in the DA neurons in the mPFC to determine insulin sensitivity in response to prenatal adversity throughout development; 2) measure miR-218 levels in the PFC throughout development; 3) track DA neuron development in response to prenatal

adversity throughout development; 4) assess executive functioning skills, such as impulsivity and attention, in response to prenatal adversity throughout development. These studies will need to be performed in both males and females to inspect the sex differences involved in the development of the DA pathway.

There are certain limitations within this thesis. The human studies done within this project highlight results in European ancestry and it needs to be confirmed whether the results would remain in different ancestries. The FI GWAS allows us to determine the risk for having high fasting insulin levels but that does not necessarily equate to insulin action and even more specifically, insulin action in a specific brain area. An expression based PRS in the PFC can be a way to address this limitation to determine a more specific association between insulin and adversity interaction. But the ePRS will only be establishing an association, therefore, future studies will need to be done to establish causation. While the relationship between adversity and stress on glucocorticoids has been well established, as discussed in chapter 2 of the thesis, it would be interesting to further explore the direct impacts of glucocorticoids on insulin levels and insulin signaling/sensitivity. It is also important to note that all rodent work reported in this thesis was performed in males while our human studies reported results in both males and females. Our sex-specific studies in human highlighted that certain EF are associated by the interaction between adversity and fasting insulin at specific ages. For example, in early childhood, attention and impulsivity in addition to other EF were highlighted in both males and females. Meanwhile in adolescence, only females exhibited attention in association with the interaction effect. And lastly, in adulthood, addiction and depression, which are often mapped to EF behaviors, were highlighted in both males and females.

The studies reported in this thesis were done in newborn rodents and in adults in males, which align with our human studies. But future studies where both males and females should be analyzed in rodents should also inspect whether the same sex-specific interactions will exist in adolescence. Additional future studies to bring this work forward would require investigating whether rodents exposed to prenatal adversity exhibit impulsive behavior throughout development and how that behavior changes with insulin signaling in the PFC. To establish the role of miR-218 within the pathway, the micro-RNA will need to be measured at varying ages in the PFC of rodents. It would also be important to test different interventions in young children and in adolescents exposed to early adversity to investigate the effectiveness of improving EF through interventions.

Chapter VIII. Conclusion

This thesis encompasses studies done to inspect the link between early adversity, brain insulin signaling, and DA related executive functions. Through this work, we wanted to investigate how variations in insulin function modulate the effects of early life adversity on executive functions. In our first study, we discussed how the genetic background linked to fasting insulin interacts with postnatal adversity to predict the DA-related behavior impulsivity. In our second study, we were able to show that the same genetic background that is linked to impulsive behavior in childhood is also associated with adulthood addiction behavior. Following that, we investigated how the genetic background linked to fasting insulin interacts with early life adversity to predict executive function psychopathology, throughout development in males and females separately and we were able to establish a causal association between fasting insulin and impulsive behavior in females exposed to adversity. And lastly, in the last study, we concluded that insulin action on miR-218, which is a negative DCC regulator, could be mediating the metabolic effects of adversity on DCC expression in the mPFC, which could potentially explain the resulting altered executive functioning behaviors. The findings reported here have implications for identification of executive function psychopathology at different ages. Furthermore, our last study brings us one step closer to understanding the mechanism through which insulin sensitivity could be modulating the effects of early life adversity on DA-related executive function behaviors. These findings pave the path forward to the treatment of executive function psychopathology.

Chapter IX. Reference for Non-Manuscript Chapters (Chapters I, VII, and VIII)

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