THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

The Role of Cytokines and Inflammatory Markers in Depression in Adolescents

Natalie Therese Mills

MBBS

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

School of Medicine

Abstract

Aims: The overarching hypothesis is that circulating levels of pro-inflammatory cytokines and other inflammatory markers are genetically associated with depression in adolescents. Specifically, it is hypothesised that there will be a correlation between variation in genetic risk of depression and genetic variation of circulating pro-inflammatory cytokines and inflammatory markers.

Methods: A systematic review of the literature was conducted to establish the current understanding of the relationship between inflammatory markers and depression in adolescents and to inform study design. Multiple approaches (including different types of genetic analyses and multiple data sets) were used to address the overarching hypothesis. A pilot study measuring cytokines, inflammatory markers, and other biomarkers involved in immune regulation (including Vitamin D, antibodies to infectious agents, and gliadin antibodies (found in coeliac disease)) was conducted in 107 monozygotic (MZ) and 160 dizygotic (DZ) twin pairs (mean age 16.2 years, standard deviation (SD) 0.25 years) from the Brisbane Adolescent Twin Study. A clinical study was undertaken to collect biological samples and clinical data from an in-patient adolescent mental health unit. Investigation of the relationship between iron measures (altered in inflammatory states) and measures of depression was undertaken in community cohorts of twins and their parents (3,416 adolescents from 1,688 families, and 9,035 adults from 4,533 families). In the adolescent cohort, depressive measures were assessed through the Somatic and Psychological Health Report (SPHERE) (mean age 15.1 years (SD 3.2 years)). In the adult cohort, a quantitative score of depression was measured by the Delusions Symptoms State Inventory (DSSI) (mean age 23.2 years (SD 2.2 years)). Heritabilities of, and phenotypic and genetic correlations between, traits were estimated. Association analyses, genetic profile risk score analyses, and LD score analyses were also used to investigate the genetic relationship between the iron and depression measures. The phenotypic and genetic relationship between the inflammatory marker C-reactive protein (CRP) and depression and anxiety was investigated in a community sample from the Australian Twin Registry. Mean age at CRP measurement was 45.3 years (SD 10.1 years), with 14,750 individuals with CRP measures; 8,234 individuals with DSM-IV MDD data; 8,679 individuals with DSM-IV anxiety disorders data; 8,847 individuals with genome-wide SNP data. Analyses were stratified based on experience of childhood trauma.

Genetic profile risk score analyses were used to explore the genetic relationship between these variables.

Results: In the pilot study, cytokines that were successfully measured in plasma were found to be moderately heritable (transforming growth factor- β1 (TGFβ1), 0.57 (95% CI 0.26 – 0.80) and tumour necrosis factor-receptor type 1 (TNFR1), 0.50 (95% CI 0.11-0.63)). A negative correlation between Vitamin D and the cytokine IL-18 (-0.14) was not statistically significant (p=0.054). However, major difficulties were encountered in measuring cytokines, in particular due to the low levels of circulating cytokines in healthy adolescents. Challenges were also encountered in the cytokines study of the clinical sample of adolescents, which were able to be broadly divided into patient factors, blood collection factors, and other data collection factors.

Iron measures were found to be highly heritable in both adolescents and adults: Adolescents: iron 0.46 (95% CI 0.15-0.66), transferrin 0.64 (95% CI 0.42-0.81), transferrin saturation 0.61 (95% CI 0.39-0.70), and log10 ferritin 0.56 (95% CI 0.28-0.72); Adults: iron 0.35 (95% CI 0.25-0.41), transferrin 0.52 (95% CI 0.38-0.56), transferrin saturation 0.50 (95% CI 0.44-0.55), and log10 ferritin 0.42 (95% CI 0.27-0.49). Heritabilities calculated for depressive measures were 0.46 (95% CI 0.29-0.52) and 0.30 (95% CI 0.11-0.40) for adolescents and adults respectively. In adolescents, depression measures were significantly higher in those in the middle 10th percentile versus top 10th percentile of transferrin saturation measures (p=0.002). No evidence was found for a genetic contribution to the relationship between measures of iron and depression in adolescents or adults.

The relationship between CRP and Major Depressive Disorder (MDD) appears to be moderated by age, sex, and body mass index (BMI). No evidence was found that this relationship reflected genetic differences between individuals in CRP. Rather, BMI is likely to be the key factor mediating the relationship between circulating CRP, MDD and anxiety disorders.

Conclusion:

- Cytokines measured in plasma were found to be moderately heritable implying that there is a genetic contribution to variation between individuals in circulating levels of these inflammatory markers.

- Collection of blood inflammatory markers in an in-patient adolescent mental health unit is challenging.

- We found no evidence for a genetic relationship between blood inflammatory markers and measures of depression in community samples.

- Recommendations for future research are:

  o Longitudinal study designs are likely to improve the understanding of the role of cytokines / inflammatory markers in adolescent mental illness in relation to genetics, stress, early childhood adversity, and neurodevelopment.

  o A less invasive way is needed to accurately measure cytokines in low concentrations

  o Additional treatment options of adolescent mental illness are needed, particularly for those individuals who have associated raised pro-inflammatory markers.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Publications during candidature

Peer-reviewed papers:

Mills NT, Scott JG, Wray NR, Cohen-Woods S, Baune BT. Research Review: The role of cytokines in depression in adolescents: a systematic review. The Journal of Child Psychology and Psychiatry. 2013; 54(8): 816-835

Mills NT, Wright MJ, Henders AK, Eyles DW, Baune BT, McGrath JJ, Byrne EM, Hansell NK, Birosova E, Scott JG, Martin NG, Montgomery GW, Wray NR, Vinkhuyzen AAE. Heritability of Transforming Growth Factor-β1 and Tumor Necrosis Factor-Receptor Type 1 Expression and Vitamin D Levels in Healthy Adolescent Twins. Twin Research and Human Genetics. 2015; 18(1): 28-35

Schmaal L, Veltman DJ, van Erp TGM, Samann PG, Frodl T, Jahanshad N, Loehrer E, Tiemeier H, Hofman A, Niessen WJ, Vernooij MW, Ikram MA, Wittfeld K, Grabe HJ, Block A, Hegenscheid K, Volzke H, Hoehn D, Czisch M, Lagopoulos J, Hatton SN, Hickie IB, Goya-Maldonado R, Kramer B, Gruber O, Couvy-Duchesne B, Renteria ME, Strike LT, Mills NT, de Zubicaray GI, McMahon KL, Medland SE, Martin NG, Gillespie NA, Wright MJ, Hall GB, MacQueen GM, Frey EM, Carballedo A, van Velzen LS, van Tol MJ, van der Wee NJ, Veer IM, Walter H, Schnell K, Schramm E, Normann C, Schoepf D, Konrad C, Zurowski B, Nickson T, McIntosh AM, Papmeyer M, Whalley HC, Sussmann JE, Godlewska BR, Cowen PJ, Fischer FH, Rose M, Penninx BWJH, Thompson PM and Hibar DP for the ENIGMA–Major Depressive Disorder Working Group. Subcortical brain alterations in major depressive disorder: findings from the ENIGMA Major Depressive Disorder working group. Molecular Psychiatry. 2015; doi:10.1038/mp.2015.69

Publications included in this thesis

Mills NT, Scott JG, Wray NR, Cohen-Woods S, Baune BT. Research Review: The role of cytokines in depression in adolescents: a systematic review. The Journal of Child Psychology and Psychiatry. 2013; 54(8): 816-835 – incorporated as Chapter 2.

Contributor	Statement of contribution

Natalie Mills (Candidate)	Conducted literature search (100%) Wrote and edited the paper (75%)
James Scott	Wrote and edited the paper (5%)
Naomi Wray	Wrote and edited the paper (5%)
Sarah Cohen-Woods	Wrote and edited the paper (5%)
Bernhard Baune	Wrote and edited the paper (10%)

Mills NT, Wright MJ, Henders AK, Eyles DW, Baune BT, McGrath JJ, Byrne EM, Hansell NK, Birosova E, Scott JG, Martin NG, Montgomery GW, Wray NR, Vinkhuyzen AAE. Heritability of Transforming Growth Factor-$\beta 1$ and Tumor Necrosis Factor-Receptor Type 1 Expression and Vitamin D Levels in Healthy Adolescent Twins. Twin Research and Human Genetics. 2015; 18(1): 28-35 – incorporated as Chapter 3 (Part 1).

Contributor	Statement of contribution
Natalie Mills (Candidate)	Data analysis (65%) Wrote and edited the paper (75%)
Margie Wright	Collected twin sample (70%) Edited paper (2%)
Anjali Henders	Generation of laboratory measures (20%) Edited paper (1%)
Darryl Eyles	Generation of laboratory measures (15%) Edited paper (1%)
Bernhard Baune	Generation of laboratory measures (25%) Edited paper (1%)
John McGrath	Generation of laboratory measures (10%)

	Edited paper (1%)
Enda Byrne	Data analysis (5%)
Narelle Hansell	Data analysis (5%)
	Edited paper (1%)
Eva Birosova	Generation of laboratory measures (20%)
James Scott	Edited paper (1%)
Nicholas Martin	Collected twin sample (30%)
	Edited paper (1%)
Grant Montgomery	Generation of laboratory measures (10%)
	Edited paper (1%)
Naomi Wray	Data analysis (5%)
	Wrote and edited paper (5%)
Anna Vinkhuyzen	Data analysis (20%)
	Wrote and edited paper (10%)

Contributions by others to the thesis

In addition to the contributions detailed in the above section ("Publications included in this thesis"), Professor Naomi Wray has assisted in editing and critically revising Part 2 of Chapter 3 ("Evidence of genetic variance in putative biomarkers for depression in a cohort of healthy adolescent twins"). This has included teaching of data analysis, assisting with data analysis, and interpretation of results.

Anjali Henders had substantial input into the preparation of the application to the Human Research and Ethics Committees (HREC) for the study in a clinical sample of adolescents (Chapter 4: "Measuring cytokines in inpatients of an adolescent mental health unit: study designs and lessons learned"). Associate Professor James Scott and Professor Naomi Wray both supervised and reviewed the design of this study. Associate Professor James Scott assisted with the editing of Chapter 4.

Chapters 3, 5 and 6 use data collected by Associate Professor Margie Wright and Professor Nick Martin in twin studies conducted at QIMR Berghofer Medical Research Institute (study participants were from QTwin and / or the Australian Twin Registry).

Dr Beben Benyamin: Dr Benyamin assisted with editing and critically revising Chapter 5 ("Investigating the Relationship between Iron and Depression"). Dr Benyamin has also supervised data analysis (including preparation of data files and teaching the analysis required). This chapter is about to be submitted to a peer reviewed journal, with Dr Benyamin as senior author.

Dr Narelle Hansell: in addition to the contributions detailed in "Publications included in this thesis", Dr Hansell provided substantial teaching of the skills needed for data analysis in Chapter 5 (in particular the estimation of heritabilities of traits and genetic correlations between traits).

Robert Maier, PhD candidate: assisted with the LD score analyses in Chapter 5, and with plotting Figures 5-2 and 5-3 (Chapter 5).

Dr Enda Byrne: Dr Byrne has assisted with the editing of Chapter 6 ("Investigating the relationship between C-reactive protein, depression and anxiety"). Dr Byrne has also supervised the data analysis. This chapter is also about to be submitted to a peer reviewed journal, with Dr Byrne as senior author.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Acknowledgements

My sincere gratitude to all my supervisors, for their patience, teaching and support:

Professor Naomi Wray

Associate Professor James Scott

Professor Gerard Byrne

Professor Nick Martin

Associate Professor Margie Wright

I would also like to express sincere thanks to:

Dr Enda Byrne and Dr Beben Benyamin: for teaching of data analysis skills, including skills needed for genetic profile risk scoring.

Dr Anna Vinkhuyzen and Dr Narelle Hansell: for teaching of data analysis skills for twin and family studies.

Professor Bernhard Baune, Dr Sarah Cohen-Woods, Dr John Whitfield, Professor Elliot Nelson, Robert Maier, Baptiste Couvy-Duchesne, and Dr Lucia Colodro Conde: for their academic input and support.

Anjali Henders: for assistance with the preparation and submission of the HREC application.

Lorelle Nunn: for assistance with data collection in the clinical sample of adolescents.

I am also very grateful for the following financial support:

Australian Twin Registry (ATR): ATR Research Travel Grant – this provided assistance for attending the course "International Workshop on Statistical Genetics and Methodology of Twin and Family Studies (Introductory Course) (2012, Boulder, Colorado).

QIMR Berghofer Higher Degree Committee Travel Award: this provided assistance to present my research at the Australasian Society of Psychiatric Research Conference (2012, Perth, Australia).

Keywords

cytokines, inflammation, immune system, depression, genes, stress, anxiety, adolescents

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 110319, Psychiatry (incl. Psychotherapy), 50%

ANZSRC code: 060412, Quantitative Genetics (incl. Disease and Trait Mapping Genetics), 50%

Fields of Research (FoR) Classification

FoR code: 1103, Clinical Sciences, 50%

FoR code: 0604, Genetics, 50%

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AX = anxiety study

BDNF = brain-derived neurotrophic factor

BMI = body mass index

CD = conduct disorder

CD cells = Cluster of Differentiation cells

CHARGE = Cohorts for Heart and Aging Research in Genomic Epidemiology

CI = Confidence Interval

CMV = cytomegalovirus

CPSS = Child Post-traumatic stress disorder Symptom Scale

CRH = corticotrophin-releasing hormone

CRP = C-reactive protein

CSA = childhood sexual abuse

DBS = dried blood spots

ELISA = enzyme-linked immunosorbent assay

GAD = generalized anxiety disorder

GIANT = Genetic Investigation of Anthropometric Traits

GM-CSF = granulocyte macrophage-colony stimulating factor

GPRS = genetic profile risk scores

GWAS = genome-wide association study

HAA = hydroxyanthranilic acid

HHV = human herpes virus

HK = hydroxykynurenine

HPA = hypothalamic-pituitary-adrenal

HREC = Human Research Ethics Committee

HSV = herpes simplex virus

HT = hydroxytryptamine

IDO = indoleamine 2-3-dioxygenase

IFN-γ = interferon-γ

IL = interleukin

JEPQ = Junior Eysenck Personality Questionnaire

KYN = kynurenine

LPS = lipopolysaccharide

MCP-1 = monocyte chemo-attractant protein-1

MDD = major depressive disorder

Mip-1a = macrophage inflammatory protein-1a

M-MDD = major depressive disorder with melancholic features

MMP-9 = matrix metallopeptidase-9

NAG/IRPG = NAG and Inter Related Project Grant studies

NK cells = natural killer cells

NMDA = *N*-methyl-D-aspartate

NT = neurotrophin

OCD = obsessive compulsive disorder

PFC = prefrontal cortex

PGC = Psychiatric Genomics Consortium

PUFAs = polyunsaturated fatty acids

QUIN = quinolinic acid

RANTES = regulated and normal T-cell expressed and secreted

SPHERE = Somatic and Psychological Health Report

SSAGA = Semi-Structured Interview for the Assessment of the Genetics of Alcoholism

SSRI = selective serotonin re-uptake inhibitor

tCho = total choline

TGF-β = transforming growth factor-β

TNF-α = tumour necrosis factor-α

Toxo = toxoplasmosis

TREM-1 = triggering receptor expressed on myeloid cells-1

TRP = tryptophan

TRYCATS = tryptophan catabolites along the IDO pathway

wbc = white blood cells

CHAPTER 1:

Background

Cytokines are small proteins with immune modulating activity (1, 2). They can be pro-inflammatory or anti-inflammatory (3, 4), or have a combination of pro-inflammatory and anti-inflammatory effects (5). At the commencement of this thesis, there was emerging evidence that pro-inflammatory cytokines (specifically tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)) were associated with Major Depressive Disorder (MDD) in adults (6), however there were few studies in adolescents. The small number of studies in adolescents suggested there would be evidence of immune system dysregulation, however the role of cytokines in adolescent MDD could possibly be influenced by factors such as neurodevelopment and hormonal changes.

During the course of the thesis, further literature emerged on cytokines and other inflammatory markers, including the potential for these to act as biomarkers of illness in MDD. In addition to cytokines, and the inflammatory marker C-reactive protein (CRP) (7), other potential blood biomarkers of depression include biochemical measures altered in inflammatory states. These include iron measures (8, 9), antibodies to infectious agents (10), and antibodies present in coeliac disease (11).

Vitamin D has also been found to have a role in the immune system, through modulation of cytokines (12) and has also been identified as a potential biomarker in MDD (13). This role in the immune system, combined with changes in plasma levels observed in MDD, suggested Vitamin D may be a useful biomarker of illness in MDD, particularly in adolescents. This thesis has aimed to examine the role of these biomarkers involved in immune regulation (or altered in inflammatory states) in adolescent MDD.

Biomarkers can potentially be used as markers for illness or diagnosis, or as markers of response to treatment (14). Throughout this thesis, biomarkers are examined in the context of biological markers of illness. However, changes in inflammatory markers can be seen in other mental illnesses besides MDD, including in anxiety disorders and psychosis (15, 16). This non-specificity produces challenges, especially as non-specific symptoms (such as irritability) can also be seen across mental illnesses (17).

Figure 1-1 provides an overview of the biomarkers examined in this thesis. Inflammatory markers can include pro-inflammatory cytokines, CRP, and biochemical measures altered in inflammatory states.

Figure 1-1: Overview of biomarkers involved in immune regulation, or altered in inflammatory states
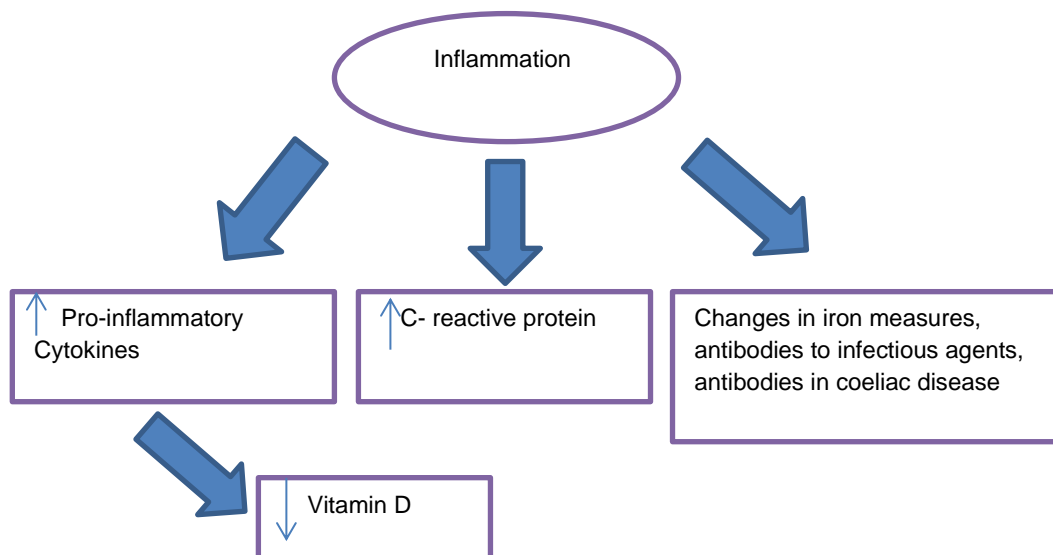
When commencing this thesis, there was very little literature on how identification of biomarkers in MDD might translate into clinical practice (18, 19). Recently studies have emerged examining particular sub-types of MDD and associated biomarkers (18), although this line of research is in its infancy. This is addressed further in Chapter 6, which includes an investigation of the association of CRP and MDD in those with co-morbid anxiety disorders.

Overall thesis aims

This thesis focuses on cytokines and other inflammatory markers in depression in adolescents. The starting hypothesis of the thesis was that pro-inflammatory cytokines, other inflammatory markers, and biomarkers altered in inflammatory states would be phenotypically and genetically associated with measures of depression. To examine this, some exploration of cytokines and inflammatory markers in depression in adults was required as few studies had previously been conducted in adolescents. This provided guidance for study design, and also allowed comparison of cytokines and inflammatory markers in adolescent versus adult depression.

The aims of this thesis are:

1) To review systematically the literature so as to identify differences in the role of cytokines in depression in adolescents compared to adults. This review would also assist in informing study design.

2) To investigate the genetic variance of cytokines, inflammatory markers, and biomarkers altered in inflammatory states in healthy adolescents.

3) To investigate the phenotypic and genetic relationship between other biomarkers altered in inflammatory states (specifically iron measures) and depression, using several different strategies.

4) To investigate the phenotypic and genetic relationship between the inflammatory marker CRP, MDD, and anxiety disorders.

Chapter overview

Chapter 2 is a systematic review critically analysing all studies available at the time examining the role of cytokines in adolescent major depressive disorder. This review identified potential barriers to testing the overarching hypothesis that pro-inflammatory cytokines and other inflammatory markers would be phenotypically and genetically associated with measures of depression. Importantly, the review showed there were some emerging differences in cytokines in depression in adolescents compared to adults (20). This had not been clearly identified previously and informed the need to consider the similarities and differences in the roles of cytokines and other inflammatory markers between adolescents and adults with MDD.

The literature review also demonstrated the paucity of studies examining the role of cytokines and inflammatory markers in adolescents. Important areas of research were identified including the role of cytokines in relation to stress, early childhood adversity, and genetics (20). In addition to identifying gaps in the research that needed addressing, this systematic review identified other issues pertaining to the project, such as technical difficulties associated with validity of the measurement of cytokines. This literature review was published in 2013 and has 34 citations (Google Scholar 26/04/16) at the time of submission.

Chapter 3

This chapter reports on the measurement and analysis of cytokines, inflammatory markers, and other biomarkers altered in inflammatory states in healthy adolescent twins. This study highlighted that measuring cytokines presents significant challenges. Some cytokines are present in low levels in physically healthy adolescents and young adults (15), so assays need to be sufficiently sensitive to detect cytokines. Cytokines are fragile and measurement of circulating levels is complicated by inter-laboratory variation and timing of blood collection (21, 22). Unfortunately the plasma cytokines included in the multiplex assays (rather than measured individually) did not generate useable results. In view of these difficulties, the cytokines were also measured in dried blood spots (DBS). This posed other challenges, so this chapter also reports on some of the difficulties in measuring cytokines and inflammatory markers.

In spite of these challenges, there was evidence that the association between antibodies (gliadin antibodies and antibodies to infectious agents) and mental illness was in the context of the immune response / inflammatory response (including raised levels of pro-inflammatory cytokines) (23, 24). There have been very few studies examining this, particularly in adolescents. Therefore, this chapter also reports analyses of antibodies to infectious agents and gliadin antibodies measured in DBS in the same study participants in whom cytokines were measured.

The role for Vitamin D in the immune system through modulation of cytokines (12) had not been examined in adolescents. To address this gap in the literature, this chapter also explored a potential association between Vitamin D and inflammation by measuring Vitamin D in DBS (same study participants in whom cytokines and antibodies were measured). Part one of this chapter was published in February 2015 (25).

Chapter 4

Following on from the study of cytokines in healthy twins, I set out to study cytokines in a clinical setting. Undertaking a clinical study involved all aspects of study design, from ethics approval to recruiting of study participants, data collection, and meticulous data entry. The challenges in measuring cytokines, and the need for an improved understanding of cytokines in adolescents in the context of early childhood adversity, also needed to be considered in this study, which aimed to study cytokines and inflammatory markers in a clinical group.

Challenges of implementing a protocol enabling the collection, processing and storage of blood in an acute clinical setting as well as collecting clinical data are discussed in this chapter. These challenges are broadly divided into patient factors (such as degree of morbidity), blood collection factors (including co-ordinating blood collection and the low acceptability of venepuncture by adolescents), and other data collection factors (such as timing the obtaining of consent from parents).

Chapter 5

Difficulties in measuring cytokines in plasma and DBS as outlined in Chapters 3 and 4 presented potential barriers to testing the overarching hypothesis that I had set out to investigate. However, questions relevant to the hypothesis could be addressed using data collected on large community samples of twins. The review of the literature had shown other biochemical measures associated with depression in adults, including iron measures. Specifically, decreases in serum transferrin and increases in serum ferritin are seen in inflammatory states (8). These changes of decreased serum transferrin and increased serum ferritin have also been found in adults with MDD (9). There were few studies examining the relationship between iron measures and MDD in adults. To the best of this candidate's knowledge, there were no studies that had examined the relationship between iron and MDD in adolescents. To address this gap in the literature, Chapter 5 investigates the phenotypic and genetic relationship between measures of iron and depression in adolescents and adults. In the chapter, several different strategies are used to investigate the genetic relationship between these measures.

Chapter 6

As work on the thesis progressed, literature on the interaction between stress and depression was emerging and expanding. There were a number of studies investigating cytokines in those with a history of childhood maltreatment, both in healthy adults (26) and in adults with MDD (27). There were also more studies investigating the role of the inflammatory marker C-reactive protein (CRP) in adults with MDD and a history of early childhood adversity (28, 29). A small number of studies had investigated cytokines and inflammatory markers (specifically interleukin-6 (IL-6) and CRP) in adolescents with depression and a history of childhood adversity (30, 31).

Studies investigating the relationship between CRP and MDD had shown evidence of a complex interplay between body mass index (BMI), CRP, and MDD (32). This association between BMI and CRP extended to anxiety disorders, with a longitudinal study showing

that the relationship between CRP and Generalised Anxiety Disorder (GAD) appeared to be mediated by BMI (33). Investigating the relationship between BMI, CRP, anxiety disorders and a history of early childhood adversity had not been previously undertaken. There were also very few studies examining the genetic relationship between CRP, BMI, MDD, and anxiety.

Chapter 6 addressed these important gaps in the literature. The clinical study (Chapter 4) demonstrated the high frequency of early childhood adversity in this sample, and the systematic review (Chapter 2) highlighted the need to better understand the relationship between CRP, BMI, and MDD / anxiety disorders. This chapter addressed these gaps by investigating the phenotypic and genetic relationship between CRP, MDD and anxiety and included analyses that stratified based on experience of childhood trauma.

Chapter 7

In this discussion chapter I assess how the findings reported in this thesis add to the existing literature, consider limitations of the studies undertaken, discuss implications for clinical practice (with the aim of improving treatment for MDD in adolescents), and recommend areas for future research. Recently, a number of groups have discussed examining the association between these potential biomarkers and stage of mental illness (34, 35), and how this may translate into clinical practice. These recent discussions on biomarkers and stage of illness have focussed on adults. However, review of the adult literature provided guidance for study design in the previous chapters, and suggested areas for future research in adolescents.

A small number of recently published studies investigated the relationship between cytokines or inflammatory markers in anxiety disorders in adolescents. Therefore, this chapter includes some discussion of directions for future research in adolescents with mental illnesses other than MDD.

CHAPTER 2: THE ROLE OF CYTOKINES IN DEPRESSION IN ADOLESCENTS: A SYSTEMATIC REVIEW

Natalie T Mills[1], James G Scott[2,3], Naomi R Wray [4], Sarah Cohen-Woods[5,] Bernhard T Baune[5]

[1]*Genetic Epidemiology, Queensland Institute of Medical Research, Brisbane, QLD, Australia*

[2] *The University of Queensland Centre for Clinical Research, Brisbane, Australia*

[3] *Metro North Mental Health, RBWH, Brisbane, Australia*

[4] *Queensland Brain Institute, University of Queensland, Brisbane, Australia*

[5]*Discipline of Psychiatry, School of Medicine, University of Adelaide, Adelaide, SA, Australia*

Abstract

Background: Cytokines have been implicated in the pathophysiology of depression in adults, however the potential role in younger age groups such as adolescents is less clear. We review the literature (i) exploring the relationship between cytokines and depression in adolescents and (ii) examining how cytokines may be related to adolescent depression in the context of other neurobiological theories of depression.

Method: A systematic review of the scientific literature on the subject was conducted in February 2013, searching the Web of Knowledge, PubMed (Medline), PsycInfo, and Cochrane electronic databases.

Results: Eighteen studies were identified measuring both depression or depressive symptoms and cytokines or immune markers in adolescents. Adolescents with depression show age specific characteristics of the immune and inflammatory system, specifically in NK cell activity and in pro-inflammatory cytokines (such as IL-1β and TNF-α). In addition, the role of cytokines in adolescent depression is influenced by neurodevelopment, hormonal changes, stress, and trauma.

Conclusions: There may be differences in the neurobiology of adolescent MDD compared to adult MDD. Increased understanding of the role of cytokines in adolescent MDD may lead to improved outcomes in the treatment of adolescent depression.

Abbreviations: MDD=Major Depressive Disorder; CD=Conduct Disorder; HPA=Hypothalamic-Pituitary-Adrenal; IL=interleukin; TNF-α=tumor necrosis factor-α; IFN-γ=interferon-γ; NK cells =natural killer cells; TRP=tryptophan; KYN=kynurenine; 3-HAA=3-hydroxyanthranilic acid; QUIN=quinolinic acid; tCho=total choline; PUFAs=polyunsaturated fatty acids; CRH= corticotrophin-releasing hormone; CRP=C-reactive protein; LPS=lipopolysaccharide; SSRI=selective serotonin re-uptake inhibitor

Conflict of Interest:

All authors declare no conflict of interest.

Introduction

Major depression is a leading cause of disability worldwide (36), with lifetime prevalence in most countries ranging between 8 to 12% (37). It is responsible for the greatest proportion of disease burden attributable to non-fatal health outcomes, accounting for almost 12% of total years lived with disability worldwide (38). Major Depressive Disorder (MDD) in young people is estimated to be experienced by approximately 2% of children and 4% to 8% of adolescents (39) and carries its own burden of disadvantage often persisting or reemerging in adulthood (40, 41). The Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM IV) considers major depression in adolescents and adults to be similar, although adolescents may show irritability rather than report depressed mood (42). The 5th edition of the DSM (DSM 5) maintains these criteria (43). Similarities in the clinical presentation of MDD in children, adolescents and adults are supported by additional evidence (e.g. (44, 45)), yet there is also support for differences in risk factors, clinical outcome, and biological correlates between adults and adolescents (44, 46-49). Apparent differences in underlying aetiologies between adolescent- and adult- onset depression (44) may be attributable to developmental differences, or biologic changes that are sub-syndromal and too subtle for studies designed to detect the effect sizes reported in adult studies. Alternatively, it could be speculated that biologic changes observed in depressed adults but not in adolescents or children might be attributable to medication intake since adults with depression often have a prolonged medication history as compared to adolescents and children with depression.

Although there are differences in risk factors for depression in adolescent- and adult- onset depression, family history and stressful life events including childhood maltreatment are significant risk factors for both (49, 50). A family history of depression does not automatically indicate a genetic contribution to the disorder, as it reflects both shared genetic and common (family) environmental effects. However, twin studies designed to tease out the heritability (proportion of variance that can be attributed to genetic factors), consistently indicate a substantial genetic component, particularly in clinical samples (e.g. (51)). Yet, identification of gene variants that robustly associated with depression have remained elusive, with environmental factors that interact with genetic predisposition being of potential relevance. A particularly important environmental mediator in depression is stressful life events, particularly childhood maltreatment (52). Such experience may result in dysregulation of the adaptive stress response system (53). To investigate the interaction of genetic and environmental risk factors requires large data sets in which participants

have provided DNA and have been consistently measured, preferably longitudinally, for environmental risk factors. Unfortunately, such data sets are rare. The on-going debate about the interaction between stressful life events and the serotonin transporter length polymorphism illustrates how differences in the measurement of environmental risk factors might, in part, be responsible for inconsistent findings between studies (54-58).

In adults, accumulating evidence suggests that MDD may be associated with immune system dysregulation (1), at times occurring in the absence of specific immune challenges such as infections. Cytokines are signaling molecules that mediate key steps in cellular and humoral immunity, and a biological relationship with MDD is supported by a large number of mechanistic studies in vivo and in vitro (59). Cytokine genes, such as variants of the *IL-1β* and *TNF-α* genes, have been implicated in impaired emotion processing in major depression and in hippocampus formation in recent functional magnetic resonance imaging (fMRI) studies (60, 61). Furthermore, a recent review suggested that genetic variants of cytokines are possibly involved in the pathophysiology of depression (62). Of particular relevance, cytokines have also been implicated in the stress response, important in depression (63). In both adults and adolescents, childhood maltreatment has been associated with an elevation in CRP levels, indicating a dysregulation of the immune system, further compounded by concurrent depressive symptoms (28, 53). In addition, cytokines are implicated in neurodevelopment and with stress regulation since pro-inflammatory cytokines may exert direct effects on the hypothalamic-pituitary-adrenal (HPA) axis (64, 65). Prolonged periods of excess corticosteroids following chronic stress are particularly important during adolescence, as brain structures such as the hippocampus are susceptible to adverse stress-associated effects (66). Also, biological susceptibility to stress related brain damage may be heightened during adolescence when myelination processes within the central corticolimbic circuitry of the brain occur (67).

The primary purpose of this review is to assess literature on the role of cytokines in adolescent depression by identifying studies that measured both cytokines and symptoms of depression in adolescents. By doing this, we aim to clarify if cytokines have a differential role in depression in adolescents compared to adults. The secondary purpose of this review is to examine how cytokines may be related to adolescent depression in the context of other neurobiological theories of depression.

Method

The literature search included published articles until February 2013 according to the PRISMA (preferred reporting items for systematic reviews and meta-analyses) guidelines as they apply to systematic reviews (68). The primary purpose was to identify all studies that provide empirical data on the measurement of cytokines and symptoms of depression in adolescents. In order to compare findings in adolescents to other age groups, the search was expanded to all age groups. In addition, as some studies in older patients with MDD found a relationship between cytokines and cognition and cognitive dysfunction which may present as a symptom in depression, we also searched for studies that measured cytokines and cognitive performance in adolescents. As a family history of depression is also considered a risk for MDD, 'genes' was included as a search term. To help identify publications considering a differential role for cytokines and immune markers in adolescent MDD, we conducted a further literature search applying the following combination of search terms – Cytokines, Immun*, and Depression and Stress. The final search strategy is illustrated in Figure 2-1.

Figure 2-1: Study inclusion flowchart

Step 1 — Search of all years of Web of Knowledge, PubMed, PsycInfo, Cochrane electronic databases, including reference lists of the articles obtained in the search.

1714 articles were identified and 1438 were excluded (based on relevance to search aims) after a review of the body and / or abstracts.

Step 2 — 276 articles

84 articles were excluded based on lack of relevance to the review.

Inclusion — 192 articles → Full text articles were sought and read.

Legend:

Inclusion criteria: Search was carried out applying the following combinations of terms – 1. Cytokines AND adolescents AND depression, 2. Cytokines AND adolescents AND cognition, 3.Cytokines AND depression AND genes, 4. Cytokines AND cognition AND genes, 5.Cytokines AND depression AND cognition, 6.Cytokines AND immun* AND depression AND stress.

Exclusion criteria: articles written in languages other than English

Results

Several detailed reviews have reported on the cytokine theory of depression (2, 6, 59, 64, 69-74), with a primary focus on adults, or older age groups. Our review comprises three

main sections: (i) the cytokine theory of depression as reported in adults, (ii) evidence specifically relevant to adolescent-onset depression, and (iii) clinical implications.

I.   The Cytokine Theory of Depression

Biological Properties of Cytokines

Cytokines are small pleiotropic proteins (4). The term cytokine includes a large and diverse family of signaling molecules that primarily have immune modulating activity, and are produced widely throughout the body by cells of diverse embryological origin (75, 76). Cytokines can be viewed as either 'pro-inflammatory' or 'anti-inflammatory', depending on their primary effects on target cells (4). Pro-inflammatory cytokines include interleukin-1α and β (IL-1α and IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). These molecules are believed to co-ordinate the local and systemic inflammatory response to microbial pathogens (59). Others, such as interleukin 10 (IL-10), are considered 'anti-inflammatory'(77), however this distinction of pro and anti-inflammatory cytokines has been regarded as an over-simplification in the context of depression (64). In the brain, as in systemic organs, the natural balance between pro- and anti-inflammatory cytokines regulates the intensity and duration of the response to immune stimuli (59).

Heritability of cytokine levels (IL-1β, IL-1ra, IL-6, IL-10, and TNF-α) have been estimated to exceed 50% (78). However, in this study cytokine levels were measured with an ex-vivo whole blood assay in response to lipopolysaccharide (LPS) stimulation, so heritability estimates may more accurately reflect "immune-response" and not necessarily naturally circulating levels of cytokines in the blood. Nonetheless, other studies based on circulating levels of cytokines implicate an important role for genetic factors (e.g.(79-81)). Dysfunction in genes controlling key proteins in cytokine production have been identified as vulnerability factors for cytokine-induced depression (71), and recent studies have reported that variation in *IL-1* and *TNF-α* genes, and elevated levels of *TNF-α,* are associated with reduced responsiveness to antidepressant treatment (82-84). Overall, the molecular genetic basis of cytokine production in humans in the context of depression is currently not well understood.

Cytokines and Depression

Increasing evidence suggests that pro-inflammatory cytokines play a major role in the pathophysiology of depression. A role of cytokines in depression was first proposed by Smith in the form of the 'macrophage theory of depression' stating that excessive secretion of macrophage monokines cause depression (85). Although no consistent association between cytokines and MDD has been reported (86, 87), a recent meta-analysis that included 24 studies reported an association between elevated levels of two pro-inflammatory cytokines, IL-6 and TNF-α, and major depression (6).

Additional research in humans is required to clarify whether cytokines are causally involved in clinical depression since the majority of studies have been cross-sectional (73), with only a few prospective studies in humans allowing for causal inferences. For example, findings from a 12 year study of a large British occupational cohort in adults concluded that inflammation predicted cognitive symptoms of depression (88). Moreover, in support of a role of inflammation in the etiology of depression, Baune et al. showed in older adults that the pro-inflammatory cytokine IL-8 predicted first onset of mild to moderate depressive symptoms over a 2 year period, indicating IL-8 could be a marker of first onset of depressive symptoms in the elderly (89). Such studies are sparse in adolescents with clinical depression, and would require a longitudinal study design.

Both biological and environmental factors can precipitate an episode of depression, possibly through an increase in circulating plasma cytokines. For example, external factors such as psychosocial stressors and medical conditions such as organic inflammatory disorders or physiological conditions (i.e. the postpartum period), may trigger clinical depression via inflammatory processes (74). Clinical depression may also be induced by a therapeutic administration of interferon in hepatitis C (90). Experimentally, peripheral administration of lipopolysaccharide (LPS), or of recombinant cytokines, such as IL-1β or TNF-α, induces nonspecific symptoms of sickness, including fever, activation of the HPA axis, reduction of food intake, and withdrawal from the physical and social environment, termed as *sickness behaviour* (71).

Further mechanistic studies demonstrate that LPS not only causes a peripheral inflammatory response, but also induces a neuroinflammatory reaction with increased production of pro-inflammatory cytokines such as TNF-α in the brain (91, 92). Pre-treatment with antidepressant drugs have also been found to abrogate LPS- or IL-1β-behaviour related to reduced consumption or rewards of sweetened solutions or sucrose in

rats (93, 94). Brain circuits involved in depression-like behavior have also been identified using LPS induction of cytokines in animal models (e.g., in the amygdala, hippocampus, and hypothalamus) (95). However, a functional dissociation between those brain structures that underlie cytokine-induced sickness behavior and cytokine-induced depressive-like behavior has been reported, indicating the need for further research on the temporal relationship between cytokine elevation, structural and behavioural changes (95). Figure 2-2 shows the cellular and humoral immune factors implicated to have a role in depression. Specifically, cellular neuroimmune mechanisms implicated in the pathophysiology of depression include dysfunction of T helper (Th 17) cells and CD4+CD25+ T regulatory (Treg) cells (70, 96). Findings on the possible involvement of natural killer (NK) cells in adult and adolescent MDD are discussed later.

Figure 2-2: Immunological Factors involved with Depression
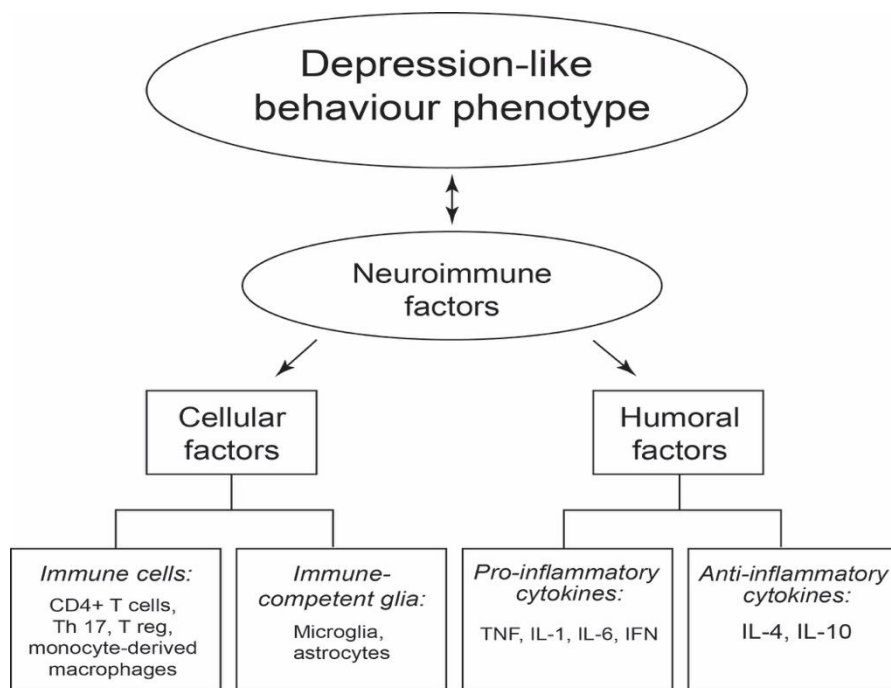
Key: IL= interleukin; IFN= interferon; NK= natural killer cells; TNF= tumor necrosis factor; Th = T helper cell; T reg = regulatory T cells

Cytokines in the brain

Cytokines can exert direct and indirect effects on brain function through their influence on neurotransmitters, neurogenesis, and the HPA axis influencing neuroplastic changes relevant to depression (97). While cytokines do not readily pass through the blood-brain

barrier (69), five potential pathways for cytokine signals to reach the brain have been described: 1) passive transport of cytokines into the brain at circumventricular sites lacking a blood-brain barrier (98-100); 2) activation of the cerebral vascular endothelium, thereby releasing cytokines and inducing the generation of secondary messengers such as prostaglandins and nitric oxide (101-103); 3) carrier-mediated transport of cytokines into the brain across the blood-brain barrier (104); 4) activation by cytokines of peripheral afferent nerve terminals, which then relay cytokine signals to relevant brain regions (100, 105, 106); and 5) recruitment of activated cells such as monocytes / macrophages from the periphery to the brain, where these cells can produce cytokines (107). These mechanisms are not mutually exclusive, and depend in part on the location of the inflammatory stimulus and the disease state of the organism (108). In addition, most cytokines can be synthesized and released within the CNS (108) such as by microglia that are a primary source of pro-inflammatory cytokine production in the brain (2). However, the brain circuitry that mediates the various behavioural responses to cytokines remains elusive (59).

Clinical and experimental studies indicate that stress and depression are also associated with increased circulating concentrations of cytokines, such as TNF-α and IL-1 (109). Increased levels of these cytokines have the potential to impair synaptic plasticity (structurally and functionally), modulate long-term potentiation (LTP) and glutamatergic-dependent synaptic plasticity (110-117), and to induce fear learning, thus contributing to progression of a depressive disorder (4). It has also been postulated that antidepressants indirectly modulate synaptic plasticity as a mechanism of antidepressant action, and that selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants may induce changes in TNF-α expression and function in the brain (4).

Cytokines influence on biological pathways of depression

Cytokines affect biological pathways that have been associated with depression, and so the cytokine theory in depression can be viewed as complimentary rather than competitive to other hypotheses of depression, such as the monoamine theory of depression. Tryptophan is an essential amino acid required for protein synthesis and is a precursor for the monoamine serotonin and a lowered availability of plasma L-tryptophan has been associated with depression (118). Interestingly, decreased levels of L-tryptophan are also correlated with inflammation, indicating that systemic inflammation may contribute to clinical depression via a decrease in the serotonin precursor L-tryptophan (119-121).

As depicted in Figure 2-3, pro-inflammatory cytokines induce IDO (indoleamine 2-3-dioxygenase), an enzyme that mediates the catabolism of tryptophan into kynurenine (KYN) (122). KYN is further metabolized into 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), and quinolinic acid (QUIN), which may induce neuronal damage (123, 124). Hence, it is suggestive that increased levels and activity of cytokines may lead to depressive symptoms by inducing a reduction in important neurotransmitters such as serotonin and by stimulating neuronal damage both implicated in clinical depression.

Figure 2-3: Tryptophan pathways
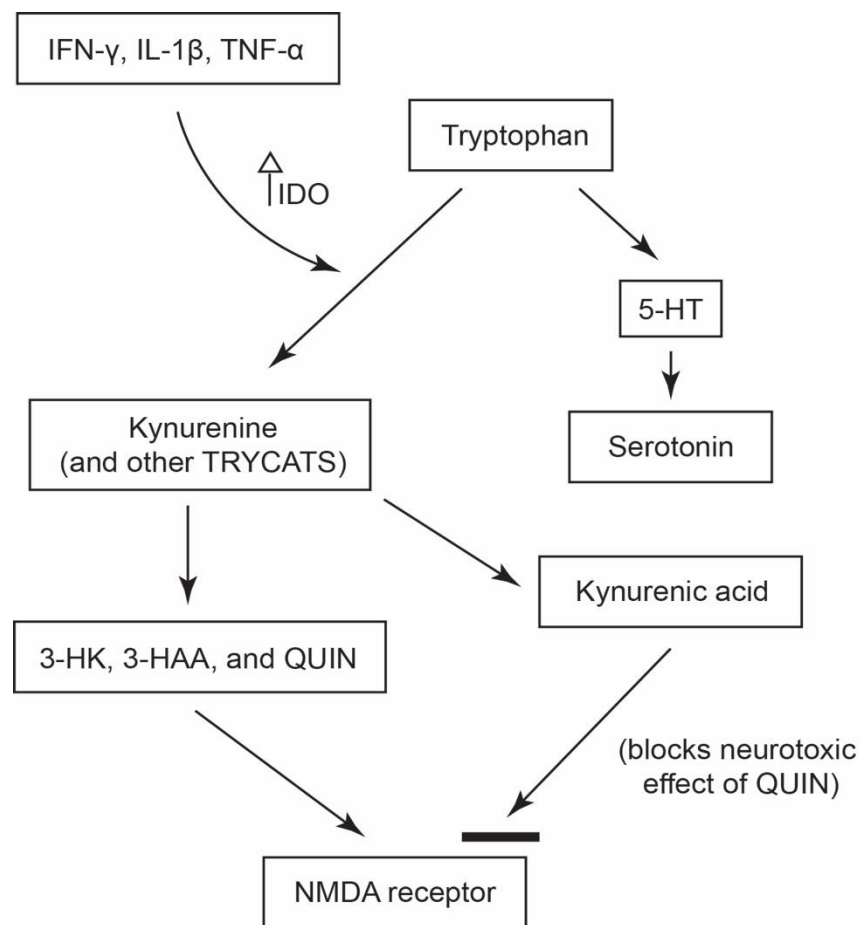
Key: 5-HT= 5-hydroxytryptamine; HAA=hydroxyanthranilic acid; HK= hydroxykynurenine; IDO= indoleamine 2-3-dioxygenase; ↑IDO= increased levels of IDO; IFN= interferon; IL= interleukin; NMDA= N-methyl-D-aspartate; QUIN= quinolinic acid; TNF= tumor necrosis factor; TRYCATS=tryptophan catabolites along the IDO pathway

As cytokines, among other humoral and cellular immune factors, have the potential to influence systems heavily implicated in depression (97), it is a clinically relevant question to determine if a genetic predisposition, or environmental factors that frequently associate with depression, or a combination of both, contribute to the pathophysiology of depression. It has been frequently reported that stress elevates levels of pro-inflammatory cytokines by activating their signaling pathways (125-128). Moreover, cytokines influence the hypothalamic pituitary axis (HPA)(129) and IL-1, IL-6, TNF-α, and IFN-α may increase corticotrophin-releasing hormone (CRH) release and disrupt the function of the glucocorticoid receptor (reviewed by (130)). Indeed, since a hyperactive HPA axis has been reported in a proportion of patients with MDD (129), it is plausible that such hyperactivity could be a consequence of an elevation in cytokines, attributable to potentially both environmental and genetic moderators. Particularly genetic variants of cytokines have been suggested to be associated with clinical depression as recently reviewed by Bufalino et al. (62). However, the likely important interaction between these genetic variants and environmental factors such as stress and maltreatment has not been considered yet in original studies.

Other genes of potential relevance are polymorphisms of the glucocorticoid receptor gene (*NR3C1*), involved in the regulation of the HPA axis. While some studies have reported that genetic variants of *NR3C1* influence susceptibility to MDD and depressive symptoms (131), three further reports found no association, or associations that failed to withstand correction for multiple testing (132, 133). In addition, multiple genome-wide studies have presented no evidence for genome-wide significance (134). Failure to replicate may reflect insufficient power, due to insufficient sample size, heterogeneity of the phenotype of depression, or interaction with unidentified environmental factors. Indeed, evidence for gene-environment interaction has been reported with variation in the *NR3C1* gene and childhood maltreatment (135), and altered methylation of the *NR3C1* gene has been associated with stress exposure (136, 137). These findings may lead to the speculation that depression could be a consequence of the effect of elevated cytokine levels in response to a stressor, occurring in the context of an *NR3C1* system that was already dysregulated due to genetic or epigenetic effects earlier. Hence, levels of pro-inflammatory cytokines would be elevated in the context of the dysregulated HPA axis.

Recent work on the HPA axis and depression has also been conducted by Solomon et al., who showed a sex difference in the role of forebrain glucocorticoid receptors in regulating

HPA axis activity and depression-like behavior in mice (138). Specifically, in mice with selective deletion of glucocorticoid receptors in forebrain cortico-limbic sites (forebrain glucocorticoid receptor knockout mouse – FBGRKO), female mice did not show basal HPA axis dysregulation or exaggerated stress responses. The authors noted that in females, glucocorticoid receptor regulation of HPA axis function and behavior may be from other brain areas than those targeted in the FBGRKO mouse (138).

Another biological pathway associated with depression might be a stress-induced decrease in neurogenesis influenced by cytokines. Pro-inflammatory cytokines, such as IL-1β, have been shown to inhibit cell proliferation and promote cell death in the hippocampus (139). Furthermore, there is evidence that neurodegeneration and the defects in neurogenesis in depression are caused by inflammatory processes, related to the production of oxidative and nitrosative stress molecules and pro-inflammatory cytokines (74). Glutamate has also been implicated in MDD (140, 141), and glutamate neurotoxicity represents a pathway leading to increased apoptosis enhanced by pro-inflammatory cytokines via various pathways: (a) activation of the kynurenine pathway in microglia, with glutamate release and increased quinolonic acid; (b) reducing glial glutamate transporter activity, resulting in decreased glutamate removal from the extracellular space; and (c) by inducing long-term activation of microglia, which releases TNF-α and IL-1 (reviewed in McNally et al 2008) (4, 142).

In summary, cytokines exert wide-ranging influences on neuronal structure, function, and directly on the stress-response system, as well as in response to stress. Evidence for the role of cytokines in the brain in depression is building, and seems tantalizing, yet remains largely circumstantial. Still needed is to clarify whether effects are causal in humans, to better specify the actual molecular mechanisms operating in human depression related to cytokines, and in particular to clarify the effects in adolescence.

II.   The Role of Cytokines in Depression in Adolescents

Emerging literature has examined a possible role of cytokines in adolescent depression. A total of twenty-four articles examining the role of immune markers in depression in adolescents were identified for this review. Of these, eighteen studies report empirical data as summarized in Tables 2-1, 2-2, and 2-3. Twelve publications present case-control studies which have relatively small sample sizes; the largest study consisted of 134 cases

and 149 controls. Four studies used a prospective design (30, 32, 143, 144), assessing N=135, N=141, N=147, and N=1420 individuals respectively for depressive symptoms. The two remaining studies are a cross-sectional representative community sample (145), and an observational study of youths with diabetes (146). Key results from the studies are included in the relevant sections below. Of the remaining 6 articles examining the role of immune markers in depression in adolescents, some did not measure cytokines (for example cytokine genes were genotyped but not circulating cytokine levels (147); Pandey et al. measured serotonin receptors postmortem and discussed the interaction between the HPA axis and serotonergic system (148)). Two of these remaining 6 articles measured inflammatory markers or cytokines only when the cohort had reached adulthood (29, 53). Of these 6 articles, the studies by Danese et al. (29, 53), and Pandey et al. (148) are discussed in more detail later in the review.

Stress Response, Neurogenesis and Neurodevelopment

Increased levels of pro-inflammatory cytokines in response to acute stress has been described as characteristic (96), involving an activated HPA axis that may lead to a further rise in pro-inflammatory cytokines through complex positive feedback loops (64). These mechanisms could be particularly important during adolescence, as brain structures such as the hippocampus appear susceptible to adverse effects of prolonged periods of excess corticosteroids with consequences such as atrophy of the apical dendrites of the pyramidal cells (66). Therefore, chronic unpredictable stress in the environment is regarded as an important factor in the development and onset of depression (96).

The variation in how an individual responds to stress, including their vulnerability to depression, may be influenced by an inflammatory response of the immune system. In a recent review, Fagundes et al. proposed a model of early adversity leading to greater stress sensitivity, and so placing an individual at greater risk for immune dysregulation (149). Elevated levels of inflammatory markers, specifically C-reactive protein (CRP), have been reported not only in depressed adults exposed to childhood maltreatment (28, 53, 150), but also in adolescence (28). CRP is an acute phase protein that promotes resistance to infection and repair of damaged tissues (53). In these studies, CRP levels showed a linear increase depending on depression and/or maltreatment exposure (28, 29). Individuals with a history of depression or current depression exhibited higher CRP levels relative to individuals with no depression or exposure to maltreatment; those with a

history of maltreatment and no depression exhibited even higher levels of CRP, and finally those that had experienced both maltreatment and depression presented the most elevated profile of all the groups. The elevation in CRP levels was significant in the combined depressed and maltreated group, relative to those with no exposure to maltreatment or depression. The authors concluded that a history of childhood maltreatment has a significant role in explaining the co-occurrence of depression and inflammation through the lifespan with "biological embedding" already seen at adolescence (28, 29). The findings in adolescents (Danese et al) (28) are particularly important, as increased inflammatory markers in adulthood has been linked to increased risk of both mental and physical illness (2, 29).

In adolescents, pro-inflammatory cytokines have also been implicated in the stress response with a higher number of stressful life events associated with higher TNF-α levels (151). In support of these findings is an 18-month longitudinal study of adolescent females who showed increased IL-6 responses to two different types of threatening stimuli on the background of exposure to a harsh family environment (143). An extension of this study that investigated circulating levels of IL-6 and CRP found that among those exposed to higher levels of childhood adversity, the transition to depression was also accompanied by relative increases in both CRP and IL-6 (30). Furthermore, the authors noted that higher CRP levels remained in these subjects 6 months later, even after the episode of depression had abated. Importantly, this coupling of depression and inflammation was not apparent in those without a history of childhood adversity (30).

Cytokines also appear to be involved in neurodevelopmental processes (152); for example, IL-6 has demonstrated both neuroprotective (153, 154) and neurodegenerative properties (155). In support of a possible neuroprotective effect of IL-6 is a recently published neuroimaging study conducted in healthy individuals that showed increased hippocampus volumes associated with genetic variants of the *IL-6* gene (156). There is also some preliminary evidence suggesting that maternal psychopathology and HPA function influences fetal, infant, and adolescent HPA axis function resulting in a higher tonic setting of the HPA axis through epigenetic programming (157, 158). Psychiatric epigenetics is a relatively new field, however it does provide a biological mechanism by which stress might influence the immune response, and ultimately predispose to depression. Moreover, such mechanisms could lead to atypical early neurogenesis and vulnerable neural systems in the post-pubertal adolescent brain (50). Such a sensitization

of the neurobiological systems implicated in stress adaptation and response (as is seen in childhood maltreatment) may increase the risk of developing depression (159). Therefore, as individuals move through different stages of adolescence, the risk for depression as a consequence of stress exposure may change, and arguably so might the inflammatory stress-response profile. To date little gene-environment work has focused on stress exposure and depression onset in adolescents specifically – the stress exposure has primarily focused on either adulthood or childhood (although this at times spans adolescent years) (e.g. (54, 160)).

Cytokines and onset of Depression during Adolescence

Similarly to adult MDD, immune system dysregulation with a pro- and anti-inflammatory imbalance has been proposed in MDD in adolescents (161). The first study to examine cytokines in adolescent MDD, reported increased pro-inflammatory cytokines IFN-γ and IFN-γ/IL-4 as well as a trend for increased IL-6 in adolescents with MDD compared to healthy controls (161).

In an attempt to examine the neurobiology of clinical subtypes of depression in adolescence, Gabbay et al. examined whether adolescent MDD with melancholic features (M-MDD) has distinct biological features in the kynurenine pathway. As previously reported, pro-inflammatory cytokines induce IDO, which metabolizes tryptophan (TRP) into kynurenine (KYN), eventually decreasing TRP availability in the brain (162). The authors reported decreased plasma TRP levels and an increased KYN/TRP ratio (estimating IDO activity) in adolescents with M-MDD compared to both non M-MDD and a control group. Interestingly, the severity of episodes as measured by Children's Depression Rating Scale-Revised (CDRS-R) was associated with several KYN pathway measures (e.g., KYN and 3-hydroxyanthranilic acid (3-HAA)/KYN) in the M-MDD group (162).

Investigating the relationship between early onset of depression and anxiety, IL-10 levels have been associated with increased anxiety and depression scores, and IFNα2 levels with anxiety scores only (163). The association between IFNα2 levels and anxiety scores remained significant after controlling for familial risk of MDD, gender, current stress, and childhood trauma (163), suggesting an independent effect of cytokines in anxiety.

Of clinical relevance is the potential that cytokine levels are modified during treatment with antidepressants (64). In a clinical sample of adolescent females with MDD and/or anxiety

disorder the effects of antidepressant treatment with SSRIs on cytokine levels was compared to healthy controls (164). The overall sample showed significantly increased levels for IL-1β, IL-2 and IL-10 as compared to healthy controls, with SSRI-treatment associated with IL-6 levels in the clinical sample (164). The non-SSRI subgroup showed significantly higher levels of IL-1β, IL-2, and IL-6 compared to healthy controls. The authors concluded that pro-inflammatory cytokines are likely to be part of the pathophysiology of emotional disorders in adolescent females, and that SSRIs may exert anti-inflammatory properties in this patient group (164).

Cytokine interactions with gonadal hormones

The prevalence of major depression is known to increase during periods of changes in gonadal hormones (165). While the male-to-female ratio is 1:1 during childhood, the 1:2 sex ratio that characterizes adult MDD first emerges during adolescence (39). The timing of the change in male-to-female prevalence ratios for depression has important implications for theories about the relationship between depression and puberty. Angold et al. reported early the role of secondary sex characteristics in the development of depression (166). Characteristics of gender development (sex characteristics) as expressed in Tanner stages ranging from I-prepubertal to V-adult level of development have been suspected to better associate with the development of depression rather than age. It also appeared that this transition in gender prevalence ratios was a mid-pubertal event, occurring in Tanner stage III, generating theories about a role of gonadal hormones in the etiology of depression (166).

Research in pre-menopausal women indicates that gonadal hormones may modulate immune function (167, 168). The cytokine response of peripheral blood monocytes after LPS stimulation in premenopausal women appears to be modulated by the phase of the menstrual cycle (167). Specifically, a lower release of TNF-α (p<0.05) and IL-6 (not significant) during the luteal phase compared to the follicular phase was reported (167). Overall, however, findings on cytokine production across the normal menstrual cycle have been inconsistent (169) requiring further investigations. In addition, it remains to be examined as to whether such findings derived in adult women would generalize and apply to younger age groups such as adolescents. Systematic research is required to determine if a relationship exists in adolescence between cytokines and hormones that are important

for brain development. If such a relationship does exist, it will be important to determine if cytokines modulate gonadal hormones or vice versa.

Differences in the Role of Cytokines in Depression between Adolescents and Adults

In this section, we aim to identify evidence for possible differences and similarities on the role of cytokines in adolescent and adult MDD. It is worthwhile to summarise the findings on the possible involvement of natural killer (NK) cells in adult and adolescent depression as an expression of immune activity in this psychiatric condition. While studies in adult MDD when compared to age consistent controls consistently demonstrate lower NK cell activity (170-172) and reduced number of major lymphocyte subclasses (173), studies of NK cell activity and lymphocyte subpopulations in adolescent MDD as compared to age consistent controls have yielded contradictory findings (174-178). Some of these inconsistencies in adolescent MDD might be related to methodologies such as accounting for adverse life events in some studies (175), or sampling of younger age groups (i.e. inclusion of both children and adolescents) (174), or gender specific findings in girls (144). The inconsistent results in adolescents may also indicate a lack of any real association. However, the sample sizes for most of these studies are relatively small (174-178), and only one of the studies (144) used a longitudinal design.

Table 2-1: Studies of Immune Markers in Depression in Adolescents

Study	Objectives	Design	Immune markers	Results
Targum *et al.,* 1990 (178)	Determine if hospitalized adolescents with MDD or conduct disorder (CD) show reduced lymphocyte populations compared to controls, and whether there is an association between reduced lymphocyte nos / subpopulations and cortisol dysregulation in hospitalized adolescents	30 (11 patients with MDD, 5 males, 6 females, ages 15.5+/-1.6 yrs; 11 with conduct disorder (CD), 7 males, 4 females, ages 15.3+/-1.2 yrs; 8 controls, 4 males, 4 females, ages 14.1+/-1.5 yrs); structured diagnostic interviews using DSM-III criteria for MDD or conduct disorder; cross-	Total T cells, CD4+, CD8+, CD16, total B cells	No significant differences on any lymphocyte measure between patients and controls

		sectional study		
Shain *et al.*, 1991 (177)	To compare natural killer (NK) cell activity in depressed adolescent patients with NK cell activity in age- and sex-matched controls	32 (16 patients with MDD, 16 controls; 6 males, 10 females in each group), ages 13-18 yrs; interview by child psychiatrist, using the Schedule for Affective Disorders and Schizophrenia (SADS) Present Episode version (179); cross-sectional study	NK cell activity	No significant differences between patients and matched controls. Age significantly correlated with NK cell activity.
Birmaher *et al.*, 1994 (175)	Determine whether adolescents with MDD have disturbances in their cellular immunity, and to study whether the immunological changes detected are specific to depression or are general responses to stress	54 (20 patients with MDD, 5 males, 15 females; 17 non-depressed patients with conduct disorder (CD), 7 males, 10 females; 17 sex matched healthy controls), ages 11-18 yrs, Tanner stage III or more; SADS Present Episode version; cross-sectional study	NK cell activity, lymphocyte subtypes	Patients with CD significantly higher absolute number of B cells than healthy controls (p=0.02), and a significantly greater % of B cells than MDD group (p=0.05) and controls (p=0.02). Patients with CD (who had significantly more adverse life events) showed a trend for lower NK cell activity than those in the MDD group (p=0.08) and the control group (p=0.08).
Bartlett *et al.*, 1995 (174)	To examine for differences in immunity between children with MDD and healthy controls	36 (18 patients, 18 controls; 13 males, 5 females in each group), ages 8-12 yrs; Diagnostic Interview Schedule for Children- Revised (DISC-R) (180); cross-sectional study	Total white blood cells (wbc), lymphocytes, T cells, B cells, monocytes, NK cells, CD4+, CD8+ cells	Lowered NK cell activity in depressed subjects compared to healthy controls (p<0.001).

Schleifer et al., 2002 (176)	To determine if immune changes in MDD are age-related	72 (36 patients, 13 males, 23 females; 36 sex matched healthy controls), ages 14-20 yrs; Diagnostic Interview Schedule for Children DSM-III-R criteria; cross-sectional study	Total wbc, lymphocytes, granulocytes, monocytes, T cells, B cells, CD4+, CD8+, CD29+, CD45RA+, NK cells, HLA-DR+ cells	Increased levels of lymphocytes, T cells, B cells, CD4+, and CD29+ lymphocytes in depressed group compared to controls (p<.05). Increased NK cell activity in MDD adolescents (p< .001)
Caserta et al. 2011 (144)	To test the hypothesis that self-reported efficacy and depression would predict immunity and rate of illnesses.	141 children, 76 males, 65 females; assessed on 3 occasions, 6 months apart; depression symptoms (Children's Depression Inventory – Short Form) (181) and self-efficacy measured by self-report; parents recorded illness (mental health problems were not coded as illness); longitudinal study; age 7-13 years (median age 9.3 years)	IL-6, NK cell functional assay	Negative association between self-efficacy and IL-6 (p=0.03); depression was associated with increased NK cell function (p=0.02) and higher rates of illness (p<0.01) in girls older than 9.3 years of age;

Key: CD= Conduct Disorder; CD cells= Cluster of Differentiation; MDD= Major Depressive Disorder; NK cells= natural killer cells; wbc= white blood cells; reference numbers for studies are included in study column (in brackets)

A meta-analysis of studies examining the association between depression in adults and elevated levels of the inflammatory marker CRP have also yielded inconsistent results (182). In adolescents, a large population-based study has found no apparent association between CRP and depressive symptoms (145). Although this first study indicates there may be a difference between adult and adolescent levels of CRP in depression, problems with this study include a lack of clinical diagnosis. In a meta-analysis of the associations of depression with CRP, IL-6, and IL-1, Howren et al. noted the importance of the method used to assess depression, with larger associations noted in clinical samples and when standard clinical interviews were used to assess depression (7). Copeland et al. used a

structured interview to assess depression in a study examining longitudinal pathways between CRP and depression in adolescents and young adults (32). The authors found that cumulative depressive episodes predicted later CRP levels after adjusting for important covariates (covariates of sex, age, body mass index (BMI), current nicotine/ alcohol/ illicit drug use, current medication use, recent health ailments, and current low socioeconomic status) (32).

Heterogeneity in results from both adult and adolescent studies may be due to no consideration of other variables that influence inflammation, such as stressful life events (28, 30) . Addressing such a potential confounder, Brambilla et al examined the immune function of children with a first episode of MDD unlikely to have been preceded by stressful events. Contrary to that seen in depressed adults, those children without the experience of stressful life events with MDD had normal IL-1β levels (183). However, it is likely that several depression studies in adults have not accounted for stressful life events, limiting a direct comparison with the study by Brambilla.

Many studies in adult MDD have reported increased TNF-α compared to controls suggesting a role in the pathophysiology of depression (6, 184-187).   However, when studying suicidality in adult MDD, studies on its relationship with cytokines have not always included assessment of TNF-α (188, 189). A study which did include measurements of TNF-α in suicidal adults found increased levels of TNF-α (and IL-6) in suicide attempters compared to non-suicidal depressed patients and healthy controls (190). In adolescents, contrasting findings were reported. Gabbay et al found that plasma levels of TNF-α were significantly decreased in suicidal adolescents with MDD compared to a nonsuicidal MDD group (191). However, the authors noted their findings should be considered preliminary in view of the small sample size (30 patients, 15 controls), and the substantial percentage (57%) of patients receiving psychotropic medications (191). The authors also noted that due to the small sample size, in order to preserve statistical power, a multiple comparison correction was not applied (191).

If these findings *in vivo* hold true, postmortem studies suggesting the serotonergic system of the prefrontal cortex (PFC) is implicated in suicide in both adolescents and adults (148, 192), provide a possible pathway linking TNF-α levels to suicidality (191). A proposed mechanism to explain how cytokines may affect behavior is through activation of the enzyme IDO, which results in altered serotonin metabolism (193, 194). Specifically,

increased 5-HT2A receptor binding has been observed in the PFC of teenage (148) and adult suicide victims (195). In addition, serotonin has been observed to be depleted in multiple brain regions, including the frontal cortex, in rodents acutely administered IFN-α by intracerebroventricular injection (196).

Importantly, cytokines in the brain of suicide victims, or subjects with depression, have not been systematically studied (197), and the direction of their association remains to be fully understood. For example, Pandey et al. observed that the mRNA levels of TNF-α, IL-1β, IL-6, and protein levels of TNF-α and IL-1β were significantly increased in Brodmann area 10 of the PFC of teenage suicide victims (compared to controls) (197). Interestingly, Tonelli et al. found no significant change in TNF-α in male or female adult suicide victims, however observed increased IL-4 in female suicide victims and increased IL-13 in male suicide victims (193). It is possible that these studies are not directly comparable (Pandey et al. (197); Tonelli et al. (193)), as the pathophysiology of teenage suicide and the role of cytokines in teenage suicide may differ from that in adults (197).

Table 2-2: Clinical Studies of Cytokines / Inflammatory Markers in Depression in Adolescents

Study	Objectives	Design	Inflammatory Markers	Results
Brambilla *et al.,* 2004 (183)	To determine whether cytokine secretion is impaired at an early phase of development of depression, possibly involvement in the course of the disease	33 (22 patients, 17 males, 5 females; 11 psychologically healthy age and sex matched controls), ages 6-14 yrs; DSM IV criteria using the Kiddie SADS interview (198); cross-sectional study	Plasma IL-1β, TNF-α	IL-1β levels significantly higher (p<0.0003, z= -2.95) and TNF-α lower (p<0.01, z= -2.53) in dysthymic patients than in controls; IL-1β and TNF-α not significantly different between MDD and controls.
Gabbay *et al.,* 2009 (161)	To examine immune system dysregulation in adolescents with MDD	45 (30 patients, 19 females; 15 healthy controls, 8 females), ages 12-19 yrs; DSM-IV diagnostic criteria, then interview by child psychiatrist using the	Plasma IFN-γ, TNF-α, IL-6, IL-1β, IL-4	Significantly increased plasma level of IFN-γ (p<0.003, Bonferroni corrected p<0.02) and IFN-γ/IL-4 ratio (p=0.007, Bonferroni corrected p<0.05) in adolescents with MDD; Trend for increased IL-6 in

		Schedule for Affective Disorders and Schizophrenia – Present and Lifetime Version for Children (K-SADS-PL) (199); cross-sectional study		adolescents with MDD compared to controls (p=0.09)
Gabbay *et al.*, 2009 (191)	To examine the role of cytokines in suicidal symptomatology in adolescent MDD	Patient group as above (30 patients – 12 suicidal, 18 non-suicidal; 15 controls); cross-sectional study	Plasma IFN-γ, TNF-α, IL-6, IL-1β, IL-4	Suicidal adolescents had significantly decreased plasma levels of TNF-α compared to non-suicidal adolescents with MDD (p=0.03); Increased IFN-γ in both suicidal (p<0.02) and non-suicidal (p=0.005) adolescents with MDD compared to controls;
Gabbay *et al.*, 2010 (162)	To examine whether MDD in adolescents has distinct biological features in the kynurenine pathway in MDD cases with (M-MDD) and without (Non M-MDD) melancholic features;	20 adolescents with M-MDD, 11 females; 30 adolescents with non M-MDD, 16 females; 22 healthy controls, 13 females. Ages 12-19yrs. Interview by child and adolescent psychiatrist using the K-SADS-PL. Cross-sectional study	Plasma TRP, KYN, 3-HAA	KYN/TRP ratios significantly elevated and TRP concentrations significantly reduced M-MDD group compared to non M-MDD adolescents (p=0.001; p=0.006 respectively), and controls (p=0.008; p=0.02 respectively); Significant positive correlation between 3-HAA/KYN and MDD severity in the M-MDD group (p=0.03);
Blom *et al.* 2012 (164)	To study effects of antidepressants on systemic cytokines in post pubertal adolescent females with anxiety disorders and/or MDD compared to healthy controls	42 adolescent females with MDD, 60 healthy controls; age 14 – 18 years. Assessment by child and adolescent psychiatrist or psychologist and the Development and Wellbeing Assessment (DAWBA)-interview	Plasma IL-1β, IL-2, IL-6, IL-10, IFN-γ, TNF-α	Unmedicated subgroup of clinical sample showed significantly higher IL-2, IL-1β and IL-6 compared to controls (adjusted Z= -3.3, p<0.001; adjusted Z= -2.2, p<0.05; adjusted Z= -2.3, p<0.05 respectively); in the medicated subgroup, only IL-2 was significantly higher as

				compared to controls (adjusted Z= 2.3, p<0.05). Unmedicated subgroup of clinical sample showed significantly higher IL-6 and IL-6/IL-10 compared to medicated subgroup (adjusted Z= 2.8, p<0.001, adjusted Z= 2.5, p<0.05 respectively);
Pandey *et al.* 2012 (197)	To examine the role of pro-inflammatory cytokines in suicide	24 suicide victims (14 males, 10 females), 24 controls (17 males, 7 females). Cause of death for the controls varied (e.g heart disease, motor vehicle accident); unclear if controls ever attempted suicide; Ages 12-20 years; cross-sectional study	Protein and mRNA levels of TNF-α, IL-1β and IL-6 in prefrontal cortex	Significantly increased mRNA levels of TNF-α, IL-1β and IL-6 in Brodmann area 10 of the PFC in suicide victims (p<0.01). Significantly increased protein levels of TNF-α and IL-1β in Brodman area 10 of the PFC in suicide victims (p<0.01)
Quinones *et al.* 2012 (163)	To further understand the role of specific immune mediators early in the development of depression and anxiety	A group at high familial risk for MDD (n=134), and an age and sex matched low-risk group (n=149); no previous mood disorder or substance abuse diagnosis. Ages 12-15 years; cross-sectional study	Plasma cytokine levels	IL-10 was significantly associated with anxiety and depression scores; IFNα2 levels were correlated with anxiety scores independent of familial risk for mood disorders and environmental stressors

Key: CRP= C-reactive protein; HAA= hydroxyanthranilic acid; IFN= interferon; IL= interleukin; KYN= kynurenine; LPS= lipopolysaccharide; MDD= Major Depressive Disorder; M-MDD= Major Depressive Disorder with melancholic features; NK cell = natural killer cell; TNF= tumor necrosis factor; TRP= tryptophan; reference numbers for studies are included in study column (in brackets)

In summary, studies that provide empirical data for a role of cytokines in adolescent MDD currently show similarities and differences between adolescents and adults, not allowing

definite conclusions at this stage. Similarities include increased IL-6 levels, and decreased plasma TRP levels (161, 162). Differences in IL-1β variation have been reported, with normal levels in "un-stressed" children with MDD (183) and elevated levels in adults with MDD (201). Plasma TNF-α was noted to be significantly decreased in adolescents with MDD and suicidality (191), yet mRNA levels of TNF-α were significantly increased in the PFC of teenage suicide victims (197). A meta-analysis in adults with MDD, regardless of suicidality, suggests a consistent association between circulating levels of TNF-α and adult MDD (6). Therefore, results for TNF-α are inconsistent between studies, however cytokines have been measured from different sites (for example, plasma in Gabbay et al. (191), versus postmortem brain tissue in Pandey et al. (197)). In addition, it remains to be clarified, if suicidality alone could account for the differences in cytokines reported in MDD studies in adults and adolescents.

Small sample sizes in most of the studies on adolescents limit conclusions (e.g. 33 individuals in the Brambilla et al. study (183), and 45 individuals in the studies by Gabbay et al. (161, 191)). In addition to small sample sizes, there are a range of ages of study participants (i.e. pre-pubertal and post-pubertal) in the studies. The youngest study participant was 6 years old in the Brambilla et al. study (183), and 7 years old (median age 9.3 years) in Caserta et al. (144).

There are significant differences between many of the study protocols, such as cross-sectional studies of hospital inpatients (e.g. a proportion of patients in Gabbay et al. (161, 191) were inpatients) versus longitudinal population-based observational studies (e.g. Copeland et al. (32), Miller and Cole (30)), prohibiting a formal meta-analysis. Another significant difference between study protocols is the method used to assess for MDD or assess depressive symptoms. Some studies used DSM criteria (e.g. Gabbay et al. (161, 191), however others used self-report questionnaires (e.g. Chaiton et al. (145) used a depression subscale, Caserta et al. (144) measured depression symptoms and self-efficacy using self-report). These differences in the assessment of depression symptoms limit comparisons between studies, particularly as there are currently no diagnostic biomarkers for MDD. A further issue is the problem of an unknown number of negative results not being published. If these unpublished results are not included in meta-analyses, this acts as a source of bias. Further research using epidemiological approaches (202) in well-powered and well-designed cohorts is required to provide empirical data to consolidate and build on these results.

Table 2-3: Population based Epidemiological Studies of Cytokines / Inflammatory Markers in Adolescents

Study	Objectives	Design	Inflammatory Markers	Results
Miller and Chen, 2010 (143)	To evaluate if a harsh environment engenders a pro-inflammatory phenotype in children that is marked by exaggerated cytokine responses to bacterial stimuli and the anti-inflammatory properties of cortisol	135 adolescent females, assessed at 4 occasions over an 18 month period (prospective study). No history of chronic medical or psychiatric disorders; Ages 15-19 yrs at time of study entry; UCLA Life Stress Interview – Adolescent Version (203) administered at each visit.	Circulating serum IL-6, production of IL-6 following LPS stimulation, and resistance to glucocorticoids	Those raised in a harsh environment showed increased IL-6 response to 2 different types of threatening stimuli - in vitro LPS (p=0.01) and a stressful life event (p=0.001). Over this time, subjects also showed progressive desensitization of the glucocorticoid receptor (p=0.04)
Chaiton et al., 2010 (145)	To study the association between high-sensitivity C-reactive protein concentrations and depressive symptoms in youth	1,535 (721 aged 13 yrs, 814 aged 16 yrs); self-report questionnaires (depression subscale of the Psychological Distress Scale (204)); cross-sectional study	CRP	No apparent association between depressive symptoms and serum CRP (p=0.81)
Copeland et al., 2012 (32)	To test 1. Effect of CRP levels on later depression status; 2. Effect of depression status on later CRP levels; 3. Effect of cumulative episodes of depression on later CRP levels	1,420 children, ages 9, 11, and 13 years at intake; 48.7% of total sample female. Longitudinal study, with annual assessments to age 16 years, and again at ages 19 and 21 years; depression assessed by a structured interview: Child and Adolescent Psychiatric Assessment (205) until age 16, the Young Adult Psychiatric Assessment	CRP (measured in dried blood spot samples)	CRP levels were not associated with later depression status. CRP levels increased with number of prior depressive episodes. Only cumulative depressive episodes predicted later CRP levels after controlling for covariates (p= 0.02)

		(206) at later ages		
Miller and Cole, 2012 (30)	To aim to clarify the direction of the association between depression and inflammation To determine whether other kinds of childhood adversity (besides childhood maltreatment) also promote clustering of depression and inflammation	147 adolescent females, assessed every 6 months over 2.5 years. Ages 15-19 years at time of study entry; Structured Clinical Interview for DSM-IV-TR (SCID) Axis I Disorders - Non-Patient Edition (207); longitudinal study	Serum CRP, IL-6	High levels of IL-6 predicted risk of depression 6 months later in those with a history of childhood adversity (serum IL-6 odds ratio 1.50, 95% CI 1.10-2.06, p=0.01). Higher CRP levels remained 6 months after depressive episode had abated in those subjects exposed to higher levels of childhood adversity.
Hood, Lawrence et al. 2012 (146)	To provide preliminary evidence that the increased risk for depression in youth with diabetes is associated with metabolic and inflammatory markers To inform future examinations of the directionality of these associations	2,359 youths with diabetes from the SEARCH study – an observational study of US children diagnosed with diabetes at <20 years of age. Mean age of study participants 15.2 +/- 3.1 yrs, 53% of sample female; depression measured with the Centre for Epidemiologic Studies Depression (CES-D) Scale (208).	CRP, IL-6	CRP was significantly (p<0.006) associated with depression in youth with diabetes in bivariate analysis. In regression models stratified by diabetes type and accounting for demographic and clinical characteristics, only higher levels of apoB remained associated with higher levels of depression in youth with type 1 diabetes.

Key: CRP= C-reactive protein; IL= interleukin; LPS= lipopolysaccharide; reference numbers for studies are included in study column (in brackets)

A summary of the differences in immune and inflammatory markers between adults and adolescents with MDD is provided below in Table 2.4.

Table 2-4: Summary of Differences in Immune and Inflammatory Markers in Adult versus Adolescent Depression

Study	Age group	Immune or Inflammatory marker	Results
Kronfol *et al.*, 1989 (170), Nerozzi *et al.*, 1989 (171), Irwin *et al.*, 1990 (172)	Adult	NK cell activity	Decreased NK cell activity in patients suffering an episode of MDD
Shain *et al.*, 1991 (177)	Adolescent	NK cell activity	No significant differences in NK cell activity between patients with MDD and matched controls.
Bartlett *et al.*, 1995 (174)	Adolescent	NK cell activity	Lowered NK cell activity in depressed subjects.
Schleifer *et al.,* 2002 (176)	Adolescent	NK cell activity	Increased NK cell activity in adolescents with MDD.
Maes *et al.,* 1991 (201)	Adult	IL-1β	Elevated IL-1β levels in adults with MDD
Brambilla *et al.,* 2004 (183)	Adolescent	IL-1β	Normal IL-1β levels in children with MDD
Dowlati *et al.,* 2010 (6)	Adult	TNF-α	Elevated TNF-α in adults with MDD
Gabbay *et al.,* 2009 (191)	Adolescent	TNF-α	Decreased TNF-α in suicidal adolescents with MDD

Key: IL= interleukin; MDD = Major Depressive Disorder; NK cell = natural killer cell; TNF= tumor necrosis factor

III.   Clinical Implications

Antidepressant drugs appear to have some action on pro-inflammatory cytokines (209, 210), with attenuation of an imbalance between pro- and anti-inflammatory cytokines in patients with MDD treated with the antidepressants fluoxetine, sertraline, or paroxetine (184, 187, 211-213). Furthermore, patients who fail to respond to antidepressants have

been found to demonstrate increased plasma concentrations of IL-6 and acute phase reactants when compared to treatment-responsive patients (214-216).

Other agents and treatment programs that have anti-inflammatory actions or block actions of cytokines, such as physical exercise and omega-3 polyunsaturated fatty acids, may have a role in the treatment of depression. It is possible that physical exercise may exert similar anti-inflammatory effects beneficial to improving depressive symptoms, which are believed to be more globally mediated through various pathways of the neuroimmune system (96). As recently extensively reviewed by these authors, consistent exercise / physical activity has been shown to reduce levels of IL-1β, TNF-α, IL-6, and CRP (96), whereas studies examining the cytokine levels during or immediately after exercise have shown an upregulation of IL-6 and IL-8 (217, 218). The short-term effects of exercise with an acute transient upregulation of IL-6 appears to induce a rise in IL-10 (219), and to negate neurotoxic changes of TNF-α (220). Further research has been recommended to enhance alternative treatment approaches to depression, such as physical exercise, that might improve depression via the immune system (96).

A meta-analysis of 10 double-blind, placebo-controlled studies in adult patients with mood disorders receiving omega-3 PUFAs, indicated an antidepressant effect of omega-3 PUFAs (221). However, the authors noted that it is premature to draw firm conclusions based on the findings due to the heterogeneity of the different study methodologies (221). Similar effects have also been reported for children in a small randomized controlled trial study showing that long chain omega-3 PUFAs supplementation in the treatment of children with a first episode of depression had a benefit in reducing depressive symptoms, however not in achieving remission (222). Given the implication of omega-3 PUFAs in depression, it is therefore interesting that omega-3 PUFAs have the capacity to decrease the production of pro-inflammatory cytokines, and exert strong anti-inflammatory effects (74); studies in adolescents could add valuable knowledge to the literature.

In studies of adults with depression, cytokine antagonists have also been found to have antidepressant-like effects (223-227). TNF-α blockers such as etanercept and infliximab have been found to attenuate the depressive symptoms that accompany immune system activation in psoriasis (223-225, 227). However, conflicting results have been reported for TNF-α antagonists such as infliximab in that depressed patients with higher levels of CRP

prior to treatment may benefit from such treatment as opposed to a general benefit in depression (226).

Anti-inflammatory medications have also been found to have antidepressant-like effects. For example, acetylsalicylic acid added to fluoxetine led to increased remission rates in depressed patients previously unresponsive to fluoxetine alone (228). Furthermore, in patients with MDD adding the cyclooxygenase-2 (COX-2) inhibitor celecoxib to treatment with reboxetine (229) or sertraline (230) induced an antidepressant response. Interestingly, Abbasi et al. also showed a significantly greater reduction in serum IL-6 concentrations in the group treated with celecoxib and sertraline (compared to the group treated with sertraline) (230).

It has been noted that selectively targeting COX-2 in the treatment of depression may be problematic (231). Consistent with determining the mechanism of action of COX-2 inhibitors in depression, Maes reviewed possible detrimental effects of COX-2 inhibitors targeting pathways involved in depression. He concluded that treatments with COX-2 inhibitors may aggravate the pathophysiology of depression, through involvement in pathways which include lowering of antioxidant defenses and inducing neuroinflammation (231). As anti-inflammatory treatments and the use of cytokine antagonists are less common in adolescents than in adults, further research is required to determine if interventions that act on immune responses are effective interventions for depression in adolescent populations.

Conclusion

A substantial body of literature has now focused on the association between cytokines and depression, primarily in adults with less extensive research in adolescents (73). However, this review shows that the role of cytokines in adolescent depression is characterized by many similarities with adult MDD, although important differences of cytokines in depression between these age groups may be emerging.

The relatively small number of studies on the role of cytokines in adolescent MDD has to be taken into account alongside with several methodological issues, which limit their comparability among studies in adolescents but also with research in adults. In general, the sample sizes of these studies are small and few have employed a prospective design. This could particularly be an issue if altered cytokine levels are a consequence of illness,

rather than a cause. If this is the case, altered cytokine levels in adolescence would be more subtle, and so larger sample numbers would be required to detect them statistically, compared to adult studies where changes might be more established and pronounced.

Depression in children and adolescents show some different immune and inflammatory changes to those seen in adult depression in the current literature, with contradictory findings of NK cell activity, and differences in pro-inflammatory cytokines such as IL-1β and TNF-α (177, 183, 191, 197). In adults with MDD, NK cell activity has been found to be decreased (170-172), however in adolescents with MDD, NK cell activity has been found to be increased (176), unchanged (177), and decreased (174). The elevated levels of IL-1β seen in adults with MDD have not been found in adolescents (183). Decreased levels of TNF-α have been found in adolescents with MDD (191), however many studies in adults (including a meta-analysis) have found elevated levels of TNF-α in MDD (6, 184-187). Some of the reported differences between the role of cytokines in depression could be influenced by neurodevelopment, hormonal changes, stress, and trauma, with more direct effects in young individuals as they experience developmental changes compared to adults. An improved understanding of the role of cytokines in adolescent MDD also in relation to stress, maltreatment, hormonal changes and genetic background may inform aetiology and treatment options in adolescent depression.

Further research is warranted to explore more broadly the role of cytokines in depression of adolescents considering adequately the neurobiological, hormonal and environmental changes young individuals are undergoing. Moreover, additional treatment options might be suitable in some forms of inflammation associated depression in adolescents. Initially, the field could be progressed with the inclusion of measurements of circulating cytokines, such as IL-1β, IL-6, and TNF-α, in intervention studies in adolescent MDD. Later, interventions that change levels of circulating plasma cytokines in adolescents with MDD may prove worthy of investigation. Importantly, future research requires well-designed, well-powered clinical studies that consider environmental factors (in particular stressful life events), in addition to using genetic, developmentally, and physiologically sensitive designs with prospective community-based studies, as well as psychiatric samples (50).

Conflict of Interest

None

- Cytokines mediate key steps in cellular and humoral immunity

- Immune system dysregulation / pro- inflammatory cytokines have been implicated in adult major depressive disorder (MDD)

- Studies on the role of cytokines in adolescent MDD are few; the immune / inflammatory changes seen in adolescent MDD show specific similarities and differences to those seen in adult MDD

- Cytokines may influence neurodevelopment during adolescence

- Potential treatments that modify inflammation require further research, as this may lead to better outcomes in the treatment of adolescent MDD

Chapter review

This chapter addressed aim 1 of the thesis: to review systematically the literature so as to identify differences in the role of cytokines in depression in adolescents compared to adults. This review helped to inform the design of studies described in the chapters that follow. The literature review also identified challenges in accurately measuring cytokines and inflammatory markers. Significant gaps in the literature were also identified by undertaking this review.

CHAPTER 3

In this chapter I report analyses of measures of cytokines, inflammatory markers, and other blood measures involved in immune regulation in a pilot study of healthy adolescent twins. The purpose of the pilot study was to determine if these biomarkers could be measured accurately, to provide important preliminary data for an intended larger study in twins and for the clinical study reported in Chapter 4. Part 1 of this chapter, published in the Journal *Twin Research and Human Genetics* (February 2015), reports the measurement and investigation of genetic variance of two of the measured cytokines and Vitamin D. In fact, the pilot study highlighted important difficulties in the measurement of cytokines. Part 2 of this chapter reports the measurement and investigation of genetic variance of cytokines (measured in dried blood spots (DBS)), neurotrophins and antibodies (gliadin antibodies and antibodies to infectious agents) and the analytical steps that led to my conclusions that most of the pilot data could not be presented for publication. As a result of the analyses of this pilot study we decided that we should not progress to the intended larger study.

PART 1: Heritability of Transforming growth factor-β1 and Tumour necrosis factor-receptor type 1 expression and Vitamin D levels in healthy adolescent twins

Total Word Count: 6,759

Word count excluding abstract, acknowledgements, references, and tables: 4,337

Heritability of Transforming growth factor-β1 and Tumour necrosis factor-receptor type 1 expression and Vitamin D levels in healthy adolescent twins

Natalie T Mills[1,2]; Margie J Wright[1]; Anjali K Henders[1]; Darryl W Eyles[2,3]; Bernhard T Baune[4]; John J McGrath[2,3]; Enda M Byrne[2]; Narelle K Hansell[1]; Eva Birosova[5]; James G Scott[6]; Nicholas G Martin[1]; Grant W Montgomery[1]; Naomi R Wray[2]; Anna AE Vinkhuyzen[2]

[1]*Genetic Epidemiology, QIMR Berghofer Medical Research Institute, Brisbane, 4006, Australia*

[2]*Queensland Brain Institute, University of Queensland, Brisbane, 4072, Australia*

[3]Queensland Centre for Mental Health Research, The Park Centre for Mental Health, Brisbane, 4076, Australia

[4]Discipline of Psychiatry, School of Medicine, University of Adelaide, Adelaide, 5005, Australia

[5]School of Medicine, James Cook University, Townsville, 4811, Australia

[6]Metro North Mental Health, Royal Brisbane and Women's Hospital, Brisbane, 4006, Australia

Running Title: Heritability of potential biomarkers of mental illness

Abstract

Cytokines and vitamin D both have a role in modulating the immune system, and are also potentially useful biomarkers in mental illness such as major depressive disorder and schizophrenia. Studying the variability of cytokines and vitamin D in a healthy population sample may add to understanding the association between these biomarkers and mental illness.

To assess genetic and environmental contributions to variation in circulating levels of cytokines and vitamin D (25-hydroxy vitamin D: 25(OH)D3), we analysed data from a healthy adolescent twin cohort (mean age 16.2 years; standard deviation 0.25). Plasma cytokine measures were available for 400 individuals (85MZ, 115DZ pairs), dried blood spot sample vitamin D measures were available for 378 individuals (70MZ, 118DZ pairs).

Heritability estimates were moderate but significant for the cytokines transforming growth factor-β1 (TGF-β1), 0.57 (95% CI 0.26 – 0.80) and tumour necrosis factor-receptor type 1 (TNFR1), 0.50 (95% CI 0.11 – 0.63), respectively. Measures of 25(OH)D3 were within normal range and heritability was estimated to be high (0.86, 95% CI 0.61 – 0.94). Assays of other cytokines did not generate meaningful results.

These potential biomarkers may be useful in mental illness, with further research warranted in larger sample sizes. They may be particularly important in adolescents with mental illness where diagnostic uncertainty poses a significant clinical challenge.

Keywords: cytokines, vitamin D, major depression, psychosis, heritability

Introduction

Emerging evidence suggests a range of blood measures may be useful biomarkers in mental health disorders such as Major Depressive Disorder (MDD) and schizophrenia. Potential blood biomarkers include measures of Vitamin D, cytokines, C-reactive protein (CRP), and antibodies to infectious agents (7, 13, 232). Blood biomarkers may be particularly relevant in adolescence, where depression is often comorbid with other disorders (17) resulting in diagnostic uncertainty. Genetic and environmental influences both contribute to the aetiology of psychiatric disorders (233). Heritability is estimated around 40% for MDD (234, 235) and around 80% for schizophrenia (236). The presence of plasma pro-inflammatory cytokines (20, 161) and variation in Vitamin D levels (13) in adolescents with mental illness is especially interesting, with both also having a role in the modulation of the immune system (12). At present these biomarkers are not specific to a particular mental illness (MDD or schizophrenia). However, understanding how putative biomarkers vary in a healthy population of adolescents may help in guiding the study of these blood measures in psychiatric disorders in adolescents. Examining the link between psychiatric disorders and immune system dysregulation (161), and the genetic contribution to these biomarkers, are both required to understand the role of plasma cytokines and vitamin D levels in adolescent psychiatric disorders.

Cytokines:

Cytokines are small proteins that mediate key steps in cellular and humoral immunity (64), and have been shown to act as neuromodulators. Examples of pro-inflammatory cytokines include Interleukin-1β (IL-1β), Interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) (6, 15), (with tumour necrosis factor-receptor type 1 (TNF-R1) the key mediator of TNF signalling in most cells) (237). Some cytokines, such as transforming growth factor-beta (TGF-β) and TGF-β1, have both pro-inflammatory and anti-inflammatory effects (5, 238).

Cytokines, in particular pro-inflammatory cytokines, have been associated with both MDD and schizophrenia. MDD is thought to be associated with immune system dysregulation (1, 28, 53, 161). In adults, a recent meta-analysis reported an association between elevated levels of two pro-inflammatory cytokines, IL-6 and TNF-α, and MDD (6). It is also possible that symptoms of depression may be a result of natural variation between individuals in

levels of circulating cytokines even in the absence of specific immune challenge such as infection.

A role for immune system dysregulation in schizophrenia has also been hypothesized (239, 240). Levels of circulating cytokines have been associated with acute exacerbations of schizophrenia. Specifically, a recent meta-analysis reported an increase in the cytokines IL-1β, IL-6, and TGF-β during acute relapse and/or first episode psychosis which normalised with antipsychotic treatment (15). The same meta-analysis found IL-12, TNF-α, Interferon-γ (IFN-γ) and soluble IL-2 receptor (sIL-2R) appeared to be trait markers, with levels elevated in first episode psychosis, and remaining so following antipsychotic treatment. Levels of IL-12, TNF-α, and IFN-γ were also found to be elevated in acute relapses of schizophrenia, and remained elevated following antipsychotic treatment (15).

Some studies have measured cytokine response in stimulated cells, for example ex vivo stimulation with amyloid-β in whole blood (241), or antigen-stimulated whole-blood assays (242). This is likely to reflect the heritability of the immune response rather than natural variation. Few studies have estimated the heritability of circulating levels of cytokines, particularly in adolescents. Heritabilities of circulating levels of the cytokines IL-1β, IL-6, IL-10 and TNF-α have been estimated in adult females, with the cytokines IL-1β and IL-10 found to be moderately heritable (range 0.27 – 0.32 and 0.3 for IL-1β and IL-10, respectively), and the cytokines IL-6 and TNF-α found to be less heritable (range 0.15 – 0.16 and 0.17 – 0.23 for IL-6 and TNF-α, respectively) (80). It should also be noted that measurement of cytokines is known to present difficulties and quantification of cytokine levels has been found to depend on a number of factors. For example, concentrations of several inflammatory markers have been found to change depending on whether serum or plasma is collected (243), and healthy adolescents should have very low levels of circulating inflammatory cytokines (15). Furthermore, circulating levels of some cytokines vary throughout a 24 hour period (for example IL-2) (21), or a 7 day period (for example TNF-α) (169). These challenges all need to be considered in the design of studies involving the measurement of cytokines.

Vitamin D:

Vitamin D is best known for its role in skeletal health. More recently, vitamin D has also been implicated in non-skeletal health such as cancers, immunology and psychiatric

disease (12, 244, 245). Activation of cells of the immune system results in upregulation of the Vitamin D receptor (246). Furthermore, Vitamin D is a direct inhibitor of the pro-inflammatory cytokine IL-17 (247). With regard to psychiatric disease, an increased risk of depression has been associated with low Vitamin D in cross sectional studies (248), yet this may reflect individuals with depression spending less time outdoors with less sun exposure. However, a prospective study of 2,759 individuals found a significant association between low Vitamin D measured at 9 years of age, and higher scores on depressive symptoms at 11 and 14 years of age (13). With regard to schizophrenia, an association has been observed with increased risk of this syndrome later in life in neonates who had either low levels or high levels of Vitamin D (249).

Heritability estimates for Vitamin D show much variation and some also seem to vary across season of measurement and sex. Heritability of Vitamin D ranges from 0.43 in European adult twins (98.3% of these twins were female) (250) to as high as 0.80 in the German Asthma Family Study Group (251). A study of male twins found Vitamin D levels to be highly heritable during winter, but not heritable in summer (252), whilst a study of male and female twins found Vitamin D to be heritable in summer, but not during winter (253). Sex differences have been observed with a study of Vitamin D in rural Chinese adolescent twins estimating heritability at 0.86 for males and 0.17 for females (254).

With the clinical diagnosis of both MDD and schizophrenia likely to represent a biological heterogeneous group of disorders, identification of biomarkers that stratify patients for application of "stratified medicine" is a direction worthy of further research (18). The study reported here represents preliminary research towards the goal of understanding the role of potential blood biomarkers (specifically plasma cytokine and vitamin D measures) of depression and psychosis. We could not find prior published heritability estimates for some of these cytokines. We use a population based sample of healthy adolescent twins to assess the genetic and environmental contributions to variation in circulating levels of these potential biomarkers.

Methods

Samples:

Participants were 16 year old twins (mean 16.2 years, standard deviation (SD) 0.25 years) from the Brisbane Adolescent Twin Study (255). In this sample, both plasma cytokine and dried blood spots (DBS) Vitamin D measures were collected as part of a study focussing on health and well-being in an adolescent population sample. Plasma cytokine measures were available for 400 individuals (48.25% males, 51.75% females, 85 MZ twin pairs and 115 DZ twin pairs), DBS Vitamin D measures were available for 378 individuals (50.13% males, 49.87% females, 70 MZ twin pairs and 118 DZ twin pairs, two twin pairs were incomplete). Of these twin pairs, 49 MZ twin pairs and 74 DZ twin pairs had data available for both plasma cytokine and Vitamin D measures.

Blood was collected when twins came in for testing as close as possible to their 16th birthday. To explore impact of age of biological sample in this pilot study we selected individuals who participated either between the years of 1997 – 2000 (wave 1) or between the years of 2006 – 2009 (wave 2). Blood samples were available from these years both as plasma (for measurement of cytokines) and DBS (for measurement of Vitamin D). DBS for the measurement of Vitamin D were stored on Whatman 903 filter paper for wave 1, and on Whatman FTA cards impregnated with antibacterial agents to preserve DNA for wave 2. Blood of two members of a twin pair (i.e. co-twins) was always collected within the same wave.

Assay of plasma cytokine and Vitamin D measures

Plasma cytokine measures: A total of 25 plasma cytokines and inflammatory markers were measured at the James Cook University, Townsville, Australia. Of these 25 markers, TGF-β1 and TNFR1 were assayed successfully. Specifically, plasma cytokines and inflammatory markers were divided into 4 separate groups, based on multiplexing compatibility with other cytokines and inflammatory markers with the groups as follows: Group 1: TGF-β1; Group 2: Interleukin-2 (IL-2), IL-4, IL-7, IL-11, IL-12, IL-13, TNF-α, IL-17A, IL-21, Exotaxin, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF). Group 3: IL-1α, IL-1β, IL-3, IL-5, IL-6, IL-8, IL-9, IL-10, IFN-γ-inducible protein 10 (IP-10),

Monocyte Chemo-attractant protein-1 (MCP-1), Macrophage inflammatory protein-1a (Mip-1a), IFN-γ; Group 4: TNFR1.

TGF-β1 and TNFR1 were measured individually (not part of the multiplex) due to not being compatible with other cytokines. For TGF-β1 (560429) we used the Human TGF-β1Duoset ELISA kit (R&D). We used BD Biosciences flex set multiplex assays at half strength (allowing double the number of samples) compared to the manufacturers recommendations, supported by pilot analyses. Plasma samples were diluted 4 times before testing, consistent with observations from the kit manufacturer (and our own pre-experiment measurements) that undiluted plasma causes high background signal that interferes significantly with specific signals. Standard curves were generated using technical controls of lyophilised standard protein at a known concentration. In addition, for all cytokines and inflammatory markers an intra-assay experiment (to evaluate accuracy of the method) was run on 20 samples created from tissue culture medium spiked with standards. The experimental design for the plasma samples ensured that co-twins (two members of a twin pair) were randomised across plates.

Of the cytokines measured in plasma we found that only the two assays conducted for individual cytokines (TGFβ1 and TNFR1) not included in the multiplex, generated meaningful results. For the multiplexed cytokines (groups 2 and 3) we found that although the technical controls (the intra-assay experiment) were measured accurately, the assay was not sufficiently sensitive to the low circulating levels in healthy adolescents following the recommended dilution of sample.

Given the poor outcome of cytokine assays from plasma samples we also attempted to assay cytokines from DBS (256). Inflammatory markers are more stable in DBS, with measurable concentrations of cytokines found to be stable in DBS stored at different temperatures for many days when compared to DBS frozen immediately after preparation (although long-term storage at -20°C is considered optimal) (256). Whilst measurable amounts of most cytokines have been found to be nearly constant in DBS stored up to 23 years, a marked decrease over time has been noted in measurable concentrations of IL-1β, IL-8, sIL-6ra, Matrix Metallopeptidase-9 (MMP-9), Triggering Receptor Expressed on Myeloid cells 1 (TREM-1), CRP, Brain-derived Neurotrophic Factor (BDNF), and Neurotrophin-4 (NT-4) in stored DBS (257). We found that the cytokine measures were sensitive to paper type (often generating null reads for one or other paper) and were

subject to plate effects which could not be adjusted appropriately in an experimental design in which co-twins within a twin pair were present on the same plate. We also attempted to measure antibodies to infectious agents (258), another potential biomarker for MDD, but also found the assays to be highly sensitive to paper type and plate effects, and few individuals had non-null measures.

Vitamin D measures: the main circulating form of Vitamin D is 25 hydroxyvitamin D3 (25[OH]D3) (244). In our study, 25(OH)D3 was measured from DBS by liquid chromatography tandem mass spectroscopy (249, 259) at the University of Queensland, Brisbane, Australia. The assay method is highly sensitive, and can measure 25(OH)D3 in DBS stored for between 8 to 22 years (259). With this method, assay calibrants are extracted and derived in the same way as clinical samples, using multi-point calibration curves (259). Co-twins were plated in adjacent wells on the same plate. As Whatman FTA paper is thicker than 903 and therefore absorbs more blood, standards were prepared on both paper types in accordance with previous studies (260).

For the measurement of 25(OH)D3 the experimental design for the DBS was not ideal for partitioning of variance as co-twins of the same pair were plated in neighbouring wells of the same plate. Under this design, batch effects are common to the twin pair so will be partitioned into the common environmental component. In contrast, if co-twins of a twin pair are plated randomly, batch effects will partition into the unique environment variance component. The DBS plating layout had been established for a different study interested in differences between twin pairs in blood markers. The static associated with tiny blood spots meant that replating was not practical. However, the extensive technical controls implemented in the 25(OH)D3 assay eliminated plate effects and so confounding of plate and twin pair was not an issue.

Statistical analyses:

To account for non-normality of the data, a square root transformation was applied to TGFβ1. Subsequently, TGFβ1 and TNFR1 were standardized and effects of plate and year of blood sampling were regressed out. For 25(OH)D3, which is largely affected by seasonal differences in ultra violet exposure, a cosinor regression model was fitted to the data to adjust for seasonality, additionally possible effects of sex, year of blood sampling, and paper type (261) were regressed out. Year of blood sampling did not have a significant effect on any measure.

A saturated model was fitted to the residuals to estimate MZ and DZ twin correlations for cytokines TGFβ1 and TNFR1 and for 25(OH)D3. A saturated model does not hold any assumptions regarding the underlying variance components model. Means and variances were constrained to be equal across zygosity groups and twin correlations were constrained to be equal for same-sex DZ and opposite-sex DZ twin pairs to improve stability of the genetic model. Within the saturated model, differences in means and variances between first and second born twins and between zygosity and sex (and BMI for 25(OH)D3) were tested using likelihood ratio tests.

To investigate the relative contributions of genetic and environmental factors to the observed variation in TGFβ1, TNFR1, and 25(OH)D3 univariate variance components models were specified. The analysis of 25(OH)D3 was done in the full sample as well as separately including individuals which had their blood collected in summer or winter (where the months May through October inclusive were considered winter and November through April inclusive were considered summer in the Southern hemisphere). Within these models, the variance was partitioned into additive genetic (A), common environmental (C), and unique environmental (E) variance components. 'A' represents the additive genetic effects of alleles summed up over all genetic loci in the genome, 'C' represents shared or common environmental factors that render offspring of the same family more alike, and 'E' represents environmental factors that result in differences between family members, 'E' also includes measurement error.

To examine the significance of estimated variance components, the fit of a nested (increasingly more restricted) model was compared to the fit of the full model. Two sub models were considered, (i) a model including A and E and (ii) a model including C and E. Goodness-of-fit of these sub models was assessed using likelihood ratio tests. The difference in log-likelihoods between the full (ACE) model and the reduced (AE or CE) model follows a χ^2-distribution and was evaluated using a χ^2-difference test. If the χ^2-difference test is significant the estimate of the variance component that is removed from the model (e.g., C in an AE-model) is considered significantly larger than zero. If the χ^2-difference test is not significant, the estimate of the variance component that is removed from the model is not significantly larger than zero. Twin correlations and variance components were estimated in the statistical software package Mx (262).

Results

Means and variances of raw values for TGFβ1, TNFR1, and 25(OH)D3 are shown in Table 3-1. For TGFβ1 and TNFR1 we were not able to find an agreed normal reference range to which we could compare our results, Vitamin D levels were within the normal range (263). For TNFR1, we observed a significant difference in means between males and females with males having higher levels than females ($p < 0.001$). For 25(OH)D3, we observed significant differences in variances ($p = 0.001$) between MZ and DZ twins (with MZ twins having on average higher levels and larger variance compared with DZ twins) and a birth order effect in both means ($p < 0.05$) and variances ($p < 0.01$) (with first born twins having on average lower levels of vitamin D and smaller variance). We expect the effects of zygosity and birth order to be a result of the sampling variance. The phenotypic correlations between the Vitamin D and the two cytokine measures were not significantly different from zero.

MZ correlations were significantly higher than DZ correlations for TGFβ1 and 25(OH)D3, and approached significance for TNFR1 (Table 3-1) suggesting a genetic component in the variance decomposition. Given the small sample size we did not estimate heritability separately for males and females. Twin correlations, however, were very similar for males and females suggesting no differences in heritability estimates across sex in our data (Table 3-1).

Table 3-1: Means, Variances (of raw values), and Twin Correlations (with 95% Confidence Intervals) for transformed values of TGFβ1, TNFR1, and 25(OH)D3.

	Mean	Variance	rMZ (95% CI)	rDZ (95% CI)
TGFβ1	A: 30.54	A: 327.75	A: 0.72 (0.62,0.80)	A: 0.45 (0.29,0.58)
	M: 29.66	M: 339.30	M: 0.68 (0.50,0.79)	M: 0.60 (0.30, 0.76)
	F: 31.45	F: 316.41	F: 0.77 (0.63,0.85)	F: 0.54 (0.31, 0.70)
TNFR1	A: 809.25	A: 85039.67	A: 0.52(0.35, 0.65)	A: 0.21 (0.04,0.37)
	M: 890.70	M: 82972.81	M: 0.53 (0.23, 0.70)	M: 0.27 (0.00, 0.48)
	F: 725.01	F: 73712.50	F: 0.52 (0.29, 0.67)	F: 0.25 (-0.10, 0.52)
25(OH)D3	A: 69.51	A: 752.87	A: 0.91 (0.87,0.94)	A: 0.49 (0.33,0.62)
	M: 71.84	M: 824.77	M: 0.92 (0.87,0.95)	M: 0.76 (0.60,0.85)
	F: 67.16	F: 673.56	F: 0.91 (0.84,0.94)	F: 0.71 (0.49, 0.82)

Sex was included as a covariate in the means model in the genetic analyses. We did not include sex as a covariate in the variance model, neither did we consider birth order effects in our genetic models. Including those effects in our genetic models would greatly reduce power given the current sample size, which in turn would probably lead to spurious results.

Within the genetic model, the two cytokines were found to be moderately heritable (Table 3-2), with heritabilities estimated at 0.57 (95% CI 0.26 – 0.80, χ^2 =12.49, p=<0.001) and 0.50 (95% CI 0.11 – 0.63, χ^2 =5.9, p=0.008) for TGFβ1 and TNFR1, respectively. Heritability of 25(OH)D3 (all months combined) was estimated to be high (0.86, 95% CI 0.61 – 0.94, χ^2 = 55.98, p=<0.001) (Table 3-2). Heritability of 25(OH)D3 for individuals who had blood collected in the summer months was also high (0.90, 95% CI 0.60 – 0.94, χ^2 = 25.43, p=<0.001), whilst heritability for individuals who had blood collected in winter was moderate (0.56, 95% CI 0.31 – 0.92, χ^2 = 23.023, p= 0.001). For the cytokines TGFβ1 and TNFR1, any plate or batch effects would tend to increase the unique environment variance component.

Table 3-2: Heritabilities of TGFβ1, TNFR1, and 25(OH)D3 (with 95% Confidence Intervals)

	A	**C**	**E**
TGFβ1	0.57(0.26,0.80)	0.16(0.00,0.42)	0.27(0.20,0.38)
TNFR1	0.50(0.11,0.63)	0.00(0.00,0.29)	0.50(0.37,0.66)
25(OH)D3 - overall	0.86(0.61,0.94)	0.06(0.00,0.30)	0.09(0.06,0.13)
25(OH)D3 - summer	0.90 (0.60,0.94)	0.00 (0.00,0.30)	0.10(0.06,0.17)
25(OH)D3 - winter	0.56(0.31,0.92)	0.36(0.00,0.60)	0.08(0.05,0.14)

Key: A= standardized additive genetic variance; C= standardized common environmental variance; E= standardized unique environmental variance

Discussion

We set out to determine if potential biomarkers for MDD and schizophrenia were heritable in a community cohort of healthy adolescent twins. Mean levels of circulating Vitamin D, measured as 25(OH)D3, were in the normal range for healthy Australian adolescents (263). The differences in variances observed for 25(OH)D3 may have been due to chance. The heritability estimates of the two cytokines that could be measured in plasma were found to be moderate (57% and 50% for TGFβ1 and TNFR1, respectively) whilst the heritability of vitamin D was found to be high (86%).

The heritability estimate for the standardised values of Vitamin D was similar to the heritability of 0.80 found for Vitamin D (specifically also 25(OH)D3) in the German Asthma Family Study Group (251), and 0.86 found for 25(OH)D (as 25(OH)D3) in male rural Chinese adolescent twins (254), but greater than the estimate of 0.43 estimated in adult twins from the St Thomas UK Adult Twin Registry (Hunter, De Lange et al. 2001). Similar to Snellman et al. (253), we found the heritability estimate of Vitamin D to be higher in the summer months. This was in contrast to another study, which observed heritability of Vitamin D to be higher in winter months (252). The only previous published report we could find estimating the heritability of TGFβ1 found a heritability of 0.28, however the levels of this cytokine were estimated in an antigen-stimulated whole-blood assay (242). We could not find published reports of heritabilities of TNFR1 to which to compare our results.

A genetic contribution to variation in circulating levels of cytokines TGFβ1 and TNFR1 and Vitamin D is potentially relevant in the aetiology of MDD and schizophrenia, as each of these biomarkers may contribute to a role in the aetiology of these disorders. Here we show the variance of these biomarkers is largely genetic, whilst changes in environmental factors appear to have less influence. Variants in the Major Histocompatibility Complex (MHC) region have been associated with schizophrenia (240, 264), and many of the genes of this region code for cytokines (265). This association does not appear to be driven by the high linkage disequilibrium in the extended MHC (240). With the complexity of the MHC region presenting challenges in understanding its role in schizophrenia (266), further study of shared genetic factors between these biomarkers and schizophrenia is warranted.

The possibility of shared genetic factors between these biomarkers and MDD could also be investigated in future studies.

It should also be noted that although we did not find a strong positive phenotypic association between cytokines and vitamin D measures, this was to be expected. Stimulation of human CD4+CD25- T lymphocytes in the presence of 1,25(OH)2D3 has been shown to inhibit production of pro-inflammatory cytokines, including IFN-γ and IL-17 (267). Low levels of plasma 25(OH)D3 have been associated with chronic disease where inflammation has been found to play an important role, such as type 2 diabetes (268). This finding may not diminish the potential usefulness of these blood biomarkers, rather it suggests that the role of Vitamin D deficiency in inflammation may be an area where further research is warranted (12).

A limitation of our study is that healthy adolescents are expected to have very low levels of circulating inflammatory cytokines (15). This may explain why our initial ELISA multiplex assay performed on plasma samples was not sufficiently sensitive (i.e. did not generate useable results) to the circulating levels of cytokines in our healthy community cohort of adolescents. This was despite the assay being calibrated to standardised adult samples (269). To address the limitation of assay sensitivity, high-sensitivity assays (such as ELISA single assays or high sensitivity kits based on the Luminex 200 system) can be used. ELISA assays utilize undiluted samples, but they usually need high volume of sample (up to 500µL per sample). A limitation of the TGF-β1 assay was the volume of sample required. The human TGF-β1 Duoset ELISA used required 100 µl of sample. This was higher than the volume of aliquots available, so we decreased the sample volume used to 50 µl. In addition, the release of TGF-β1 from platelet granules upon platelet activation also needs to be considered. Our protocol should remove platelets from the plasma sample but incomplete removal of platelets, which can cause variable and irreproducible results (270), would serve to reduce estimates of heritability.

Recommendations for future studies could include extending this research to adolescents with MDD or schizophrenia (or psychotic symptoms) and conducting both univariate and bivariate analyses to investigate to what extent genetic impact on circulating levels of cytokines and vitamin D are heritable and whether the same genes affect these measures in people with psychiatric disorders such as MDD and schizophrenia. However, high sensitivity assays (such as ELISA single assays), with larger sample sizes would be

required. This could be followed by investigating the role of other potential blood biomarkers (such as CRP and antibodies to infectious agents) in adolescents with MDD or psychosis.

Conclusion

This study reports heritability estimates of cytokines/inflammatory markers and Vitamin D in a community based cohort of adolescent twins. The heritability estimate for Vitamin D was high whereas the two cytokines that could be measured in plasma showed moderate heritabilities. As presented in the published literature, we found measurement of cytokines at the levels circulating in healthy adolescents to be difficult, perhaps limiting their utility. Further research (which takes into account the limitations of our present study) is warranted to explore the potential uses of these biomarkers, in particular in adolescents with mental health disorders.

Conflict of interest

None

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

PART 2: Evidence of genetic variance in putative biomarkers for depression in a cohort of healthy adolescent twins

Abstract

Introduction: Part one of this chapter reported on two cytokines measured in plasma and vitamin D measured in dried blood spots (DBS) in a pilot study of healthy adolescent twins. Other cytokines were measured (in both plasma and DBS) in the same samples of twins, as were other potential biomarkers (measured in DBS) that may be associated with MDD, including neurotrophins, gliadin antibodies, and antibodies to infectious agents. Here I report on the analysis of these data.

Methodology: Study participants are 16 year old twins (mean age 16.2 years, standard deviation 0.25 years) from the Brisbane Adolescent Twin Study. For cytokines, neurotrophins, and antibodies measured in DBS, there were 87 monozygotic (MZ) twin pairs and 126 dizygotic (DZ) twin pairs. Of these twin pairs, 65 MZ twin pairs and 81 DZ twin pairs (total of 146 twin pairs) had plasma measures of cytokines and inflammatory markers (reported in part one of this chapter), and 128 individuals were participants in the Brisbane Systems Genetics Study.

Results: All cytokines, neurotrophins, and antibodies measured in DBS suffered from batch effects and from confounding of plate and twin pair. Common and unique environmental factors were not able to be estimated. Cytokines and neurotrophins measured through gene expression showed large unique environmental effects.

Conclusions: The study highlighted important limitations to consider when further exploring the potential use of these biomarkers in adolescent mental illness. Due to these limitations, variance component estimations of the data were not reliable, and hence heritability estimates are not able to be reported.

Introduction

Identifying potential blood biomarkers of Major Depressive Disorder (MDD) is a direction worthy of further research (18). Reasons for this include the potential to assist in

diagnosis, and to improve treatment by re-classifying illness (19). As discussed in part one of this chapter, a range of blood measures may be useful as biomarkers of illness not only in MDD, but also in other mental illnesses such as psychosis. Potential blood biomarkers include cytokines, neurotrophins, antibodies to infectious agents, and Vitamin D (13, 232, 271, 272). A preliminary question is how putative biomarkers vary in a healthy population of adolescents. Since genetic factors are important in MDD (heritability 0.37) (234) and particularly adolescent onset MDD, the contribution of genetic factors to natural variation in biomarkers is of interest.

Evidence that MDD may be associated with immune system dysregulation has been provided for both adults (1, 6) and adolescents (28, 53), although there are considerably fewer studies on the immune system in adolescent MDD (161, 164, 273). It is possible that natural variation between individuals in levels of circulating cytokines or other inflammatory markers, even in the absence of specific immune challenge (such as infection), is associated with depression. Evidence for this comes from a recent meta-analysis that reported an association between elevated levels of two pro-inflammatory cytokines, Interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), and MDD in adults (6).

Neurotrophins regulate differentiation and survival of neurons, neurotransmitter release and synaptic plasticity (274). Examples of neurotrophins include brain-derived neurotrophic factor (BDNF),Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4) (275). Plasma and serum BDNF levels have been found to be reduced in adults with MDD (272). In adolescents with MDD, one study investigated BDNF, and found lower levels of BDNF in those with MDD (276). With regard to the role of NT-3 and NT-4 in MDD, one study in adults investigated expression levels of NT-3 mRNA and NT-4 mRNA in peripheral blood. The study found reduced expression of NT-3 mRNA in those with a current episode of MDD, but did not find a significant difference in expression levels of NT-4 mRNA (277).

Exposure to infectious agents during childhood and adolescence may influence the development of mental illness in adult life (278). A recent systematic review and meta-analysis found significant associations between schizophrenia and the viruses Human Herpes Virus 2, Borna Disease Virus, and Human Endogenous Retrovirus W (HERV-W) (232). Among the bacteria and parasites examined in this systematic review and meta-analysis, there was a statistically significant association between schizophrenia and

infection by *Toxoplasma gondii, Chlamydophila pneumonia,* and *Chlamydophila psittaci* (232). Prenatal depression and anxiety have also been associated with latent toxoplasmosis (10, 279). The associated immune response following exposure to infectious agents such as *T. gondii* has been postulated to be a factor in the development of mental illnesses such as schizophrenia (280). For example, astrocyte activation during toxoplasma infections increases brain kynurenic acid production (281, 282). Furthermore, it has been hypothesized that dopamine is increased by activated pro-inflammatory cytokines (such as IL-2) (283) as a consequence of toxoplasma infection (284). With regard to MDD, activated pro-inflammatory cytokines are part of the body's immune response to infection (59).

The body's immune response to non-infectious agents has also been hypothesized as a possible link with mental disorders such as MDD and schizophrenia. For example, the presence of antibodies to gliadin signifies an immune response to glutens in dietary cereal grains, and usually indicates celiac disease or wheat allergy (285). Celiac disease has been associated with a subsequent risk of MDD, and MDD has been associated with a subsequent diagnosis of celiac disease (11). With regard to schizophrenia, an increased risk has been reported in patients with celiac disease (286). An association has been observed between gliadin antibodies and schizophrenia (287), and between high levels of maternal gliadin antibodies and the subsequent development of non-affective psychosis in offspring (288).

Previous heritability estimates for cytokines have sometimes been estimated on stimulated cells, reflecting the heritability of the immune response (241) rather than natural variation. A twin study estimating heritabilities of circulating levels of the cytokines IL-1β, IL-6, IL-10 and tumour necrosis factor-α (TNF-α) found heritabilities to be moderate for IL-1β (range 0.27 – 0.32) and IL-10 (0.30), and low for IL-6 (range 0.15 – 0.16) and TNF-α (range 0.17 – 0.23) (80). Heritability estimates for antibodies to infectious agents have shown a strong influence of shared environment, presumably from exposures shared by twins living in the same family. However, genetic factors have also been found to have a strong influence on antibody levels for some pathogens (289).

The objectives of this study are i) to measure cytokines / inflammatory markers, neurotrophins, antibodies to common infections, and gliadin antibodies in a community

based population of adolescent twins and ii) to determine genetic contribution to the variation of these biomarkers in a cohort of healthy adolescent twins.

Methods

Samples:

Participants are 16 year old twins (mean 16.2, standard deviation (SD) 0.25 years) from the Brisbane Adolescent Twin Study. Blood was collected either between the years of 1997 – 2000 (blood collected during these years was stored on Whatman 903 filter paper), or between the years of 2006 – 2009 (blood collected during these years was stored on Whatman FTA® cards impregnated with antibacterial agents to preserve DNA). For dried blood spot (DBS) cytokine, other inflammatory markers, neurotrophin, and antibody measures, the sex distribution of the sample was 49.77% male and 50.23% female, with 87 monozygotic (MZ) twin pairs and 126 dizygotic (DZ) twin pairs. Of these twin pairs, 65 MZ twin pairs and 81 DZ twin pairs (total of 146 twin pairs) previously had cytokines measured in plasma (two of which were reported in part one of this chapter). Of the twins who had cytokines and other inflammatory markers measured in DBS, 128 individuals are also participants in the Brisbane Systems Genetics Study (290). The study was approved by the Human Research and Ethics Committee of the QIMR Berghofer Medical Research Institute.

Assay of cytokines / inflammatory markers, neurotrophins and antibodies

The cytokines measured in plasma (not reported on in part one of this chapter) were measured at the James Cook University, Townsville, Australia. The cytokines were measured in a multiplex assay, where they were grouped based on compatibility with other cytokines as follows: Multiplex 1: Interleukin-2 (IL-2), IL-4, IL-7, IL-11, IL-12, IL-13, TNF-α, IL-17A, IL-21, Exotaxin, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF). Multiplex 2: IL-1α, IL-1β, IL-3, IL-5, IL-6, IL-8, IL-9, IL-10, Interferon-γ-inducible protein 10 (IP-10), Monocyte Chemo-attractant protein-1 (MCP-1), Macrophage inflammatory protein-1a (Mip-1a), Interferon-γ (IFN-γ). The preparation of plasma samples, generation of standard curves, and intra-assay experiment (to evaluate accuracy of the method) are

described in part one of this chapter. The experimental design for the plasma samples ensured that co-twins (two members of a twin pair) were randomised across plates.

A multiplex immunoassay (25-plex xMAP assay for analysis of DBS) was used to measure cytokines and other inflammatory markers in DBS (243). Measurement was completed by the Statens Serum Institut, Copenhagen, Denmark. Cytokines, other inflammatory markers, and neurotrophins measured in DBS were: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, Interleukin receptor antagonist of IL-6 (IL-6ra), TNF-α, TNF-β, transforming growth factor-β (TGF-β), IFN-γ, Regulated and Normal T-cell Expressed and Secreted (RANTES), MCP-1, NT-4, BDNF, C-reactive protein (CRP), Macrophage inflammatory protein-1a (Mip-1a), Matrix Metallopeptidase-9 (MMP-9), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), Triggering Receptor Expressed on Myeloid cells-1 (TREM-1).

Antibodies were measured in DBS, using enzyme immunoassays (ELISA) at the John Hopkins University (258, 291). Antibodies to the following infectious agents were measured: Toxoplasma (Toxo), Cytomegalovirus (CMV), Herpes Simplex Virus 1 (HSV1), Herpes Simplex Virus 2 (HSV2), and Human Herpes Virus 6 (HHV6). In addition, gliadin antibodies were measured.

Preliminary statistical analysis

Preliminary analyses of the biomarkers assayed from DBS identified biomarkers that had failed or generated no or very limited variation between individuals. These biomarkers were excluded from analyses and included MMP-9 and IL-2. Biomarker measures from DBS were sensitive to paper type, often generating null reads for one or other paper, and were subject to plate effects as twin pairs were plated in adjacent wells on the same plate. Specifically, only measures from paper type 2 for IL-12, IL-18, IL-6ra, BDNF, CRP, HSV1, HHV6, CMV, and toxoplasma were variable between individuals. Therefore, measures from paper type 1 of these corresponding biomarkers were excluded from analyses. Paper type influenced both mean and variance of measures and so analyses were conducted on winsorised residuals after regression of within-paper rank normalised measures on sex and plate (Figure 3-1, Figure 3-2).

Estimation of Genetic Parameters:

As described in part one of this chapter, a saturated model was fitted to estimate MZ and DZ twin correlations for all cytokines, other inflammatory markers, neurotrophins, and antibodies. Within the saturated model, differences in means and variances between first and second born twins and between zygosity, sex and body mass index (BMI) were tested using likelihood ratio tests. The univariate variance component models described in part one of this chapter were used to investigate the relative contributions of genetic and environmental factors to the observed variation in the cytokines, other inflammatory markers, neurotrophins, and antibodies. Twin correlations and variance components were estimated using the statistical software package Mx (262).

For those individuals who are participants in both the Brisbane Adolescent Twin Study and stage II of the Brisbane Systems Genetics Study (BSGS), the correlation of cytokines measured in DBS with those measured by gene expression was calculated. BSGS is a family based study, which provides genome-wide expression and genotype data for 962 extensively phenotyped individuals (290). In stage II of BSGS, transcript expression levels in whole blood for 862 individuals were measured with over 47,000 genome-wide probes using the Illumina HT-12 v4.0 microarray chip (290).

Results

For the cytokines measured from plasma in a multiplex, the assay was not sufficiently sensitive to the low levels of circulating cytokines in healthy adolescents. This was despite the technical controls having been measured accurately (the intra-assay experiment). Therefore, no cytokines measured from plasma in either multiplex generated meaningful results.

Of the biomarkers measured in DBS, MMP-9 and IL-2 failed the assay. For MMP-9, the same value was obtained for every study participant. IL-2 was a tri-modal distribution, which could not be transformed to a more normalised distribution.

Figure 3-1 and Figure 3-2 show the distribution of the cytokines TNF-α, TNF-β, MCP-1, TGF-β, Mip-1a, and the inflammatory marker TREM-1. The diagonal shows the distribution of each of these cytokines and TREM-1. The off-diagonals contain all the pairwise scatter plots of the cytokines and TREM-1, with each row and column defining a single scatter

plot. Each single scatter plot shows the correlations between a pair of cytokines (or between a cytokine and TREM-1). So in Figures 3-1 and 3-2, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokines TGF-β and Mip-1a. Owing to the skewed distributions (Figure 3-1), measures were rank-normalised within paper, and then regressed on sex and plate (Figure 3-2). The residuals that were more than 3 standard deviations from the norm were winsorised, with the resulting distribution shown in Figure 3-2. Analyses were conducted on residuals after the regression (on sex and plate). The distribution of other cytokines, inflammatory markers, and biomarkers are presented in the Appendix section of the thesis (pages 178-185).

Figure 3-1: Distribution and correlations of TNF-α, TNF-β, MCP-1, TGF-β, Mip-1a, and TREM-1 (measured in DBS)
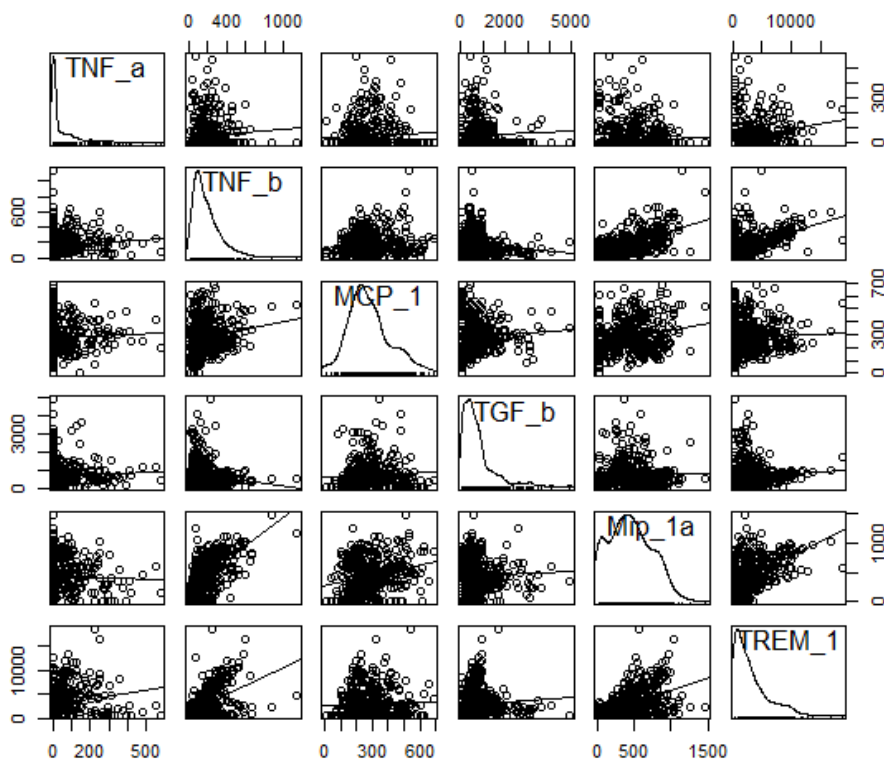
Key: MCP-1=Monocyte Chemo-attractant protein-1; Mip-1a=Macrophage inflammatory protein-1a; TGF-b=transforming growth factor-β; TNF=tumour necrosis factor; TREM-1=Triggering Receptor Expressed on Myeloid cells-1

Note: diagonal shows distribution of each cytokine and the inflammatory marker TREM-1; off-diagonals show correlations between a pair of cytokines (or between a cytokine and TREM-1, for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokines TGF-β and Mip-1a).

Figure 3-2: Distribution and correlations of TNF-α, TNF-β, MCP-1, TGF-β, Mip-1a, and TREM-1 after regression of within-paper rank normalised measures on sex and plate (measured in DBS)

Key: MCP-1=Monocyte Chemo-attractant protein-1; Mip-1a=Macrophage inflammatory protein-1a; TGF-b=transforming growth factor-β; TNF=tumour necrosis factor; TREM-1=Triggering Receptor Expressed on Myeloid cells-1

Note: diagonal shows distribution of each cytokine and the inflammatory marker TREM-1; off-diagonals show correlations between a pair of cytokines (or between a cytokine and TREM-1, for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokines TGF-β and Mip-1a).

Figure 3-3 represents the phenotypic correlations of the transformed cytokines, other inflammatory markers, neurotrophins and antibodies in DBS. Several pro-inflammatory cytokines and other inflammatory markers correlated highly with each other. IL-6 had high correlations with IL-17 (0.72) and TREM-1 (0.72). GM-CSF correlated highly with TNF-β (0.77), and BDNF correlated highly with TGF-β (0.74). The anti-inflammatory cytokine IL-10 had negative or very low correlations with pro-inflammatory cytokines, with correlations

of -0.15 with IL-18, and 0.07 with both TNF-α and CRP. Correlations of Vitamin D with the cytokines and inflammatory markers are not represented in Figure 3-3, as Vitamin D is discussed in part one of this chapter. However a negative correlation between Vitamin D and IL-18 (-0.14) was not significant (p=0.054).

Figure 3-3: Phenotypic correlations of transformed cytokines, other inflammatory markers, neurotrophins, and antibodies
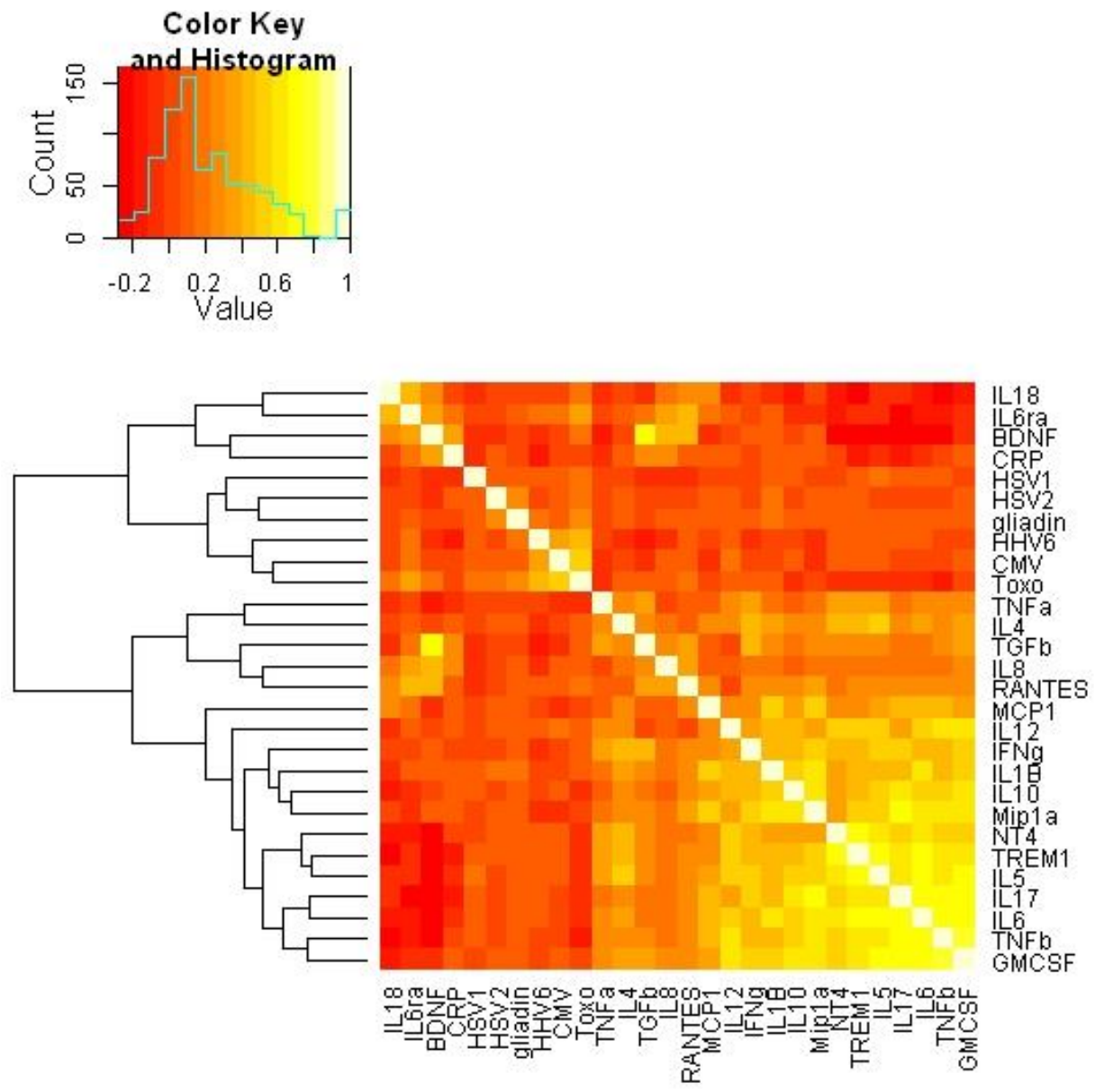
Key: BDNF=brain-derived neurotrophic factor; CMV=cytomegalovirus; CRP=C-reactive protein; GMCSF=Granulocyte Macrophage-Colony Stimulating Factor; HHV6=Human Herpes Virus 6; HSV=Herpes Simplex Virus; IFNg=Interferon-γ; IL=interleukin; MCP1=Monocyte Chemo-attractant protein-1;

Mip1a=Macrophage inflammatory protein-1a; NT4=neurotrophin-4; RANTES= Regulated and Normal T-cell Expressed and Secreted; TGFb=transforming growth factor-β; TNF=tumour necrosis factor; Toxo=toxoplasmosis; TREM1=Triggering Receptor Expressed on Myeloid cells-1

The study design, which saw co-twins of the same pair plated in neighbouring wells of the same plate, had been established for another study (where interest was differences between co-twins). This meant that plate effects were common to the twin pair, and therefore partitioned into the common environmental component. Re-plating was not practical due to the static between blood spots. Despite these concerns about batch effects that could not be removed by statistical methods because of confounding, we continued on to conduct the variance component analysis. As will become clear, the conclusion of the analysis was that the batch effects compromised the results and extreme caution is needed in interpreting the results. For completeness, the analyses conducted that led to these conclusions are detailed below.

In the model assumption testing prior to the estimation of variance components, for the cytokine RANTES there was a significant difference in means between males and females, with females having higher levels than males (p=0.04). For the antibody HSV-2, a birth order effect was observed in means (p=0.006, with first born twins having on average lower levels of HSV-2). Means were able to be constrained to equal for all other cytokines, inflammatory markers, neurotrophins, and antibodies. For IL-8 and BDNF a birth order effect was observed in variances (p=0.014 and p=0.036 respectively), with first born twins having on average larger variance. Variances were able to be constrained to equal for all other cytokines and biomarkers. These effects of birth order are likely to be a result of the sampling variance.

For the majority of the DBS measures both MZ and DZ correlations were of similar magnitude, and the DZ correlation was significantly higher than the MZ correlation for IL-17 (Table 3-3). Hence the estimates of the common environmental variance effects for these biomarkers were large (Table 3-4). These results reflect technical confounding factors (in particular plating of co-twins of the same pair in neighbouring wells), which mean the variance component estimations are not reliable.

Table 3-3: Twin Correlations (with 95% Confidence Intervals in brackets)

		MZ	DZ	MZF	MZM	DZF	DZM	DZO
A	GM-CSF	0.47 (0.29,0.60)	0.65 (0.54, 0.73)	0.50 (0.30, 0.65)	0.39 (0.04, 0.61)	0.75 (0.59, 0.84)	0.61 (0.39,0.75)	0.59 (0.38,0.73)
A	TNF-β	0.50 (0.33,0.63)	0.49 (0.35, 0.61)	0.50 (0.28, 0.65)	0.51 (0.19, 0.69)	0.53 (0.28, 0.70)	0.54 (0.26,0.71)	0.43 (0.20,0.60)
A	IL-6	0.39 (0.20,0.54)	0.53 (0.40,0.64)	0.44 (0.17,0.62)	0.34 (0.06,0.55)	0.64 (0.44, 0.77)	0.60 (0.37,0.74)	0.35 (0.05,0.57)
A	IL-17	0.31 (0.11,0.47)	0.61 (0.49, 0.70)	0.37 (0.10,0.57)	0.23 (-0.07, 0.48)	0.65 (0.44, 0.77)	0.62 (0.39,0.75)	0.58 (0.37,0.71)
A	IL-5	0.53 (0.35,0.66)	0.53 (0.40,0.64)	0.62 (0.42, 0.75)	0.36 (-0.06, 0.61)	0.57 (0.36, 0.71)	0.56 (0.33,0.71)	0.46 (0.20,0.63)
A	TREM-1	0.54 (0.38,0.66)	0.51 (0.37, 0.63)	0.55 (0.34, 0.69)	0.51 (0.26, 0.68)	0.58 (0.36, 0.73)	0.51 (0.25,0.68)	0.52 (0.24,0.69)
A	NT-4	0.47 (0.29,0.61)	0.51 (0.38, 0.62)	0.61 (0.40, 0.74)	0.30 (-0.02, 0.54)	0.48 (0.24, 0.65)	0.40 (0.14,0.59)	0.69 (0.50,0.80)
A	Mip-1a	0.48 (0.29,0.62)	0.54 (0.41, 0.64)	0.50 (0.24, 0.67)	0.45 (0.16, 0.64)	0.71 (0.52, 0.82)	0.48 (0.26,0.64)	0.47 (0.23,0.64)
A	IL-10	0.24 (0.04,0.42)	0.38 (0.22, 0.52)	0.35 (0.06,0.56)	0.15 (-0.14, 0.39)	0.38 (-0.01, 0.61)	0.34 (0.06,0.55)	0.42 (0.17,0.60)
A	IL-1β	0.49 (0.33,0.62)	0.58 (0.46,0.68)	0.52 (0.30,0.68)	0.46 (0.18, 0.64)	0.52 (0.27, 0.68)	0.68 (0.48,0.79)	0.56 (0.33,0.71)
A	IFN-γ	0.25 (0.03,0.44)	0.44 (0.29, 0.56)	0.42 (0.13, 0.62)	0.10 (-0.20, 0.37)	0.62 (0.37, 0.76)	0.38 (0.15,0.56)	0.39 (0.11,0.59)
B	IL-12	0.49 (0.24,0.66)	0.42 (0.18, 0.61)	0.50 (0.18,0.70)	0.46 (0.02, 0.71)	0.65 (0.31, 0.82)	0.17 (-0.40,0.58)	0.38 (-0.02,0.64)
A	MCP-1	0.56 (0.40,0.67)	0.59 (0.47, 0.69)	0.47 (0.23, 0.64)	0.68 (0.48, 0.79)	0.74 (0.57, 0.84)	0.67 (0.47,0.79)	0.45 (0.22,0.62)
A	RANTES	0.68 (0.56,0.77)	0.57 (0.43, 0.67)	0.81 (0.70,0.88)	0.56 (0.32, 0.71)	0.53 (0.26, 0.70)	0.68 (0.48,0.79)	0.51 (0.25,0.68)
A	IL-8	0.80 (0.72,0.86)	0.70 (0.60, 0.77)	0.84 (0.75,0.90)	0.74 (0.59, 0.83)	0.83 (0.73, 0.89)	0.59 (0.25,0.75)	0.63 (0.41,0.76)
A	TGF-β	0.65 (0.53,0.74)	0.66 (0.55, 0.74)	0.69 (0.53,0.79)	0.60 (0.38, 0.74)	0.74 (0.57, 0.84)	0.64 (0.42,0.77)	0.62 (0.41,0.74)
A	IL-4	0.34 (0.12,0.51)	0.17 (0.00,0.32)	0.34 (0.06,0.55)	0.34 (-0.03,0.58)	0.08 (-0.19, 0.35)	0.13 (-0.12,0.35)	0.41 (0.06,0.62)
A	TNF-α	0.23 (0.03,0.41)	0.43 (0.27,0.55)	0.20 (-0.06,0.43)	0.27 (-0.04, 0.51)	0.47 (0.19, 0.65)	0.50 (0.24,0.68)	0.30 (-0.02,0.54)
B	Toxo	0.37 (0.09,0.58)	0.36 (0.11, 0.55)	0.60 (0.26,0.78)	0.16 (-0.23, 0.49)	0.19 (-0.29, 0.55)	0.36 (-0.03,0.62)	0.53 (0.11,0.75)
B	CMV	0.68 (0.49,0.80)	0.64 (0.47, 0.75)	0.77 (0.54, 0.88)	0.59 (0.27, 0.77)	0.60 (0.22, 0.79)	0.50 (0.08,0.72)	0.78 (0.59,0.88)
B	HHV6	0.32 (0.05,0.53)	0.26 (-0.02,0.48)	0.78 (0.56, 0.88)	0.04 (-0.28, 0.35)	0.36 (-0.45, 0.71)	0.27 (-0.09,0.54)	0.19 (-0.29,0.56)
A	Gliadin	0.15 (-0.07,0.34)	0.21 (0.04, 0.36)	0.34 (-0.01,0.57)	0.05 (-0.21, 0.30)	0.18 (-0.17, 0.46)	0.39 (0.01,0.62)	0.16 (-0.07,0.37)
A	HSV2	0.32 (0.13,0.48)	0.43 (0.27, 0.56)	0.32 (0.09, 0.51)	0.33 (-0.02, 0.56)	0.32 (0.00, 0.55)	0.51 (0.18,0.70)	0.46 (0.23,0.63)
B	HSV1	0.37 (0.09,0.58)	0.51 (0.29, 0.66)	0.71 (0.43, 0.84)	0.12 (-0.25, 0.45)	0.56 (0.12, 0.78)	0.54 (0.20,0.74)	0.38 (-0.14,0.69)
B	CRP	0.53 (0.25,0.70)	0.41 (0.19, 0.58)	0.67 (0.35, 0.82)	0.38 (-0.09, 0.66)	0.56 (0.02, 0.78)	0.43 (0.05,0.67)	0.35 (0.02,0.59)
B	BDNF	0.56 (0.32,0.71)	0.52 (0.30, 0.67)	0.68 (0.42, 0.82)	0.35 (-0.19, 0.66)	0.64 (0.26, 0.81)	0.45 (-0.02,0.70)	0.47 (0.11,0.70)
B	IL-6ra	0.46 (0.21,0.64)	0.57 (0.36, 0.71)	0.65 (0.35, 0.80)	0.30 (-0.07, 0.57)	0.67 (0.23, 0.84)	0.51 (0.17,0.72)	0.57 (0.15,0.77)
B	IL-18	0.45 (0.19,0.64)	0.62 (0.43,0.75)	0.69 (0.42, 0.83)	0.18 (-0.24, 0.52)	0.55 (-0.06, 0.78)	0.54 (0.24,0.73)	0.78 (0.54,0.88)

Key: MZ = monozygotic twin pairs; DZ = dizygotic twin pairs; MZF = monozygotic female twin pairs; MZM = monozygotic male twin pairs; DZF = dizygotic female twin pairs; DZM = dizygotic male twin pairs; DZO =

opposite sex dizygotic twin pairs; BDNF=brain-derived neurotrophic factor; CMV=cytomegalovirus; CRP=C-reactive protein; GM-CSF=Granulocyte Macrophage-Colony Stimulating Factor; HHV6=Human Herpes Virus 6; HSV=Herpes Simplex Virus; IFN-γ=Interferon-γ; IL=interleukin; MCP-1=Monocyte Chemo-attractant protein-1; Mip-1a=Macrophage inflammatory protein-1a; NT-4=neurotrophin-4; RANTES= Regulated and Normal T-cell Expressed and Secreted; TGF-β=transforming growth factor-β; TNF=tumour necrosis factor; Toxo=toxoplasmosis; TREM-1=Triggering Receptor Expressed on Myeloid cells-1

Notes: A: Number twin pairs = 87 MZ, 126 DZ; **B:** Number twin pairs = 41 MZ, 61 DZ

Table 3-4: Estimates of the proportion of variance attributable to additive genetic effects (A) or heritabilities, common environmental effects of families (C) and unique environmental effects (E) (Univariate model), with 95% Confidence Intervals in brackets:

		A	**C**	**E**
A	**GM-CSF**	0.00 (0.00,0.12)	0.57 (0.44,0.65)	0.43 (0.35,0.53)
A	**TNF-β**	0.02 (0.00,0.39)	0.48 (0.18,0.59)	0.50 (0.37,0.62)
A	**IL-6**	0.00 (0.00, 0.19)	0.47 (0.30, 0.57)	0.53 (0.43,0.64)
A	**IL-17**	0.00 (0.00, 0.11)	0.48 (0.36, 0.58)	0.52 (0.42,0.63)
A	**IL-5**	0.01 (0.00,0.35)	0.52 (0.24,0.62)	0.47 (0.34,0.58)
A	**TREM-1**	0.00 (0.00,0.35)	0.53 (0.24,0.62)	0.47 (0.35,0.57)
A	**NT-4**	0.00 (0.00,0.31)	0.49 (0.23,0.59)	0.51 (0.39,0.62)
A	**Mip-1a**	0.00 (0.00,0.26)	0.51 (0.30,0.61)	0.49 (0.38,0.59)
A	**IL-10**	0.00 (0.00,0.27)	0.32 (0.09,0.43)	0.68 (0.56,0.81)
A	**IL-1β**	0.00 (0.00, 0.20)	0.54 (0.36,0.63)	0.46 (0.37, 0.56)
A	**IFN-γ**	0.00 (0.00,0.22)	0.37 (0.17,0.48)	0.63 (0.52,0.76)
B	**IL-12**	0.12 (0.00,0.63)	0.36 (0.00,0.59)	0.52 (0.34,0.71)
A	**MCP-1**	0.00 (0.00,0.26)	0.58 (0.36,0.66)	0.42 (0.32,0.52)
A	**RANTES**	0.23 (0.00,0.53)	0.45 (0.18,0.67)	0.32 (0.23,0.44)
A	**IL-8**	0.19 (0.00,0.39)	0.61 (0.41,0.76)	0.21 (0.15,0.29)
A	**TGF-β**	0.00 (0.00,0.26)	0.65 (0.43,0.73)	0.35 (0.25,0.43)
A	**IL-4**	0.34 (0.00,0.50)	0.00 (0.00,0.31)	0.66 (0.51, 0.87)
A	**TNF-α**	0.00 (0.00,0.21)	0.34 (0.15,0.45)	0.66 (0.55,0.79)
B	**Toxo**	0.00 (0.00,0.53)	0.36 (0.00, 0.52)	0.64 (0.44, 0.82)
B	**CMV**	0.10 (0.00, 0.49)	0.58 (0.22, 0.74)	0.33 (0.21, 0.48)
B	**HHV6**	0.11 (0.00, 0.52)	0.20 (0.00, 0.45)	0.69 (0.48, 0.90)
A	**Gliadin**	0.00	0.18	0.82

		(0.00,0.34)	(0.00, 0.31)	(0.65, 0.95)
A	HSV2	0.00 (0.00, 0.25)	0.36 (0.14, 0.47)	0.64 (0.53, 0.76)
B	HSV1	0.00 (0.00,0.37)	0.44 (0.11, 0.59)	0.56 (0.41, 0.73)
B	CRP	0.20 (0.00,0.67)	0.31 (0.00,0.58)	0.49 (0.31,0.71)
B	BDNF	0.06 (0.00,0.57)	0.49 (0.05,0.66)	0.45 (0.29,0.62)
B	IL-6ra	0.00 (0.00, 0.34)	0.52 (0.20, 0.65)	0.48 (0.35, 0.64)
B	IL-18	0.00 (0.00, 0.24)	0.54 (0.30,0.66)	0.46 (0.34,0.62)

Key: A=additive genetic variance; **C**=common environmental variance; **E**=unique environmental variance; BDNF=brain-derived neurotrophic factor; CMV=cytomegalovirus; CRP=C-reactive protein; GM-CSF=Granulocyte Macrophage-Colony Stimulating Factor; HHV6=Human Herpes Virus 6; HSV=Herpes Simplex Virus; IFN-γ=Interferon-γ; IL=interleukin; MCP-1=Monocyte Chemo-attractant protein-1; Mip-1a=Macrophage inflammatory protein-1a; NT-4=neurotrophin-4; RANTES= Regulated and Normal T-cell Expressed and Secreted; TGF-β=transforming growth factor-β; TNF=tumour necrosis factor; Toxo=toxoplasmosis; TREM-1=Triggering Receptor Expressed on Myeloid cells-1

Notes: A: Number twin pairs = 87 MZ, 126 DZ; **B:** Number twin pairs = 41 MZ, 61 DZ

Estimates of heritabilities of the cytokines RANTES and the inflammatory marker TREM-1 when measured through gene expression were moderate (0.31 and 0.23 respectively, Table 3-5). Heritability estimates of the other cytokines and neurotrophins measured through gene expression were low (Table 3-5). The BSGS investigated heritability distribution of all gene expression probes, and showed that the majority of genetic variance is additive (292). Estimation of the common environmental variance effect of IFN-γ was very large (Table 3-5), with the remaining cytokines and neurotrophins measured through gene expression showing very large unique environmental effects.

Table 3-5: Estimates of proportion of variance attributable to additive genetic effects (or heritabilities), and common environmental effects (measured through gene expression); and correlation of cytokines and other biomarkers measured in DBS with those measured by gene expression

	A	C	r	N
TREM-1	0.23	0.05	-0.04	126
Mip-1a	0.13	0	0.08	85
IL-10	0.11	0	-0.04	128
IL-1β	0.17	0	0.03	128

IFN-γ	0	0.82	N/A	6
RANTES	0.31	0	-0.07	128
IL-4	0	0	-0.06	22
TNF-α	0.17	0.05	0.07	126
BDNF	0	0	-0.28	21
IL-18	0.06	0.1	0.08	126

Key: **A=**additive genetic variance; **C=**common environmental variance; **N** = number of individuals with cytokines and other biomarkers measured in both DBS and by gene expression; **r** = correlation between cytokines / other biomarkers measured in DBS and cytokines / other biomarkers measured by gene expression; BDNF=brain-derived neurotrophic factor; IFN=interferon; IL=interleukin; Mip-1a=Macrophage inflammatory protein-1a; RANTES= Regulated and Normal T-cell Expressed and Secreted; TNF=tumour necrosis factor; TREM-1=Triggering Receptor Expressed on Myeloid cells-1

Discussion

Important limitations in the measurement of these biomarkers were encountered in this study, to the extent that the variance component estimations from these data are not reliable. Hence, heritability estimates are not able to be reported.

For the cytokines, other inflammatory markers, neurotrophins, and antibodies measured in DBS, common environmental components were high. While high estimates of common environment may reflect factors such as a response to exposures common to family members such as infection, here we believe the low heritability estimates and high common environment estimates reflect deficiencies in the experimental design, detailed below.

This study has highlighted important technical problems with the measurement of circulating cytokines and other biomarkers in healthy adolescents. The DBS plating layout had been established for another study examining differences between twin pairs in blood markers, however this experimental design is not appropriate for partitioning of variance as twin pairs were plated in neighbouring wells of the same plate. Hence, batch effects are partitioned into the common environmental component, and interpretation of the twin correlations and variance components is unclear. The high estimates of common environmental components of variance for most biomarkers may reflect inflation of common environmental effects because of this experimental design or it may reflect an important common environmental contribution reflecting shared family environment in

which immune and bacterial challenges may be shared between family members. A further problem was the limited variation between individuals in circulating levels of some biomarkers (IL-12, IL-18, IL-6ra, BDNF, CRP, HSV1, HHV6, CMV, and toxoplasma), which led to these biomarkers from paper type 1 being excluded from further analyses.

There are advantages as well as disadvantages with measuring biomarkers in DBS. The multiplex assays for DBS allow measurement of the concentrations of several inflammatory markers simultaneously in small amounts of sample, and the use of internal assay markers may improve the analysis (243). However, the potential interactions in bead-based multiplex assays between multiple antibodies and cytokines can be a disadvantage. Specifically, these antibodies can then prevent binding of the capture ± detection antibody, and so produce a falsely low result (243). A further limitation of this method is that the extraction of analytes may be less complete from old DBS (257).

In view of the limitations of measuring biomarkers in a multiplex, an alternative method is high sensitivity Enzyme-linked immunosorbent assay (ELISA). ELISA is the most widely used analytical method for measuring circulating inflammatory markers, where the concentrations measured depend on the antibodies and curve-fits that are used for calibration curves (293, 294). Results of high sensitivity ELISA are highly quantitative (295). However, ELISA allows only a single cytokine to be measured at a time, often requires higher volumes of sample, and generally costs more (295). Further sources of uncertainty within the ELISA methodology include pipetting and plate variation (22).

Another study, which measured the cytokines IL-1β, IL-6, IL-10, and TNF-α in serum, found heritabilities of IL-1β and IL-10 to be moderate, but also found unique environmental effects to be important (80). Interestingly, the authors also found that heritability of some cytokines changed with age, with heritability of TNF-α increasing with age, and heritability of IL-1β decreasing with age (80). With regard to antibody levels, the low heritability estimates measured were in contrast to the observations of another study. Specifically, heritabilities for CMV and HHV-6 antibody levels were observed to be 0.39 and 0.28 respectively, with small common environmental contributions to the variance of levels of these particular antibodies (289).

Conclusion

This study has reported on DBS measurements of cytokines, other inflammatory markers, neurotrophins, antibodies to common infectious agents, and gliadin antibodies in a community based cohort of adolescent twins. Important limitations in the measurement of these biomarkers were encountered in the study, reflecting poor experimental design (confounding of plate and twin pair), batch and DBS paper effects. We concluded that the variance component estimation from these data were not reliable. These challenges and limitations need to be addressed in further research that explores the potential uses of these biomarkers in adolescents with mental health disorders.

Chapter review

The chapter addressed the aim of investigating the genetic variance of cytokines, other inflammatory markers, and biomarkers altered in inflammatory states in healthy adolescents. This chapter also explored the difficulties in measuring cytokines and other inflammatory markers. Those cytokines that were able to be measured in plasma generated meaningful results, and provided evidence of cytokines that could be measured (individually, using ELISA) in low concentrations. It was important to have established that cytokines could be measured prior to attempting to measure these in a clinical sample in the following chapter. However, most of the pilot data in part 2 of this chapter suffered from important limitations, making the variance component estimations from the data not reliable.

CHAPTER 4: Measuring cytokines in inpatients of an adolescent mental health unit: study design and lessons learned

This chapter describes a study undertaken in a clinical sample of adolescents. The design of the study, particular challenges encountered in implementing the study, and potential solutions to overcome these challenges (when planning future studies) are all discussed.

Introduction:

Mental disorders are highly prevalent in youth (296) however their assessment and management during this developmental period is complicated by the presence of considerable co-morbidity. Depression and anxiety disorders commonly co-occur or emerge sequentially (17) and psychotic symptoms frequently accompany these disorders (297). This phenotypic overlap challenges diagnosis and management (297, 298).

These challenges in diagnosis are also considered in the Research Domain Criteria (RDoC) project of the National Institute of Mental Health (NIMH) (299). RDoC uses dimensional constructs, which include symptoms and potential biomarkers of mental illness (300). RDoC is currently a framework for organising research (299), and by attempting to link symptoms of mental illness to underlying neurobiology, recognises that identifying potential biomarkers is a direction worthy of further research (18).

These potential biomarkers include cytokines, small proteins with immune modulating activity (75). Increasingly, cytokines have been associated with Major Depressive Disorder (MDD) in adults through immune system dysregulation, even in the absence of infection (1). A meta-analysis of 24 studies reported an association between elevated levels of 2 pro-inflammatory cytokines (tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6)) and MDD in adults (6). Emerging evidence suggests that circulating levels of blood cytokines may also be a useful biomarker of MDD in adolescents (20, 301), with immune system dysregulation also observed in adolescent MDD (161).

A systematic review identified 18 studies measuring cytokines or immune markers in adolescents with depression or depressive symptoms (20). A more recent systematic review of inflammatory markers in MDD in children and adolescents identified 27 (including 7 longitudinal) studies (301). Compared to studies of adults, some differences in the

immune response of adolescents with depression have been identified. For example, Brambilla et al. (2004) examined the immune function of children with a first episode of MDD unlikely to have been preceded by stressful events. Contrary to that seen in depressed adults, those children with MDD who had not experienced stressful life events had normal IL-1β levels (183). Furthermore, in contrast to adults with MDD, plasma TNF-α was noted to be significantly decreased in adolescents with MDD and suicidality (191).

At the time of commencing the study that forms the basis of this thesis chapter, only three studies (30, 31, 143) of cytokines in adolescent MDD had included measures of childhood adversity. The paucity of studies of cytokines in adolescents is an important issue for further investigation, as a coupling of depression and inflammation has been noted during this developmental phase (28, 30), with 'biological embedding' of stress already seen in adolescence (28, 29). Specifically, individuals with MDD or a history of MDD have been found to have higher levels of C-reactive protein (CRP) compared to those with no depression or maltreatment, those with a history of maltreatment and no MDD have been found to have even higher levels of CRP, and those with a history of maltreatment and MDD have been found to have the highest CRP levels (28, 29).

Circulating levels of cytokines can be measured from serum, plasma and dried blood spots (DBS). For measurement in plasma or serum, the most widely used analytical method is Enzyme-linked Immunosorbent Assay (ELISA), where the concentrations measured depend on factors such as the antibodies used for calibration curves (294). For measurement of circulating levels of some inflammatory markers, DBS are superior to plasma or serum if samples cannot be prepared and frozen quickly (256). Cytokines can also be measured through gene expression in peripheral blood cells (302). White blood cells have been proposed as a useful peripheral model to study inflammatory processes in the central nervous system (CNS) (302), especially for genes encoding stress mediators and cytokines (302, 303).

Many factors affect circulating levels of cytokines and / or gene expression of cytokines. Circulating levels of some cytokines and other inflammatory markers vary both throughout a 24 hour period, and a 7 day period (21, 169). Levels of circulating cytokines are also influenced by medications such as selective serotonin re-uptake inhibitors (SSRIs) (212) and infections such as influenza. Furthermore, measurement of cytokines is complex, with particular cytokines degrading or alternatively increasing if blood is not separated and

stored by the laboratory within several hours (256). In addition, the same pattern of change in the concentration of cytokines is not seen for all individuals, with production and / or release of antigens and the subsequent degradation of antigen affecting concentration of cytokines (243). Therefore, whilst cytokines are potential biomarkers for adolescent MDD, many challenges exist in the measurement of these fragile proteins.

As there are a paucity of studies examining cytokines in adolescents (not only in MDD, but also in anxiety disorders and psychosis), we aimed to measure the levels of circulating plasma cytokines in adolescents admitted to an Adolescent Inpatient Unit (AIU). We hypothesised that levels of circulating IL-6 would be higher in adolescents with MDD compared to a control group, whilst circulating levels of the pro-inflammatory cytokine TNF-α may be lower in adolescents with MDD (consistent with the findings of Gabbay et al. (191)). We also hypothesised that circulating levels of the inflammatory marker CRP would be higher in adolescents with MDD compared to a control group, and would be higher in adolescents with a history of early childhood adversity (irrespective of diagnosis) compared to a control group.

However methodological challenges were encountered in recruiting subjects and collecting samples from this population of adolescent inpatients. As such, this chapter describes the study methodology and some of the practical limitations in conducting this research in an Australian acute adolescent inpatient psychiatric unit. Furthermore, we aimed to provide suggestions for future researchers in order to avoid or overcome some of the challenges encountered.

Methods:

The study was approved by the Royal Children's Hospital Human Research Ethics Committee (RCH-HREC) and the QIMR Berghofer Medical Research Institute Human Research Ethics Committee (QIMRB-HREC). Patients were recruited from the Royal Brisbane and Women's Hospital (RBWH) Adolescent Inpatient Unit. Inclusion criteria for recruitment to the study were patients who had been admitted with a diagnosis (primary or co-morbid) made by a Child and Adolescent Psychiatrist, according to ICD 10 criteria of a depressive, anxiety or psychotic disorder between June 2012 and May 2013.

The Adolescent Inpatient Unit is a 12 bed psychiatric unit which services the state of Queensland, north of the Brisbane River accepting admissions for assessment and management of adolescents with acute and serious mental illness. Prior to commencing the study, meetings were held with clinical staff on the Adolescent Inpatient Unit (AIU), to discuss the aims and methodology of the project. Staff identified individuals who met inclusion criteria and had capacity to consent. Written consent was also obtained from their legal guardian or parent by associate investigators of the study, or AIU staff. Charts were reviewed to determine previous episodes of depression, anxiety or psychosis, family history of mental illness, concurrent medical illnesses, and prescribed medications. Height and weight data were also collected from charts (to allow calculation of body mass index (BMI)). A sample size of 50 patients was needed to detect a difference of 0.5 standard deviation units between the clinical group and the control group for circulating levels of cytokines and inflammatory markers (304). The significance threshold assumed in the power calculation (based on a two sample t-test) was 0.05 for a single test. However, this threshold would be smaller for multiple testing.

Blood sample:

Bloods were collected for measurement of cytokines, cytokine genes, and routine biochemistry (2x10ml EDTA tubes, 1x10ml serum tube, and 1x1ml PAX tube). With the exception of 1 study participant, all blood samples were collected in the morning. Where possible, the blood sample was collected at the same time as clinical samples for investigations (e.g., thyroid function tests) ordered by medical staff in order to minimise venepuncture of patients. To preserve the cytokines, samples were immediately transported to the laboratory of QIMRB located on the same hospital campus. On arrival, the EDTA tubes were placed and processed on ice, separating plasma and serum before freezing at -80 degrees Celsius (256).

Psychological measures:

Participants completed the Somatic and Psychological Health Report (SPHERE) (305), the Junior Eysenck Personality Questionnaire (JEPQ) (306), and the Child Post-traumatic stress disorder Symptom Scale (CPSS) (307). We combined these questionnaires and scales into a single questionnaire, which we named the "Adolescent Behaviour and Lifestyle Questionnaire".

The SPHERE, a 34-item questionnaire (305), measures symptoms of depression, anxiety, fatigue and somatic illness (308). The 'PSYCH-6' is the 6-item subscale of the SPHERE assessing psychological symptoms of depression and anxiety (308). For the 34 item SPHERE, each item is scored as "0", "1" or "2" to correspond with responses of "sometimes / never", "often", or "most of the time" respectively (305, 308). We used the same coding employed in previous research in adolescent and young adult twins (255), where "sometimes / never" is scored as "0", and "often" or "most of the time" are both scored as "1" (309). The SPHERE has been found to be reliable in identifying depression in young persons, where co-morbid physical disease has a low prevalence (308).

The JEPQ assesses the 3 major dimensions of personality, namely Psychoticism, Extraversion, and Neuroticism (310). A fourth dimension of the JEPQ (the Lie scale) acts as a measure of social desirability (310). A negative correlation has been found between Extraversion and depression, and a positive correlation between Neuroticism and depression (311). We used the 81-item questionnaire (full version), which includes 20 items on the Neuroticism scale, as short forms have less reliability (312). The maximum score possible for the Neuroticism scale of the JEPQ is 20, as each 'yes' answer is scored as 1. Maximum scores possible for the Extraversion, Psychoticism and Lie scales are 24, 17 and 20 respectively.

Both the SPHERE and the JEPQ have also been administered to study participants in the Brisbane Adolescent Twin Study (255), a community sample of twins, enabling comparison of age-matched controls. These control samples were comprised of adolescent and young adult twins aged 12 – 25 years (mean age 15.5 years (standard deviation (SD) 2.9 years), 1,168 complete twin pairs, 53% females) for the SPHERE (309), and 16 year old twins (525 males and 570 females) for the JEPQ (313).

The CPSS assesses Post-traumatic stress disorder (PTSD) symptoms based on the 3 symptom clusters of *DSM-IV* (re-experiencing, avoidance and arousal). Answers to each question are scored as "0" ("not at all"), "1" ("once a week or less"), "2" ("2 to 4 times a week"), or "3" ("5 or more times a week"), producing a total symptom severity score ranging from 0 to 51. Seven additional items assess functional impairment (307). We asked participants if they had experienced a major loss, a life threatening event, and / or interpersonal violence in the past. Those participants who endorsed any of these

questions were then given the CPSS. The CPSS has high internal consistency for both the total score and the three subscales / symptom clusters (307).

Statistical analyses:

Responses to the combined questionnaires were analysed using the computer programme R. Means and standard deviations of each of the SPHERE, JEPQ, and CPSS were calculated for the total sample, and for each of the primary or co-morbid diagnoses (MDD, anxiety disorder, psychotic disorder, and anorexia nervosa). As 80% of the clinical sample was female, all traits in the clinical sample and community samples were corrected for sex prior to comparing mean scores of the clinical and community samples. Correlations were calculated between the SPHERE, JEPQ, and CPSS (for the total sample, as numbers were too small to allow calculations within disorders).

Results:

During the study period, 220 individuals were admitted to the Adolescent Inpatient Unit. A total of 39 individuals (31 females, 8 males) participated in the study. Reasons for non-participation included brief admissions, refusal for venepuncture and challenges accessing parents to gain consent prior to a patient's discharge. The age of study participants ranged from 14 years 3 months to 17 years 6 months (mean 16.1 years; SD 1.2 years), with the majority of study participants attending school (92%) and living at home (95%). Thirty-four of the 39 study participants provided a blood sample for the measurement of circulating cytokine levels and cytokine genes, three requested to complete questionnaires only (because of fear of venepuncture), and in two participants we were unable to process the blood sample within the time-frame required to avoid changes in cytokine levels. Thirty-eight of the 39 study participants completed the Adolescent Behaviour and Lifestyle Questionnaire resulting in complete data for 33 patients.

Of the 39 participants, 13 had a primary diagnosis of an anxiety disorder, 11 met criteria for major depressive disorder and 4 were diagnosed as having a psychotic disorder. Eleven participants had a primary diagnosis of anorexia nervosa but had co-morbid anxiety and so were also included in the study.

To detect a difference of 0.5 standard deviation units in circulating levels of cytokines and inflammatory markers between the clinical sample and the community sample of twins, the

sample size required from the Adolescent Inpatient Unit was at least 50 individuals (304). Therefore due to the small sample size, as yet cytokines and inflammatory markers have not been measured in the blood samples.

Exposure to trauma and loss were common in study participants, with 29 of the 38 (76.3%) participants endorsing at least one event. Of the 29 adolescents who endorsed exposure to trauma or loss, 20 (71%) endorsed exposure to more than one event. Twenty-eight of the 29 participants exposed to any trauma or loss then completed the CPSS based on the most distressing event they had experienced. The most common distressing events were interpersonal violence (which included being bullied; n=25), major loss (e.g. death of a parent, parental divorce; n=17) and life-threatening events (n=10).

Table 4-1 shows the average SPHERE, JEPQ (Neuroticism, Extraversion, Psychoticism and Lie scales), and CPSS scores by primary diagnosis in the clinical sample, and the JEPQ scores from the community twin sample. Compared to the community sample, the clinical samples had significantly higher scores on the Neuroticism scale (10.7 versus 16.7 respectively, $p=1.8 \times 10^{-13}$) with the group with a primary diagnosis of psychosis scoring highest (mean 19, SD 0.8) (Table 4-1). Conversely, study participants had lower scores on the Extraversion scale of the JEPQ compared to the community sample (13.8 versus 19.4 respectively, $p=6.7 \times 10^{-8}$) (Table 4-1).

Table 4-1: Mean (SD) for SPHERE, JEPQ (Neuroticism, Extraversion, Psychoticism and Lie scales), and CPSS scores (by diagnosis):

	Clinical Sample					Community Twin Sample		
	Total Sample (n=38)	Anxiety Disorder (n=12)	MDD (n=11)	Psychotic Disorder (n=4)	AN (n=11)	Total (n=1095)	Males (n=525)	Females (n=570)
SPHERE	20.6(7.1)	21.2 (7.7)	20.2 (6.9)	20.3 (3.8)	20.5 (8.2)	n/a	n/a	n/a
JEPQ-N	16.7(3.3)	16 (3.9)	17.1 (2.5)	19 (0.8)	16.2 (3.8)	10.7(20.3)	8.7 (21.1)	11.2 (20.1)
JEPQ-E	13.8(5.7)	13.8 (6.9)	14.3 (4.3)	9.3 (5.9)	14.8 (5.3)	19.4(16.4)	19 (13.1)	19 (17.2)
JEPQ-P	3.5(2.7)	4.5 (3.7)	4.4 (2.2)	2 (0.8)	1.9 (1.5)	2.6(4.2)	3.9 (6.0)	2.2 (3.8)
JEPQ-L	8.1(3.7)	7.5 (4.3)	6.8 (3.3)	8.8 (3.9)	9.7 (3.0)	7.1(12.4)	6.2 (11.2)	7.3 (12.7)
CPSS	25.0(14.0)	25.8 (18.6) (n=10)	26.6 (9.5) (n=8)	36* (n=1)	21.6(12.6) (n=9)	n/a	n/a	n/a

Key: AN = Anorexia Nervosa; CPSS = Child PTSD (post-traumatic stress disorder) scale; JEPQ-E = JEPQ Extraversion scale; JEPQ-L = JEPQ Lie scale; JEPQ-N = JEPQ Neuroticism scale; JEPQ-P = JEPQ Psychoticism scale; MDD = Major Depressive Disorder; n = number of individuals; SPHERE = Somatic and Psychological Health Report; n/a = not available

*SD not applicable as only one individual completed the CPSS from this subgroup

Mean SPHERE scores were significantly higher in the clinical sample compared to the community sample of twins (20.6 versus 8.52 respectively, p=<4.4x10^{-16}). To examine these measures further, we correlated scores from the clinical sample between the SPHERE (including the PSYCH-6 subscale), the 4 scales of the JEPQ, and the CPSS. Results of correlations between the SPHERE, JEPQ, and CPSS are listed in Table 4-2.

Table 4-2: Correlations between SPHERE, JEPQ, and CPSS:

	SPHERE	JEPQ - N	JEPQ-E	JEPQ-P	JEPQ-L	CPSS
SPHERE	1.00	0.53	-0.04	0.34	-0.55	0.74
JEPQ - N	0.53	1.00	-0.45	-0.01	-0.36	0.56
JEPQ-E	-0.04	-0.45	1.00	0.08	0.16	-0.23
JEPQ-P	0.34	-0.01	0.08	1.00	-0.61	0.32
JEPQ-L	-0.55	-0.36	0.16	-0.61	1.00	-0.53
CPSS	0.74	0.56	-0.23	0.32	-0.53	1.00

Key: CPSS = Child PTSD (post-traumatic stress disorder) scale; JEPQ-E = JEPQ Extraversion scale; JEPQ-L = JEPQ Lie scale; JEPQ-N = JEPQ Neuroticism scale; JEPQ-P = JEPQ Psychoticism scale; SPHERE = Somatic and Psychological Health Report

The correlation between the measures was high for SPHERE and PSYCH-6 (0.75, as expected for a whole-part relationship) and moderate between SPHERE and JEPQ-N (0.53), JEPQ-N and CPSS (0.56), and PSYCH-6 and JEPQ-N (0.67). Hansell et al. found the correlation between the PSYCH-14 (the 14-item subscale of the SPHERE) and the JEPQ-N to be 0.64 in the community sample of twins (309). In the clinical sample, we also found that scores on the SPHERE correlated highly with scores on the CPSS (correlation 0.74).

Discussion:

This chapter describes the study methodology and the challenges that arose in attempting to collect samples that would enable measurement of circulating cytokines in a clinical sample of adolescents admitted to an inpatient unit. The study suffered a number of challenges worthy of discussion. These challenges can be broadly divided into patient factors, blood collection factors, and other data collection factors.

Patient factors include patient selection bias, the degree of morbidity / co-morbidity of patients, and the length of admission to the unit. The selection bias of patients participating in clinical studies is systemic. Adolescents who were due to have other blood tests as part of their clinical assessment were more likely to consent to participate in the study, as potential subjects were understandably reluctant to endure extra venepuncture for research purposes. Additionally, patients were not approached about the study until they were well enough to consent. Because of pressure to ensure patient flow through acute psychiatry units, patients were already being placed on leave or on the cusp of discharge at the time they had capacity to consent to participate. Time constraints prevented consent being obtained from those patients in crisis admitted for brief admissions as other clinical priorities such as detailed assessments, psychoeducation and family meetings were prioritised ahead of research. The net effect of these selection biases was that patients with illnesses requiring extended inpatient care (with multiple medical investigations) such as anorexia nervosa and treatment refractory psychotic and affective disorders were more likely to participate in this project.

The fragility of the cytokine proteins presented a challenge to blood collection. Rapid transport, processing and freezing of blood samples were necessary in order to avoid significant changes in cytokine levels. The study used the hospital pathology service to collect the samples as funding and staff were unavailable for the exclusive collection and strict adherence to such a rigorous protocol of blood collection as used in this study. The study was able to achieve the co-operation of the pathology service in a large tertiary hospital although frequent communication was required to ensure the protocol was maintained.

Other challenges arising from data collection factors included the identification of patients and the time required to obtain consent particularly from parents. Many of the adolescents admitted to an inpatient unit have considerable family distress (314). They are more likely to come from families who are socio economically disadvantaged and access to transport, affordable parking and demands of caring for other children limit the time many parents can spend visiting their child who is unwell. Thus, when they visit, gaining a clinical understanding or spending time with their child becomes the main focus of their visit and consenting to research is understandably not a priority. As such, our observation was that those parents whose children had more persistent or treatment refractory disorders were again more likely to consent to the research.

As a result of these challenges, the sample collected was disproportionately small and biased, especially when considering the effort that went into establishing the protocol and attempting to collect the data. Although inpatient units would appear to be an ideal place to collect data from patients, especially where biological samples are required, our experience has been that the high level of clinical activity, short lengths of stay and the severity and acuity of the patients clinical presentations present significant challenges to conducting such studies in these settings. Due to the small number of study participants, the follow-up component of the study which we had planned (consisting of repeat measurement of circulating cytokines and repeat psychological measures) was not conducted.

It should be noted that other groups conducting research in an Adolescent Inpatient Unit have encountered similar challenges. A study exploring strategies to enhance patient recruitment in research involving patients with first episode mental illness noted recruitment of research participants often represents the most labour intensive component of a study (315). Furthermore, the authors noted in their study examining the benefit of citalopram in adolescents with depression that 50% of eligible participants declined enrolling in the study due to concerns from parents or family members (315). A study examining the use of Clonidine for intrusive symptoms of PTSD among patients on a Paediatric Mental Health Inpatient Unit also noted the time required to identify study participants and obtain consent from parents to be a barrier to participant recruitment (314).

As a direct result of this experience, methodology employed in current studies has changed. At the Royal Brisbane and Women's Hospital Mental Health Service, assent is now routinely used when collecting plasma samples on admission from patients. Informed consent is obtained from parents over the telephone and then later obtained from participants when they have capacity. This is done close to discharge or over the telephone once the patient has left hospital. The Human Research and Ethics Committees have approved this process of consent, having understood (including through the reporting process to the HREC) the challenges experienced in this study. This was therefore a good outcome of the study.

The findings in the clinical sample of higher scores on the Neuroticism scale (JEPQ) and lower scores on the Extraversion scale (JEPQ) (compared to the community sample) were to be expected. Likewise, the finding of higher SPHERE scores across all diagnostic groups in the clinical sample (compared to the community sample) were expected, as the majority of patients in the clinical sample had been diagnosed with depression or anxiety (primary or co-morbid). The correlation of 0.64 reported between the SPHERE and JEPQ-N in the community sample of twins (309) was similar to the correlation of 0.53 between the SPHERE and JEPQ-N in the clinical sample.

We remain of the opinion that cytokines have an important role in the mental health of adolescents either as biomarkers or as causal factors due to immune dysregulation (161, 191). It is hoped that further study participants can be obtained at a later date, with a view to then measuring cytokines and other inflammatory markers. Future studies should use the RDoC approach rather than diagnostic categories. For example, study participants could be grouped on the basis of domains such as internalizing behaviours or immunological profile.

This research experience alerts others to the challenges of conducting research in adolescents where parental consent is generally required. An awareness of the research challenges by the clinical psychiatry community is important in order to develop solutions that will progress biological psychiatry research.

Acknowledgements:

Chapter review

This study led to changes in the consent process at the Royal Brisbane and Women's Hospital Mental Health Services. Specifically, blood samples for research are now able to be collected from patients and stored on admission when other blood samples for routine clinical measures are collected. The patients are then later approached when their mental health is stabilized to determine if they consent or refuse for these samples to be used for research. These changes have allowed for collection of samples prior to or just after very limited exposure to psychotropic medication and they minimize exposure to venepuncture. This has increased participation in clinical projects that have followed on from this study.

CHAPTER 5: Investigating the Relationship between Iron and Depression

Abstract

Introduction: Measures of circulating levels of iron have been associated with depression. Our objective was to investigate the phenotypic and genetic relationship between measures of circulating levels of iron (serum iron, serum transferrin, transferrin saturation, and serum ferritin) and depressive symptoms using three strategies based on different types of analyses of genetically informative data sets.

Methodology: Data were collected from the ongoing Brisbane Adolescent Twin Study and the Health and Lifestyle Survey for Twins, both based at QIMR Berghofer Medical Research Institute (QIMRB). Here, we report data from twin adolescents (mean age 15.1 years (standard deviation (SD) 3.2 years)), and adult twins (mean age 23.2 years SD 2.2 years)). In the adolescent cohort, there were 3,416 participants from 1,688 families. Depressive measures were assessed through the Somatic and Psychological Health Report (SPHERE). In the adult cohort, there were 9,035 participants from 4,533 families. A quantitative score of depression was measured by the Delusions Symptoms State Inventory (DSSI). First, we estimated heritabilities of, and phenotypic and genetic correlations between, traits. Second, we conducted analyses that linked results from published large-scale genome-wide association studies (including iron and Major Depressive Disorder (MDD)) with our in-house adolescent and adult samples for which we also had genome-wide genotype data. We explored the genetic relationship between the traits in our samples and single nucleotide polymorphisms (SNPs) reported as associated with iron measures and MDD from published genome-wide association studies. We undertook single SNP and multi-SNP genetic risk score analyses, and LD score regression analyses.

Results: Measures of iron, transferrin, transferrin saturation, and log 10 of ferritin (L10Fer) were all found to be highly heritable in both cohorts: Adolescents: iron 0.46 (95% CI 0.15-0.66), transferrin 0.64 (95% CI 0.42-0.81), transferrin saturation 0.61 (95% CI 0.39-0.70), and L10Fer 0.56 (95% CI 0.28-0.72); Adults: iron 0.35 (95% CI 0.25-0.41), transferrin 0.52 (95% CI 0.38-0.56), transferrin saturation 0.50 (95% CI 0.44-0.55), and L10Fer 0.42 (95%

CI 0.27-0.49). Heritabilities calculated for depressive measures were 0.46 (95% CI 0.29-0.52) and 0.30 (95% CI 0.11-0.40) for adolescents and adults respectively. In adolescents, depression measures were significantly higher in those in the middle 10th percentile versus top 10th percentile of transferrin saturation measures (p=0.002). Genetic profile risk scores (GPRS) of the iron measures did not predict depression measures, and MDD GPRS did not predict iron measures in study participants of this community sample. LD score analyses showed the expected negative correlations between iron and transferrin, log ferritin and transferrin, and between transferrin saturation and transferrin, but no significant genetic relationship between iron and depression.

Conclusions: Genetic factors strongly influence iron measures in adolescents and adults. Using several different strategies we find no evidence for a genetic contribution to the relationship between measures of iron in circulating blood and measures of depression.

Keywords: iron, transferrin, transferrin saturation, ferritin, depression

Introduction:

Measures of circulating iron have been associated with depression. In adults, an association between iron measures and depressive symptoms has been demonstrated, including improvement of depressive symptoms with iron supplementation for iron-deficiency anaemia in mothers with young infants (316). Lower levels of serum ferritin (a measure of body iron stores) have also been associated with depression in adults (317, 318) although an association between iron measures and depression is not always evident (319). By contrast, very few studies of adolescents have investigated the relationship between iron and depression.

Iron is an essential component of brain growth and development, and is needed for cell differentiation, protein synthesis, hormone production and important aspects of cellular energy metabolism (320). Iron deficiency is a major health problem worldwide, and remains the leading cause of anaemia globally (321). Iron deficiency is also a problem in Australia (322), with the World Health Organization estimating the prevalence of anaemia in industrialized countries ranging from 5%, to as high as 18% in pregnant women, with iron deficiency a major cause (323).

Major Depressive Disorder (MDD) is also a major health problem worldwide, with considerable morbidity and mortality (36, 324). Major Depressive Disorder (MDD) has also been associated with inflammation (74), and changes in iron measures, such as decreased serum transferrin levels, are also seen in inflammatory states (8). Therefore MDD may also be related to iron measures indirectly (325). In view of the health burdens associated with both iron deficiency anemia and MDD, and the association between the phenotypes of these disorders, the relationship between iron and depression is an area worthy of further investigation.

It is unknown if the reported phenotypic relationship between blood iron levels and depression has a genetic component. The variation between individuals in measures of iron in serum is partly under genetic control, with heritability estimated to be ~25%-50% (326-328). Furthermore, iron absorption, absorption-diet interactions, and variation in iron loss are all potentially subject to genetic influences (327). Genome-wide association studies (GWAS) of iron phenotypes have identified highly associated single nucleotide polymorphisms, including 3 independent SNPs in the gene *TF* (transferrin) (329), and more recently SNPs affecting ferritin (330). Variants in the gene *TF,* together with the *HFE* C282Y mutation, explain approximately 40% of genetic variation in serum transferrin (329). Such large effects are unusual for complex traits (331), but have been reported for other measures such as Vitamin D (332).

Likewise, genetic influences also contribute to individual differences in MDD. Heritabilities are estimated to be approximately 31%-42% for depression (234). Genetic influences in MDD are also found in adolescents (50). The era of genome-wide association studies provides a new experimental paradigm to explore the genetic relationship between traits using data sets independently collected for different measures (333).

The aim of this study is to: 1) investigate the phenotypic and genetic relationship between measures of circulating levels of iron and depressive symptoms in two large independent community cohorts of twins, and 2) investigate if SNPs that explain variation in iron phenotypes are associated with measures of depression.

Methods:

Cohorts:

1) Adolescent Cohort

Participants are 16 year old twins from the Brisbane Adolescent Twin Study (255). Participants completed the Somatic and Psychological Health Report (SPHERE) (334), a self-report questionnaire that includes 14 anxiety and depression items. Items were recorded as binary responses, coded as 0 (less anxiety) and 1 (more anxiety) which sum to provide a quantitative measure of anxiety and depression (309) giving greater power to detect genetic influence (335) than a binary diagnostic code. Mean age at completion of SPHERE was 15.1 years (standard deviation (SD) 3.2 years). The participants also provided a blood sample (mean age at time of blood collection 16.2 years (SD 0.2 years)). This allowed quantification of a number of iron phenotypes in the serum: iron (measured in µmol/L), transferrin (g/L), transferrin saturation (measured as a percentage of transferrin saturated with iron), and ferritin (µg/L). Transferrin is an iron-binding blood plasma glycoprotein that controls the level of free iron (336). Ferritin is an intracellular protein that stores iron and releases it in a controlled manner (336). A log transformation was applied to the ferritin (L10Fer) measures to normalise the distribution. The sample size comprised between 1,363 (49.6% males, 50.4% females) and 2,890 (45.89% males, 54.11% females) adolescents from 1,688 families, depending on the measure (see Table 5-2 for twin pair numbers).

2) Adult Cohort

Participants are adult twins, taken from the Australian Twin Registry (9,035 study participants). In 1989, a Health and Lifestyle Questionnaire (HLQ) was mailed to twins born between the years of 1964-1971. The mean age of respondents was 23.2 years (standard deviation (SD) 2.2 years), with 37.14% of respondents being male, and 62.86% female. The psychiatric symptom inventory section in the HLQ contained self-report questions, consisting of 14 anxiety and depression items from the DSSI (337), as well as a 19 item subset of the 90-item Symptom Checklist (SCL-90) (338). When these 33 items are factor analysed, 4 factors are derived: depression, anxiety, somatic distress, and sleep difficulties. Study participants provided a blood sample approximately 10 years after,

allowing quantification of serum iron (µmol/L), transferrin (g/L), transferrin saturation (percentage of transferrin saturated with iron), and ferritin (µg/L).

All procedures in both the adolescent and adult cohorts were approved by the Human Research Ethics Committee of QIMR Berghofer Medical Research Institute (QIMRB).

Estimation of Genetic Parameters:

Data were analysed using the statistical program Mx. After standard testing of assumptions (equality of means and variances across zygosity and sex), twin correlations were calculated. Heritabilities were calculated initially under a univariate additive genetic (A), common environment (C) and unique environment (E) model and then under bivariate models considering all pairwise combinations of traits. To examine the significance of the estimated univariate variance components, we also considered AE and CE reduced models. Goodness-of-fit of the reduced models were assessed using likelihood ratio tests. The sample size gave at least 99% power to estimate additive genetic variance greater than zero for iron and depression measures at significance level 0.05 (adolescents and adults) (339, 340).

Percentile analysis:

We hypothesised that the relationship between iron measures and depression measures may be non-linear, and that this phenotypic relationship may only be present at the extremes of iron measures. Therefore, we also investigated whether there was a phenotypic association between the upper and lower range of circulating levels of iron measures with depressive symptoms (perhaps representing a non-linear relationship between iron and depression), by testing for differences between 1) the lowest 10th percentile and middle 10th percentile (i.e. 45th-55th percentile), 2) the highest 10th percentile and middle 10th percentile, 3) the lowest 10th percentile and highest 10th percentile, and 4) the lowest 5th percentile and highest 5th percentile (Welch Two Sample t-test). These choices reflect non-linear models that could be U-shaped (first two tests) as well as differences in the extremes (third and fourth tests). As the phenotypic relationship may only be present at the extremes of iron measures, testing for differences between the lowest 5th percentile and highest 5th percentile was included.

Association Analysis:

Single nucleotide polymorphisms (SNPs) significantly associated with iron phenotypes in genome-wide association studies (GWAS) (329, 330, 341, 342) were identified: rs2698530, rs1799852, rs1830084, rs2280673, rs3811647, rs1799945, rs8177240, rs1800562, rs7787204, rs4820268, rs855791, rs9990333, rs987710, rs744653, rs7385804, rs235756, rs4921915, rs651007, rs6486121, rs174577, rs411988. The association statistics of these 21 SNPs (or their proxies, defined as in linkage disequilibrium r^2 >0.8) were extracted from published GWAS for MDD (134). The sample size of the PGC MDD data gave at least 99% power to detect common (MAF >0.1) variants that explain 0.5% of the variance at significance level 0.05 (343).

Genomic risk profile score analysis:

Genomic profile risk scores (GPRS) for iron, transferrin, transferrin saturation and L10Fer were generated for each individual in both the adolescent and adult 'target' samples using GWAS summary statistics data from the Genetics of Iron Status Consortium (GISC) (330) 'discovery' sample. The GISC data comprise association statistics between SNP genotypes and iron markers (serum iron, transferrin, transferrin saturation, and ferritin) from approximately 24,000 individuals from a total of 19 cohorts in 9 participating centres (330). QIMRB samples used here were part of the GISC. Since genetic prediction analysis requires independence between discovery and target samples, we recalculated effect sizes from the GISC cohorts after excluding QIMRB samples. GPRS were created (separately for adolescents and adults) as the sum of associated alleles of quasi-independent SNPs (pruned so that pairwise linkage disequilibrium between SNPs was less than r^2=0.25) weighted by their effect size estimated in the GISC meta-analysis.

GPRS for MDD were generated for individuals in the adolescent and adult target QIMRB samples using GWAS data from the Psychiatric GWAS Consortium (PGC) MDD working group (134). The PGC MDD 'discovery' sample has 9,240 MDD cases and 9,519 controls (134). QIMRB samples were part of the PGC, so we recalculated effect sizes from the PGC MDD cohorts after excluding the QIMRB samples. GPRS for both iron measures and MDD were calculated using varying levels of discovery sample p-value thresholds in PLINK (344). The appropriate choice of p-value thresholds depend on the genetic

architecture of the trait and the size and hence power of the sample. For each iron measure, we selected the p-value threshold from the GWAS results that maximised variance for the same iron measure in our data. Therefore, different thresholds were selected for different traits. To help in the interpretation of results, one individual per family was selected for inclusion in the profile scoring analysis (n= 2,394 individuals for adult data; n = 1,028 individuals for adolescent data).

Linear regression models were then used to predict how much of the variation in each of the phenotypes of our samples is explained by the GPRS and the direction of association. Age was significantly associated with measures of iron and ferritin, whilst sex was significantly associated with measures of iron, transferrin saturation and ferritin. Therefore, we used an age and sex adjusted regression model to test for an association between the profile scores (iron measures and MDD) and measures in the QIMRB samples. After conducting the profile scoring analyses using one individual per family, we subsequently conducted the analyses with relatives included.

LD score analysis:

Genetic correlations based on genome-wide SNPs between iron phenotypes and depression measures were estimated using LD score regression (v1.0.0) (345), based on GWAS summary statistics (effect size, direction of effect for each SNP, and sample size). The method exploits the expectation that SNPs in high LD regions (with large LD scores) will, on average, tag more causal variants than SNPs in low LD regions. LD score regression has previously been used to estimate genetic correlations for a wide range of traits, and the resulting estimates were consistent with estimates of genetic correlations obtained by bivariate GREML, which uses full genotype data to estimate genetic correlation (346). We used LD scores for each SNP calculated from 1000 Genomes, which are available on the LD Scores Regression github page (https://github.com/bulik/ldsc), both as the independent variable in LD Score regression, as well as for the regression weights (options "--ref-ld-chr" and "--w-ld-chr"). SNPs which were located in or around the iron metabolism related genes *TF, HFE,* and *TMPRSS6*, which had previously been shown to have large effect on variance of serum transferrin levels (329) were excluded from the analysis. However, including them in the analysis did not have a large impact on the estimates of genetic correlation. Figure 5-1 shows the strategies / types of analyses

we used to investigate the genetic relationship between iron measures and depression measures.

Figure 5-1: Schematic representation of analyses
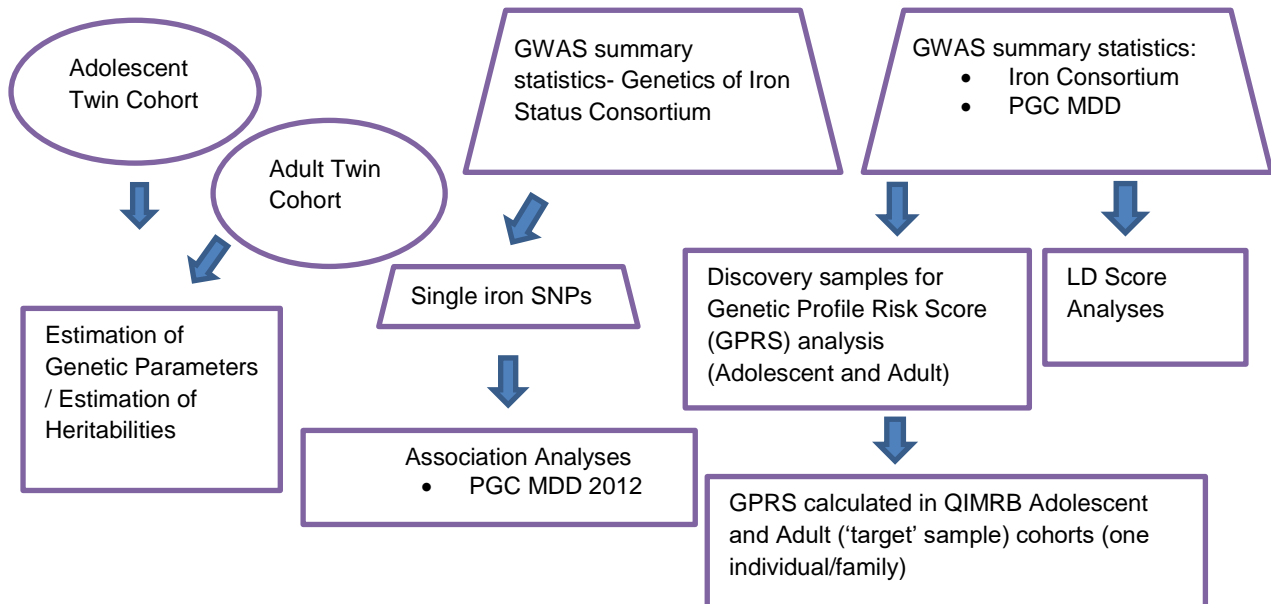
Results:

Numbers of study participants and mean iron and depression measures are shown in Table 5-1. With the exception of transferrin (in the adolescent and adult cohorts), for all iron measures males had significantly higher mean measures compared with females (Table 5-1). Mean depression measures were higher in females in both cohorts, however this difference was only significant in the adult cohort (Table 5-1).

In assumption testing analyses that are preliminary to variance component estimation in twin samples, means and variances (both within and across zygosity groups) were able to be constrained to be equal for each of the iron and depression measures in the adolescent cohort. In the adult cohort, means and variances were able to be constrained to be equal for log ferritin and depression measures, but not for other iron measures. For iron and transferrin saturation in adults, we observed significant differences in means (p=0.035 and p=0.003 respectively) between MZ and DZ twins (with DZ twins having on average higher iron and transferrin saturation levels and larger variance compared with MZ twins), and a birth order effect in means (p=0.001 for both traits), with first born twins having on average

111

lower levels. With transferrin saturation in adults, larger variance in DZ twins (compared to MZ) was observed (p=0.049). For transferrin in the adult cohort, a significant difference in means (p=0.004) and variances (p=<9.8x10^{-6}) between MZ and DZ twins (with MZ twins having on average higher transferrin levels and larger variance compared with DZ twins) was observed. A birth order effect in variances (p=0.002) was also observed for transferrin (adults), with first born twins having on average larger variance. After correction for multiple testing, only the birth order effect in means for iron and transferrin saturation, and the differences in variances for transferrin remained significant (Bonferroni corrected significance level 0.0017). In view of the differences in variances for transferrin (p=<9.8x10^{-6}), results from analysis of this trait should be considered with more caution. Sex effects were significant for all iron measures in the adolescent cohort. In the adult cohort, sex and age effects were significant for all iron and depression measures, so both sex and age were fitted as covariates in the estimation of heritability of all traits in both cohorts.

Table 5-1: Means and standard deviations for iron measures and depression measures

Trait (Adolescents)	Total		Males		Females		p-value*
	Mean	SD	Mean	SD	Mean	SD	
Iron (µmol/L)	17.47	6.76	19.04	6.64	15.93	6.52	<2.2x10^{-16}
Transferrin (g/L)	2.96	0.46	2.93	0.41	2.99	0.50	1.8 x10^{-2}
Saturation (%)	24.05	9.69	26.35	9.60	21.79	9.24	<2.2x10^{-16}
Log ferritin (µg/L)	1.62	0.34	1.74	0.26	1.51	0.38	<2.2x10^{-16}
SPHERE	7.60	6.28	8.57	6.34	8.84	6.58	0.21
Trait (Adults)	Total		Males		Females		p-value*
	Mean	SD	Mean	SD	Mean	SD	
Iron (µmol/L)	19.44	6.63	20.77	6.42	18.69	6.64	< 2.2e-16
Transferrin (g/L)	2.78	0.49	2.66	0.37	2.85	0.47	< 2.2e-16
Saturation (%)	28.42	10.59	31.38	10.44	26.73	10.30	< 2.2e-16
Log ferritin (µg/L)	2.00	0.44	2.27	0.36	1.84	0.41	< 2.2e-16
Factor 1	0.00	1.06	-0.12	0.95	0.09	1.13	< 2.2e-16

*p-value for difference between means for males and females; % = units for transferrin saturation = percentage of transferrin saturated with iron; Factor 1 = depression measure (adult cohort); SPHERE = Somatic and Psychological Health Report (depression measure adolescent cohort)

Adolescents: number individuals iron measures = 1,363 (males=676, females=687); depression measures = 2,890 (males=1,327, females=1,563)

Adults: number individuals iron measures = 4,366 (males=1,609, females=2,757); depression measures = 8,072 (males=2,998, females=5,074)

As expected, MZ correlations were higher than DZ correlations for all measures of iron (iron, transferrin, transferrin saturation, log of ferritin) in both the adolescent and adult cohort (Table 5-2). MZ correlations were also significantly higher than DZ correlations for depression measures in the adolescent cohort (Table 5-2).

Table 5-2: Twin Correlations with 95% Confidence Intervals (CI):

Trait	MZ	DZ	MZF	MZM	DZF	DZM	DZO
Adolescent sample iron measures: 266 MZ pairs, 380 DZ pairs; SPHERE: 494 MZ pairs, 852 DZ pairs							
Iron	**0.56** (0.44,0.66)	**0.33** (0.21,0.43)	**0.56** (0.38,0.68)	**0.56** (0.37,0.69)	**0.38** (0.14,0.55)	**0.56** (0.35,0.69)	**0.21** (0.04,0.37)
Transferrin	**0.75** (0.67, 0.81)	**0.47** (0.34, 0.58)	**0.72** (0.62,0.80)	**0.82** (0.71,0.88)	**0.41** (0.24, 0.54)	**0.64** (0.44, 0.76)	**0.38** (0.19, 0.52)
Saturation	**0.62** (0.51, 0.70)	**0.26** (0.14, 0.38)	**0.61** (0.46, 0.72)	**0.63** (0.47,0.73)	**0.33** (0.11,0.51)	**0.45** (0.21,0 .61)	**0.13** (-0.07,0.30)
Log ferritin	**0.65** (0.54,0.73)	**0.36** (0.24,0.46)	**0.58** (0.43,0.68)	**0.83** (0.72,0.89)	**0.36** (0.21, 0.49)	**0.61** (0.37,0.74)	**0.26** (0.04,0.44)
SPHERE	**0.46** (0.39,0.52)	**0.25** (0.17,0.34)	**0.46** (0.37, 0.54)	**0.46** (0.35,0.55)	**0.16** (0.03,0.28)	**0.35** (0.23,0.45)	**0.20** (0.10,0.29)
Adult sample iron measures: 705 MZ pairs, 957 DZ pairs; depression measures: 1630 MZ pairs, 1742 DZ pairs							
Iron	**0.37** (0.31,0.43)	**0.15** (0.07,0.22)	**0.36** (0.29,0.43)	**0.40** (0.25,0.52)	**0.15** (0.05,0.23)	**0.16** (0.01,0.30)	**0.14** (0.04,0.24)
Transferrin	**0.52** (0.49,0.56)	**0.25** (0.18,0.31)	**0.48** (0.42,0.53)	**0.67** (0.60,0.73)	**0.20** (0.13,0.28)	**0.54** (0.41,0.63)	**0.29** (0.18,0.39)
Saturation	**0.53** (0.48,0.58)	**0.17** (0.09,0.24)	**0.55** (0.49,0.60)	**0.47** (0.34,0.57)	**0.17** (0.07,0.25)	**0.18** (0.03,0.31)	**0.18** (0.08,0.28)
Log ferritin	**0.44** (0.38,0.49)	**0.27** (0.20,0.34)	**0.41** (0.34,0.47)	**0.52** (0.41,0.60)	**0.29** (0.21,0.36)	**0.23** (0.09,0.36)	**0.16** (0.06,0.25)
Factor 1	**0.34** (0.27,0.40)	**0.19** (0.11,0.27)	**0.29** (0.21,0.36)	**0.57** (0.46,0.66)	**0.18** (0.08,0.26)	**0.27** (0.09,0.42)	**0.18** (0.06,0.29)

Key: MZ = monozygotic, DZ = dizygotic; F = female, M = male, O = opposite-sex; Factor 1 = depression measure (adult cohort); SPHERE = Somatic and Psychological Health Report (depression measure adolescent cohort)

Using an ACE model, we found heritability of the iron measures in both cohorts to be moderate to high (Table 5-3). In contrast, estimates of variance attributable to the shared family environment (C) were not significantly different from zero. Heritability for depression measures in the adolescents (SPHERE) was 0.46 (95% CI 0.29 -0.52), which was in keeping with that reported from an analysis from a subset of these samples but with measures the mean of scores collected on multiple occasions (309).

Table 5-3: Estimates of proportions of variance attributable to additive genetic effects (A) or heritabilities, common environmental effects of families (C) and unique environmental effects (E) with 95% Confidence Intervals (Univariate model):

Trait	A	C	E
Adolescent sample			
Iron	0.46 (0.15,0.66)	0.10 (0.00,0.34)	0.44 (0.34,0.56)
Transferrin	0.64 (0.42,0.81)	0.11 (0.00,0.30)	0.24 (0.19,0.32)
Saturation	0.61 (0.39,0.70)	0.00 (0.00,0.18)	0.39 (0.30,0.49)
Log ferritin	0.56 (0.28,0.72)	0.09 (0.00,0.30)	0.36 (0.28,0.47)
SPHERE	0.46 (0.29,0.52)	0.00 (0.00,0.13)	0.54 (0.48,0.61)
Adult sample			
Iron	0.35 (0.25,0.41)	0.00 (0.00,0.07)	0.65 (0.59,0.71)
Transferrin	0.52 (0.38,0.56)	0.00 (0.00,0.12)	0.48 (0.44,0.53)
Saturation	0.50 (0.44,0.55)	0.00 (0.00,0.04)	0.50 (0.45,0.55)
Log ferritin	0.42 (0.27,0.49)	0.02 (0.00,0.14)	0.56 (0.51,0.61)
Factor 1	0.30 (0.11,0.40)	0.04 (0.00,0.18)	0.66 (0.60,0.73)

Key: Factor 1 = depression measure (adult cohort); SPHERE = Somatic and Psychological Health Report (depression measure adolescent cohort)

Bivariate analyses showed high phenotypic and genetic correlations between the iron phenotypes but that the phenotypic and genetic correlations between the iron phenotypes and depression measures were not significantly different from zero (Table 5-4). A bivariate AE model was used since the univariate analyses for these measures showed estimates of the common environmental components were small and not significantly different from zero.

Table 5-4: Heritabilities (diagonals) from univariate ACE models; phenotypic correlations (above diagonals) and genetic correlations (below diagonals) from bivariate AE Models (95% CI in parenthesis)

Adolescents:

Trait	Iron	Transferrin	Saturation	Log10ferritin	SPHERE
Iron	**0.46 (0.15,0.66)**	-0.03 (-0.10,0.05)	0.93 (0.92,0.94)	0.17 (0.10,0.25)	-0.06 (-0.14,0.02)
Transferrin	-0.10 (-0.34,0.07)	**0.64 (0.42,0.81)**	-0.34 (-0.41,-0.27)	-0.43 (-0.49,-0.36)	-0.02 (-0.10,0.06)

Saturation	0.94 (0.90,0.97)	-0.51 (-0.78,-0.36)	**0.61 (0.39,0.70)**	0.28 (0.21,0.35)	-0.05 (-0.13,0.03)
Log ferritin	0.32 (0.12,0.69)	-0.69 (-0.97,-0.51)	0.44 (0.27,0.68)	**0.56 (0.28,0.72)**	-0.04 (-0.12,0.04)
SPHERE	-0.19 (-0.51,0.04)	0.00 (-0.19,0.18)	-0.15 (-0.36,0.04)	-0.01 (-0.22,0.19)	**0.46 (0.29,0.52)**

Adults:

Trait	Iron	Transferrin	Saturation	Log10ferritin	Factor 1
Iron	**0.35 (0.25,0.41)**	-0.02 (-0.05,0.02)	0.90 (0.90,0.90)	0.24 (0.21,0.27)	-0.03 (-0.03,-0.02)
Transferrin	-0.15 (-0.25,-0.05)	**0.52 (0.38,0.56)**	-0.39 (-0.40,-0.38)	-0.39 (-0.39,-0.36)	0.00 (-0.04,0.03)
Saturation	0.95 (0.90,0.99)	-0.57 (-0.64,-0.57)	**0.50 (0.44,0.55)**	0.34 (0.31,0.37)	-0.03 (-0.03,-0.03)
Log ferritin	0.27 (0.15,0.41)	-0.33 (-0.42,-0.24)	0.38 (0.28,0.51)	**0.42 (0.27,0.49)**	0.03 (0.00,0.07)
Factor 1	0.02 (0.02,0.03)	0.10 (-0.01,0.24)	-0.03 (-0.17,0.10)	0.00 (-0.15,0.13)	**0.30 (0.11,0.40)**

Key: Factor 1 = depression measure (adult cohort); SPHERE = Somatic and Psychological Health Report (depression measure adolescent cohort)

Heritabilities in the adolescent and adult cohorts, and SNP heritabilities are also represented in graphical format in Figure 5-2. SNP heritabilities are from GWAS data used in the estimation of genetic correlations. Figures 5-3 and 5-4 represent phenotypic correlations (adolescent and adult twin cohorts), genetic correlations (adolescent and adult twin cohorts), and genetic correlations obtained through LD score analyses for each pairwise combination of iron traits (Figure 5-3) and iron-depression traits (Figure 5-4).

Figure 5-2: Heritabilites: adolescent and adult cohorts (univariate ACE models) & SNP heritabilities (bars represent 95% Confidence Intervals)
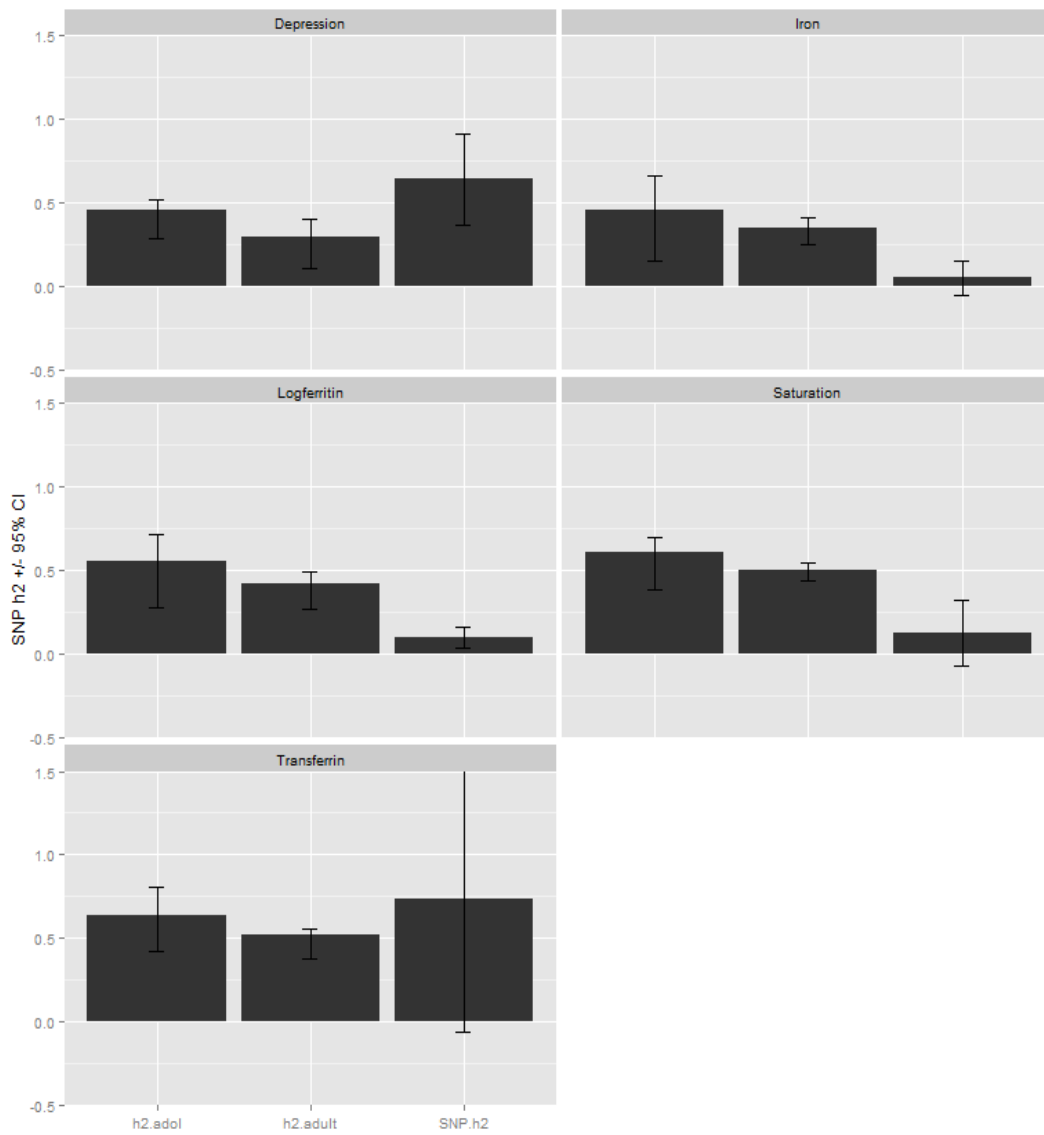
Note: name of file used for MDD SNP heritability estimate: "pgc.mdd.full.2012-04.txt" (contained in "pgc.mdd.2012-04.zip", from https://www.med.unc.edu/pgc/downloads)

Figure 5-3: Phenotypic and genetic correlations (adolescent and adult twins), and genetic correlations (LD score analyses) for pairwise combinations of iron traits; bars represent 95% Confidence Intervals
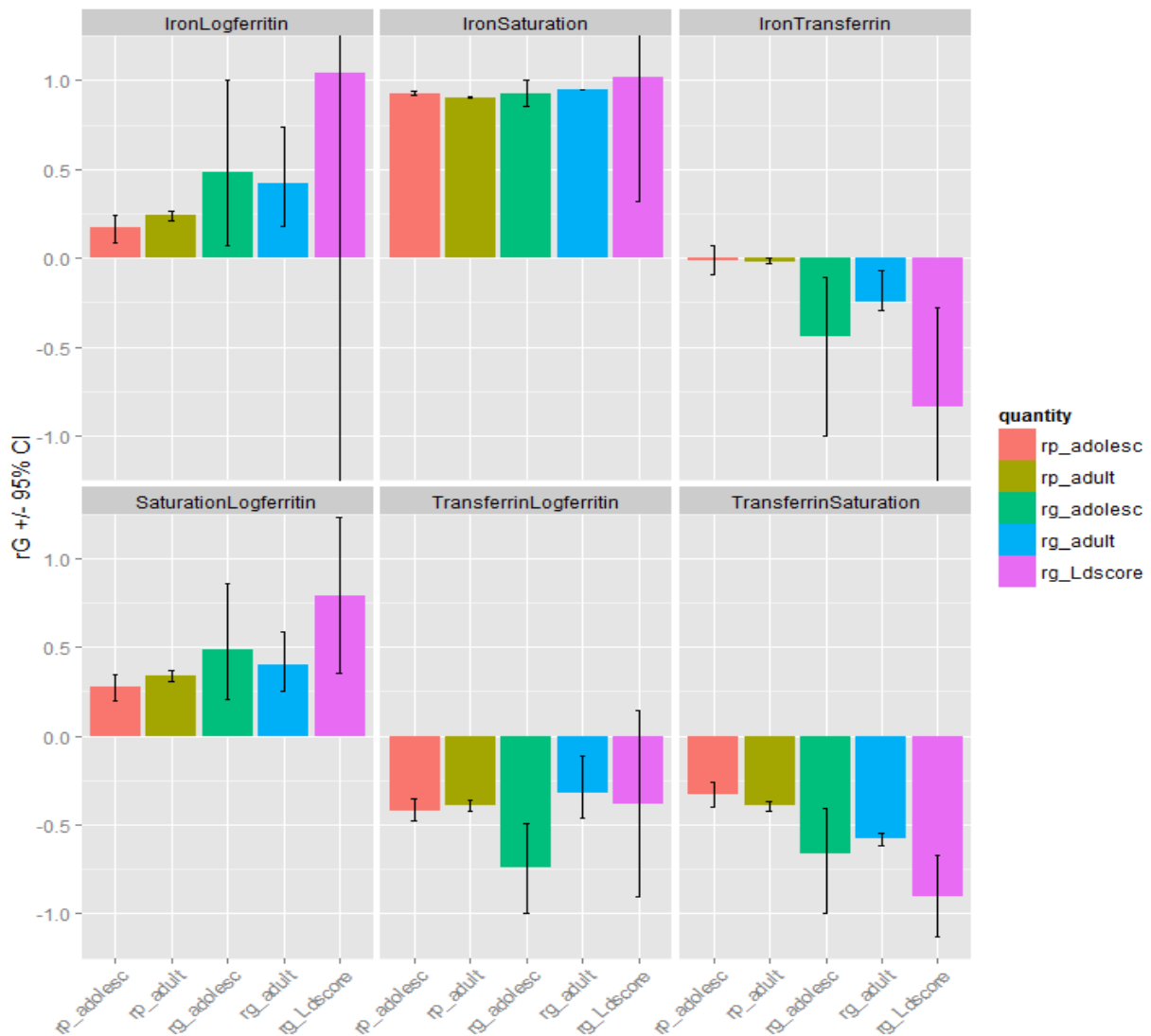
Key: rp = phenotypic correlation; rg = genetic correlation

Figure 5-4: Phenotypic and genetic correlations (adolescent and adult twins), and genetic correlations (LD score analyses) for pairwise combinations of iron-depression traits; bars represent 95% Confidence Intervals
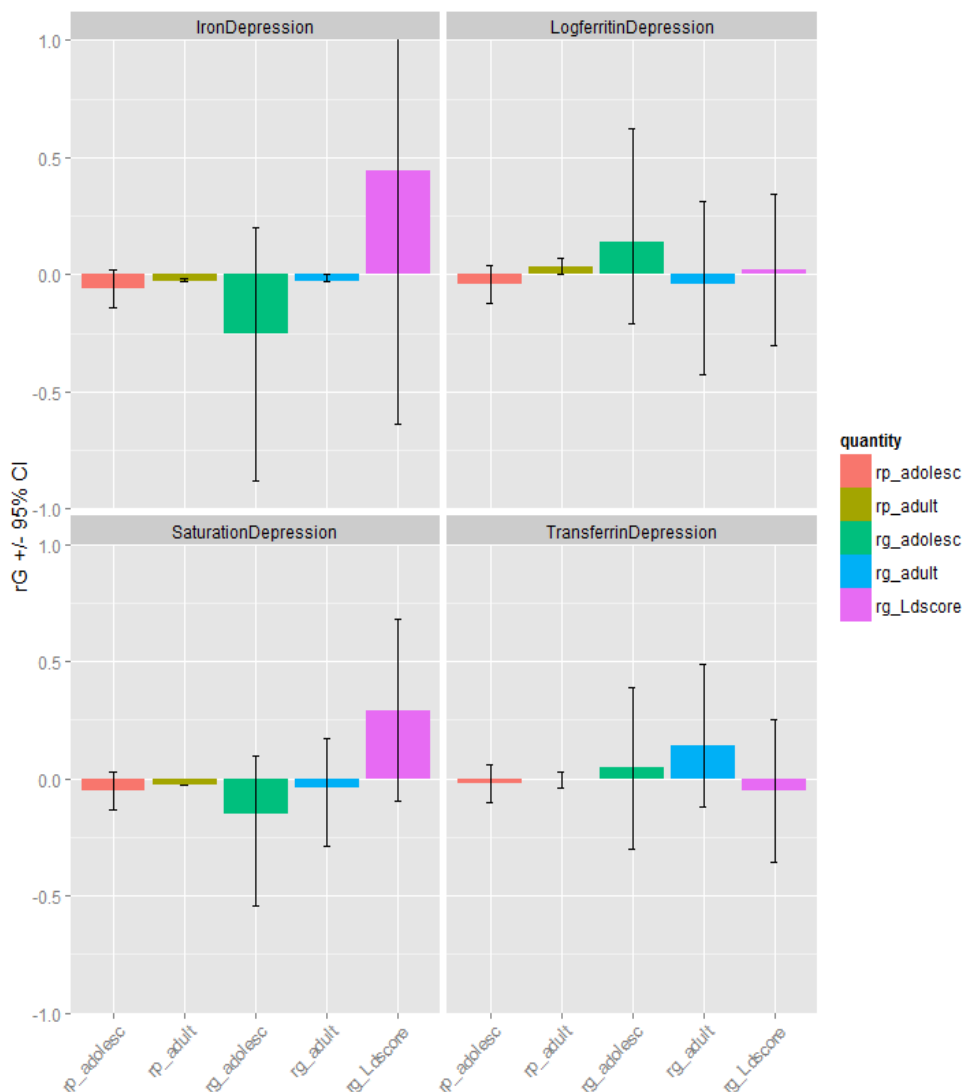
Key: rp = phenotypic correlation; rg = genetic correlation

Percentile analysis: In the adolescent cohort, depression measures were nominally significantly higher in those in the lowest 5th percentile of log ferritin measures compared to those in the highest 5th percentile of log ferritin measures (p=0.034). We also found depression measures were significantly higher in those in the middle 10th percentile of iron and transferrin saturation measures compared to those in the highest 10th percentile of iron and transferrin saturation measures (p=0.008 and p=0.002 respectively). In the adult

cohort we did not find a phenotypic association between the upper and lower range of circulating levels of iron measures with depressive symptoms. To be conservative we used two-sided t-tests. However, given the multiple testing of 4 traits and 4 tests the Bonferroni corrected significance level is 0.0031, so only the association between depression measures and transferrin saturation survives multiple testing (p=0.002).

Association Analysis: Of the 21 independent SNPs associated with iron phenotypes, association results from 15 were available in the PGC MDD GWAS. The smallest p-value of association was rs744653 for MDD (p = 0.027), and hence no association was significant after correcting for multiple testing. As expected, together the SNPs did not explain a significant proportion of the variance. Results of a sign test of direction of effect were consistent with the hypothesis that there was not a significant association between iron measures and MDD (p-values 0.94 – 1).

Genetic Profile Risk Scores (GPRS): Results of testing for an association between the profile scores (iron, transferrin, transferrin saturation, and log ferritin) and the measures of iron and depression in the QIMRB samples are shown in Table 5-5. Profile scores calculated for each individual in the QIMRB sample using GWAS association results from analyses of iron, transferrin, transferrin saturation, and log ferritin were each highly significantly associated with their respective trait measures (Table 5-5 column 3). Marginal associations were observed with genetic profile risk scores of iron and transferrin for depression measures in adults at p-value thresholds of p<0.05 and p<0.1 respectively, however these associations were not significant after correction for multiple testing (Bonferroni corrected significance level p=0.0013). In both the adolescent and adult cohort, the direction of effect was not in the expected direction between transferrin genetic profile risk scores and depression measures (Table 5-5). Neither transferrin saturation profile scores nor log ferritin profile scores predicted depression status in adolescents or adults. Furthermore, profile scores (iron, transferrin, transferrin saturation, and log ferritin) did not predict depression measures in either cohort when relatives were included in the analyses (results not shown).

Table 5-5: Iron, transferrin, transferrin saturation, and log ferritin genetic profile risk scores – prediction of iron / depression measures

Target	Adult/Adolescent	P-value Target	Variance explained Target (%)	Direction of effect
Iron discovery sample; p-value threshold P< 10^{-3}; 212 SNPs				
Iron	Adolescent	0.010	1.56	+
SPHERE	Adolescent	0.87	0	-
Iron discovery sample; p-value threshold P< 10^{-5}; 23 SNPs				
Iron	Adult	1.81×10^{-8}	5.49	+
Depression	Adult	0.10	0.57	-
Transferrin discovery sample; p-value threshold P< 10^{-5}; 27 SNPs				
Transferrin	Adolescent	1.83×10^{-7}	7.09	+
SPHERE	Adolescent	0.50	0	+
Transferrin discovery sample; p-value threshold P< 10^{-5}; 27 SNPs				
Transferrin	Adult	8.20×10^{-10}	5.93	+
Depression	Adult	0.85	0.08	+
Transferrin saturation discovery sample; p-value threshold P< 10^{-3}; 227 SNPs				
Transferrin sat	Adolescent	4.82×10^{-5}	4.30	+
SPHERE	Adolescent	1.00	0	-
Transferrin saturation discovery sample; p-value threshold P< 10^{-5}; 32 SNPs				
Transferrin sat	Adult	4.15×10^{-13}	9.07	+
Depression	Adult	0.20	0.38	-
Log ferritin discovery sample; p-value threshold P< 10^{-3} ; 212 SNPs				
Log ferritin	Adolescent	3.24×10^{-3}	2.14	+
SPHERE	Adolescent	0.52	0	+
Log ferritin discovery sample; p-value threshold P< 5×10^{-4} ; 959 SNPs				
Log ferritin	Adult	2.26×10^{-3}	1.38	+
Depression	Adult	0.83	0.08	-

The results of testing for an association between MDD profile scores and the QIMRB adolescent and adult iron measures are shown in Table 5-6. The nominal associations (p<0.05) listed (Table 5-6) do not survive correction for multiple testing. Furthermore, MDD profile scores did not predict iron measures in the adolescent or adult cohort when relatives were included in the analyses (results not shown).

Table 5-6: MDD genetic profile risk scores – prediction of iron measures

P-value of SNPs in MDD discovery GWAS	N SNPs	Target	P-value Target	Variance explained Target (%)	Direction of effect
Adolescent sample					
P <10^{-6}	11	Iron	0.17	0.24	-
P <0.05	17,699	Transferrin	0.066	0.61	+
P <10^{-6}	11	Saturation	0.25	0.08	-
P <0.10	29,840	Log ferritin	0.46	0	+
Adult sample					
P <10^{-6}	11	Iron	0.022	0.30	+
P <1.0	119,734	Transferrin	0.43	0	-
P <10^{-6}	11	Saturation	0.093	0.13	+
P <10^{-4}	733	Log ferritin	0.048	0.19	-

Key: MDD = major depressive disorder

LD Score

Results of LD score analyses showed the same pattern for SNP genetic correlations between iron and transferrin, log ferritin and transferrin, and saturation and transferrin (Figure 5-5) as expected from the whole genome genetic correlations estimated from the twin data (Table 5-4). Estimates of SNP genetic correlations can only be estimated as different from zero if they are more than twice the standard error (s.e.) of the estimate. The magnitude of the s.e in Figure 5-5, shows that despite the large sample sizes in the contributing GWAS, the data was only powered to detect very high correlations. The SNP-correlation between transferrin saturation and MDD (0.29 ± se 0.20) was not significantly different from zero after accounting for multiple testing.

Figure 5-5: Estimate of genome-wide SNP correlations (rG) between traits from LD score analyses using GWAS summary statistics

(vertical lines represent standard error (SE)); these data are also included in Figures 5-3 and 5-4.
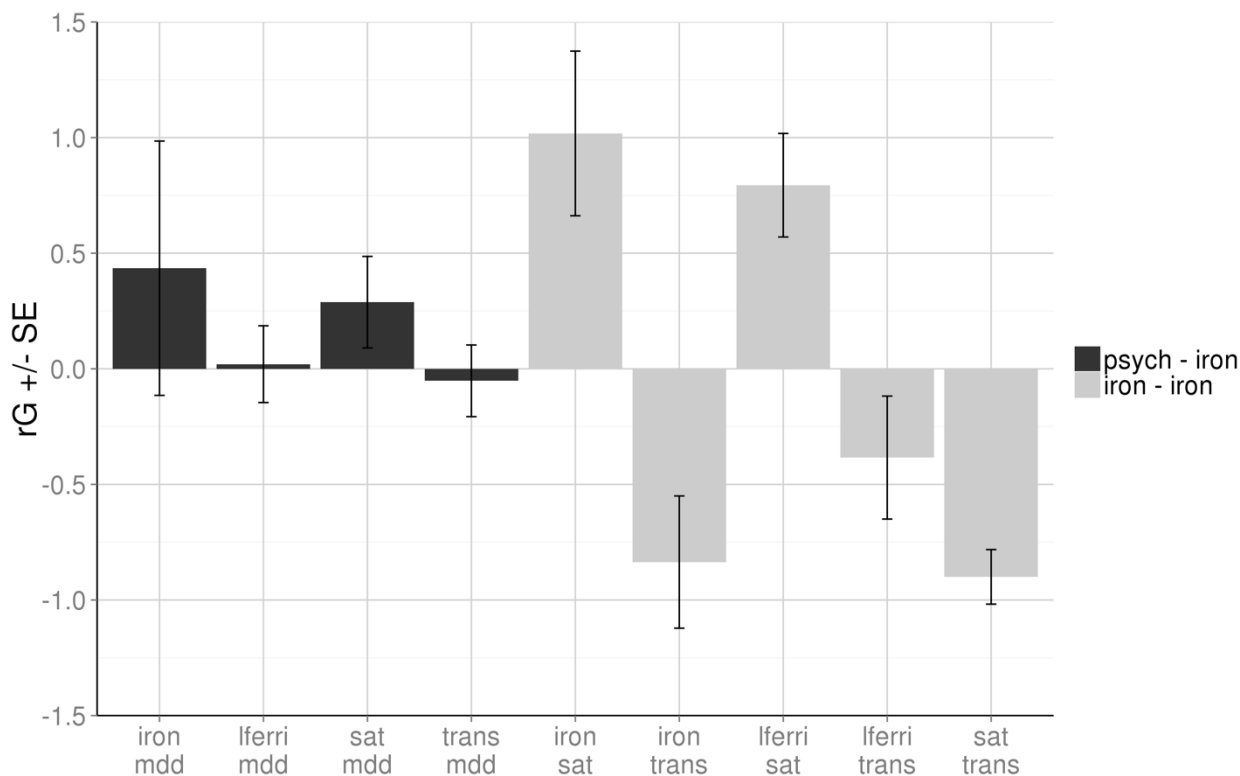
Key: lferri = log ferritin; mdd = major depressive disorder; sat = transferrin saturation; trans = transferrin

Discussion:

This study examined the phenotypic and genetic relationship between measures of circulating levels of iron and depressive symptoms using different data sets. In both the adolescent and adult cohorts, phenotypic correlations of the iron measures with depression measures were not significantly different from zero. We explored the possibility of a non-linear relationship between iron and depression by testing the differences in adolescents and adults with measures for iron categorised into (i) the lowest 10th, middle 10th, and highest 10th percentiles, and (ii) the lowest 5th and highest 5th percentiles. In adolescents, depression measures were significantly higher (after correcting for multiple testing) in those in the middle 10th percentile of transferrin saturation measures compared

122

to those in the highest 10th percentile of transferrin saturation measures (p=0.002). We could not find any studies examining the relationship between iron and depression in community samples of adolescents. However, it is possible that a phenotypic relationship of serum ferritin (or iron) with depressive symptoms is more likely to be observed in the presence of iron deficiency. In our study, the lowest iron level in adolescent females was 2.80 µmol/L, and the lowest iron level in adolescent males was 4.0 µmol/L (normal reference range 14-32 µmol/L for males and females (347)). Similarly, it may be that a phenotypic relationship between serum iron and depressive measures is more likely to be observed at the lower range of iron scores. Non-linear relationships have also been reported for other biological measures such as Vitamin D, with neonates who had either low or high levels of Vitamin D observed to have an increased risk of schizophrenia later in life (249).

To examine the genetic relationship between measures of iron and measures of depression, we first used data from a community sample of twins to estimate heritabilities and genetic correlations of these measures. Heritabilities for circulating levels of iron measures were moderate to high, and somewhat higher than heritabilities estimated from previous studies (328, 348). These differences from previous studies may simply reflect sampling, but may also reflect differences in the ages of subjects between the studies. Both previous studies were in adults, but Traglia, Sala et al. 2009 reported the effect of age to be significant in the estimation of serum transferrin heritability (328). Here, our estimates of heritability were higher in the adolescent than in the adult cohort. This study was well-powered (≥99%) to estimate additive genetic variance greater than zero for iron and depression measures at significance level 0.05 (339, 340). Genetic correlations between iron measures and depressive measures were not different from zero, however in both twin cohorts negative genetic correlations were found between transferrin and iron, transferrin and log ferritin, and transferrin and saturation.

We used LD score analyses (345) to explore the genome-wide correlation between SNP effects for different traits. This is likely the most powerful analysis based on currently available data, but still lacked power to detect small correlations as being different from zero. The SNP-correlations between the iron measures were consistent with the genetic correlations obtained using a different approach (see Table 4), with negative correlations between transferrin and iron, transferrin and log ferritin, and transferrin and saturation. We

found a positive correlation between MDD with iron and transferrin saturation, however these were not significant after accounting for multiple testing. Using LD score analyses, SNP heritability for MDD and transferrin were higher than estimated heritability of these traits in the QIMRB twin cohorts.

We used another independent strategy to explore the hypothesis of a genetic relationship between measures of circulating iron and depression. We undertook analyses using published genome-wide association studies of iron and MDD. Despite these sample sizes giving at least 99% power to detect common (MAF > 0.1) variants (343), none of the SNPs explaining variation in iron phenotypes showed significant association in the published MDD GWAS results (134), and together the SNPs did not explain a significant proportion of the variance. We also conducted analyses that linked results from published large-scale genome-wide association studies with our in-house adolescent and adult samples for which we also had genome-wide genotype data. In the resulting genetic profile score analysis we found no significant association between iron measures and depression.

A limitation of this study was the time difference between iron measures and depression measures, particularly in the adult cohort. While mean age at completion of the SPHERE was 15.1 years (SD 3.2 years) for the adolescent cohort, in the adult cohort study participants provided a blood sample approximately 10 years after completing the DSSI. This time difference was important in the investigation of the phenotypic relationship between the iron and depression measures. A further limitation was that the depression measures did not use MDD DSM-IV diagnostic criteria.

Conclusion:

We used multiple approaches to explore evidence for a genetic relationship between measures of circulating serum iron and depressive measures. Although each approach may have limitations, the results when taken together across the different approaches provide no compelling evidence for a genetic relationship between circulating iron and measures of depression, even though we were well-powered to detect a relationship through estimation of heritabilities, association analyses, and LD score analyses. The reported phenotypic relationship between iron and depression may be more likely to be observed at times when the body requires higher amounts of iron, such as during times of rapid growth. In this way it may reflect a highly non-linear relationship, in which those with

circulating levels of iron measures below an extreme threshold are more likely to be impacted.

CHAPTER 6: Investigating the relationship between C-reactive protein, depression, and anxiety

Abstract

Background: The inflammatory marker C-reactive protein (CRP) has been associated with Major Depressive Disorder (MDD), and a small number of studies have investigated the association of CRP with anxiety disorders, with inconsistent results. It has been suggested that body mass index (BMI) mediates the relationship between CRP and MDD, since both raised CRP and MDD are associated with higher BMI, but few studies have investigated the relationship.

Objectives: To investigate the phenotypic and genetic relationship between (i) CRP levels, MDD and anxiety, and (ii) CRP levels and childhood sexual abuse (CSA), and to test whether those who carry more CRP increasing alleles, are at increased risk to develop depression and / or anxiety.

Methods: Participants were from the Australian Twin Registry, in studies conducted at QIMR Berghofer Medical Research Institute. A total of 14,750 individuals had CRP data (mean age at CRP measurement 45.3 years (standard deviation (SD) 10.1 years)), of which 8,234 had completed questionnaires using DSM-IV criteria for MDD, 8,679 had completed questionnaires using DSM-IV criteria for anxiety disorders, and 8,847 individuals had genome-wide SNP data. Genetic risk profile scores were estimated for each individual based on GWAS summary statistics from the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium (for CRP) and the Genetic Investigation of Anthropometric Traits Consortium (for BMI).

Results: Mean BMI of 25.97 kg/m^2 (standard deviation 4.87 kg/m^2) was slightly above the normal range of 20-25 for adults. A point estimate of higher mean log CRP in the group (n=942) that met criteria for both MDD and a co-morbid anxiety disorder compared to those that met criteria for neither was not statistically significant (p=0.13), but was in the same direction found in previous studies of CRP and psychiatric disorders. A significant association between CRP and social phobia (p=0.0045), was not significant after adjusting

for BMI (p=0.090). CRP genetic risk profile scores did not predict MDD or anxiety disorders in this large community sample.

Conclusion: The complex relationship between CRP, BMI, MDD and anxiety disorders requires further study, but our results suggest that BMI is the key factor that mediates the reported relationship between circulating CRP and psychiatric disorders.

Introduction

Major Depressive Disorder (MDD) is associated with considerable morbidity and mortality worldwide (36, 324). There is increasing recognition of the role of inflammation in the pathophysiology of MDD. This includes the association of C-reactive protein (CRP) (32), an acute phase reactant involved in fighting infection and repair of tissue damage (349). An association between elevated CRP and MDD has been found in meta-analyses (7, 271). However, the finding of increased activation of the inflammation system in depression has not been consistently demonstrated across studies (182). Known or unknown confounding factors may explain this inconsistency, at least in part. Age, female gender and diabetes mellitus are possible confounding factors that are all known to contribute to low grade inflammation, with elevated plasma CRP levels (79, 350-353). Another potential confounder of the association between CRP and MDD is body mass index (BMI). BMI has been shown to be significantly correlated with both CRP (354) and MDD (355). Adjustment for BMI has reduced the association between MDD and CRP to non-significance in several studies (145, 356).

Other potential confounders are experience of early childhood adversity or comorbid anxiety disorders. A history of early childhood adversity is reported to be associated with inflammation (including raised CRP in later life) (29, 53, 357). Individuals with a history of early childhood adversity, who also have MDD (or a history of MDD), have been found to have even higher levels of CRP than those with MDD alone (28, 29). These findings are particularly important, as persistent inflammation may subsequently contribute to increased risk of not only mental health disorders, but also physical disorders in adult life (2, 358, 359). Anxiety disorders are often comorbid with MDD and there is evidence of shared genetic risk for anxiety and MDD (360). Despite this, the question of whether

increased levels of pro-inflammatory markers are also found in those suffering from anxiety disorders, or whether this finding is specific to depression has not been addressed.

There have been several studies investigating the relationship between inflammation and psychiatric disorders, but few have evaluated the potential mediation of that relationship by genetic factors. MDD and anxiety disorders as well as CRP levels and BMI have been demonstrated to be heritable. A meta-analysis estimate of heritability of MDD in adults is 37% (234), with evidence that much of the heritability is explained by many common risk variants of small effect (134). Both CRP and BMI are also polygenic traits with heritabilities estimated to range from 20% to 52% (79, 361-363) for CRP and from 40% to 80% for BMI (364, 365). Worns et al. hypothesized that BMI and adipose tissue mediated a major part of CRP heritability (79), but a study of healthy female twins concluded that obesity and adipose tissue influence baseline CRP levels independent of genetic influences (350). Functional allelic variants of the genes for the inflammatory marker CRP have been identified as increasing risk for depression (62). Specifically, haplotypic variation in the *CRP* locus has been found to moderate an association between CRP levels and depressive symptoms, partly through body mass index (366).

To date, no genome-wide significant loci have been found for either MDD or anxiety disorders in Caucasians. This finding may be due to limitations of sample sizes or heterogeneity of these conditions. Support for heterogeneity as a limitation comes from a study conducted by the CONVERGE consortium, which recruited 11,670 Han Chinese women (367). The study aimed to further reduce heterogeneity by recruiting only those with recurrent MDD (hence also likely to be a severe illness). The authors attributed this recruiting of more homogeneous cases to the identification (and replication in an independent sample) of two loci contributing to MDD risk (367).

Genetic variants for CRP and BMI have been identified. A recent meta-analysis of GWA studies including more than 80,000 individuals identified 18 loci associated with CRP levels. These loci included those implicated in pathways related to the immune system or metabolic syndrome (368). Genetic profile risk scores, calculated to model the effect of the identified loci on CRP levels, have been found to explain approximately 5% of the variation in CRP levels in independent samples (368). If there is a causal relationship between CRP levels and depression and anxiety disorders whereby raised CRP levels are a risk factor for development of psychopathology, we would expect to find that alleles that

increase CRP levels also show evidence of increasing risk to develop these psychiatric disorders. Furthermore, among those who have experienced abuse in childhood, individuals carrying more CRP alleles may be at greater risk of developing psychiatric disorders in adulthood. Assigning causality in the relationship between BMI and CRP from observational data is problematic, but Mendelian Randomisation analysis (369) indicates that increased BMI leads to increased levels of circulating CRP (370), consistent with the hypothesis that the CRP and MDD association is mediated by BMI. Likewise, if there is a direct causal relationship between CRP and MDD, then genetic variants that are associated with increased circulating CRP may also lead to increased risk of MDD.

Here, we first investigate the phenotypic relationship between CRP and MDD and anxiety disorders, and CRP levels and childhood trauma in a large population-based cohort of twins from the Australian Twin Registry. We attempt to elucidate the role of BMI in the association by testing how the association changes when accounting for the relationship between BMI and CRP. We then investigate the potential association at the genetic level by evaluating whether those carrying more CRP-associated alleles are at increased risk for MDD or anxiety disorders, under the hypothesis that natural variation between individuals in circulating CRP is associated with variation between individuals with respect to common mood disorders. We compare the results to those from using BMI genetic risk scores to predict MDD or anxiety.

Methods

Samples

Overview:

Our analyses use measures from participants enrolled in studies conducted at QIMR Berghofer Medical Research Institute (QIMRB) that drew participants from the Australian Twin Registry. All procedures were approved by the Human Research Ethics Committee of QIMR Berghofer. Briefly, C-reactive protein (CRP) was measured as part of the protocol of three studies, with partly overlapping samples (Figure 6-1). The protocol of two of those studies also involved a telephone-based diagnostic interview for MDD and anxiety disorders. All phenotypic analyses of CRP and MDD and anxiety disorders included only participants from those two studies. One other study measured CRP as part of the protocol, but did not include a diagnostic interview. A number of other waves of data

collection also included phone interviews diagnostic for MDD, but did not measure CRP. Genetic analyses included anyone with information on lifetime MDD and anxiety and so therefore participants were drawn from a larger number of studies. In total, CRP measurements were available on 14,750 participants (37.64% males, 62.36% females) from 3,217 families, while BMI was measured on 8,800 participants (43.67% males, 56.33% females) from 3,206 families. Across all the studies, a total of 2,609 MDD cases and 6,785 controls with genome-wide genotypes were available for analysis. With regard to anxiety disorders, a total of 1,839 cases and 7,849 controls had genome wide genotypes for analysis. From those cases and controls, 1,596 unrelated MDD cases, 1,856 unrelated MDD controls, 1,172 unrelated anxiety disorder cases, and 1,616 unrelated anxiety disorder controls were utilised for the profile scoring analysis.

Details of the contributing studies are as follows:

The "Anxiety" study and NAG/IRPG study – CRP, diagnostic interview data:

CRP was measured contemporaneously with assessment of lifetime diagnosis of MDD and anxiety disorders as part of two studies. The first study, known as the "Anxiety Study" (AX) is described in detail in Kirk et al (371). Based on previous responses to the 12-item neuroticism scale from the revised short form of the Eysenck Personality Questionnaire, twins and siblings were invited to participate in the study. Participants were selected with the purpose of maximising statistical power for a linkage analysis by identifying families where twins were highly concordant for high or low neuroticism, or where twins were highly discordant for neuroticism. Participants were interviewed over the phone by trained interviewers who administered the Composite International Diagnostic Interview (CIDI), which assessed DSM-IV diagnostic criteria for MDD, Generalised Anxiety Disorder, Social Phobia, Obsessive Compulsive Disorder (OCD), Agoraphobia, and Panic Disorder. Interviews were conducted with 2,475 individuals, of whom 1,906 also provided a blood sample from which CRP could be measured.

The second study was conducted between 2003 and 2007 and combines multiple separate studies focused on the genetics of alcohol and nicotine addiction measured in community samples. These studies are called the NAG and Inter Related Project Grant studies (NAG/IRPG). The individual studies are described in detail in Table 2 of Hansell et al. (372). A computerised version of the Semi-Structured Interview for the Assessment of

the Genetics of Alcoholism (SSAGA) was administered by trained interviewers. A total of 8,012 individuals completed the sections from which DSM-IV diagnosis of MDD and anxiety disorders could be made, and also provided a blood sample for measurement of CRP. OCD was not assessed as part of the protocol, so for individuals who had been measured in both the AX and NAG/IRPG studies (n = 537) we included only the data from the AX study, as the instrument also assessed OCD. Height and weight information were collected as part of this study. The NAG/IRPG study also included a questionnaire about childhood trauma that included a section on childhood sexual abuse (373).

SSAGA and Twin89 – diagnostic interview and GWAS data – no CRP measurement:

In addition to the AX and NAG/IRPG studies, lifetime diagnosis of MDD and anxiety disorders was also assessed in two earlier studies at QIMRB. The studies, which are described in detail elsewhere (374) and have non-overlapping samples, are known as the SSAGA and Twin89 (TW89) studies. These studies were conducted prior to the AX and NAG/IRPG studies. In both studies, a semi-structured psychiatric interview was conducted by telephone between the years of 1996 to 2000. This telephone interview contained lifetime assessments of alcohol and nicotine dependence, drug use, childhood conduct disorder, DSM-IV diagnosis of MDD and anxiety disorders, in addition to childhood sexual abuse (CSA) and childhood physical abuse (CPA) screening questions. Both CSA screening questions (375, 376) and CPA screening questions have been described previously (377). The target sample for the SSAGA study was twin pairs from the Australian Twin Registry (ATR) born between 1892 and 1963 and their siblings. The TW89 study targeted twins from the ATR born between 1964 and 1971.

The AX and NAG/IRPG studies described earlier drew a substantial proportion of participants from the SSAGA and TW89 studies, but also included new participants. However, no measures of CRP were taken until the AX study.

Childhood Sexual Abuse:

In addition to the SSAGA, TW89 and NAG/IRPG studies, two further studies assessed childhood trauma. Where one or more member of the twin pair endorsed either form of abuse in the TW89 study, the twins, full siblings and parents went on to be interviewed in the Childhood Trauma Study, between the years of 2003 – 2008. Control families (those in which twins had not reported either form of abuse in earlier studies) were also interviewed.

The Childhood Trauma Study was administered as a computer assisted diagnostic interview, and included an evaluation of trauma (both CSA and CPA) prior to age 18 years, based on the Christchurch Trauma Assessment (378-380).

A further study took place between 2010 and 2013, and consisted of a follow-up interview of the cohort, constituting the sample for a funded GWAS of alcohol dependence. Data for this survey was initially collected by a structured telephone interview, and later by online self-report questionnaire. The study collected detailed information on environmental stressors including childhood sexual and physical abuse, lifetime assessments of PTSD (DSM-IV diagnosis) and illicit drug dependence, and updated information on alcohol use and alcohol dependence (DSM-IV diagnosis).

Childhood sexual abuse was defined as any reported instance of unwanted sexual contact before the age of 16 years.

Figure 6-1: Overview of contributing studies
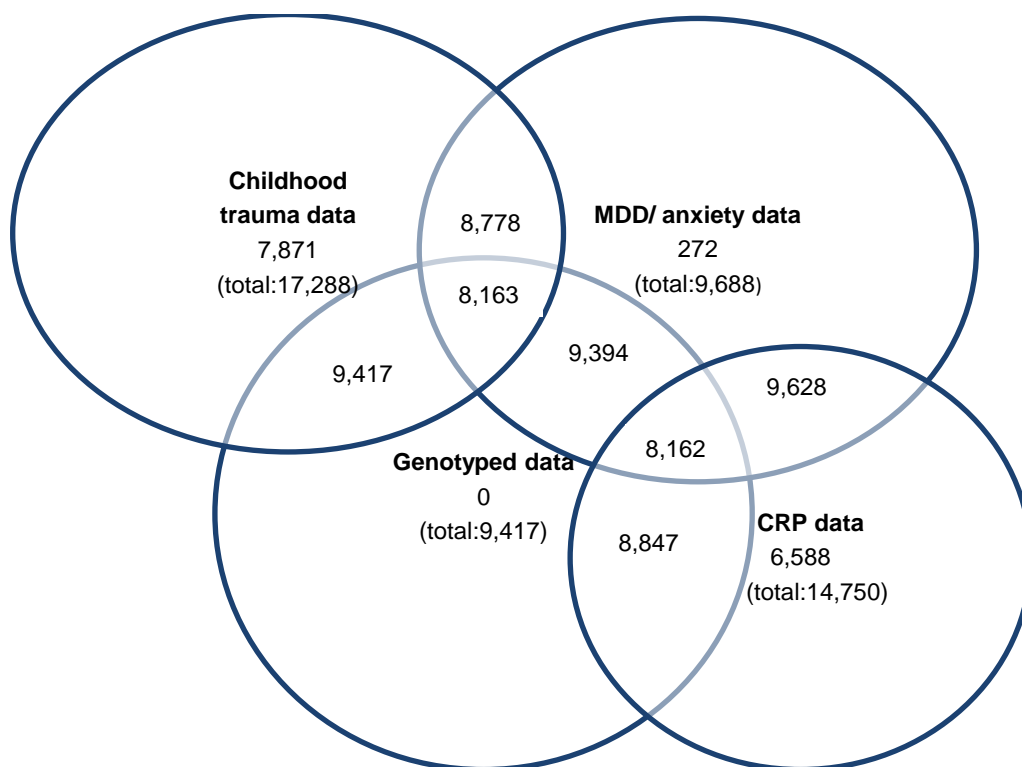
Key: **CRP data:** from Anxiety Study (AX), NAG and Inter Related Project Grant (NAG/IRPG) studies; **MDD / anxiety data:** from AX, NAG/IRPG, Semi-Structured Interview for the Assessment of the Genetics of Alcoholism (SSAGA), Twin89 (TW89) studies; **Childhood trauma data:** from NAG/IRPG, SSAGA, TW89, Childhood Trauma Studies; **Genotyped data:** from follow-up of SSAGA, TW89, and NAG/IRPG cohort.

Notes: Total numbers (in brackets) show total numbers of study participants with CRP data, MDD / anxiety data, childhood trauma data, and genotyped data. The numbers immediately above the bracketed total numbers are the number of individuals with only that type of data (e.g. 272 individuals have MDD / anxiety data but no other type of data; no individuals have only genotyped data). Numbers in overlapping parts of circles show numbers of study participants with each type of data represented by the circles that overlap (e.g. 8,847 individuals have both CRP data and genotyped data. Of these 8,847 individuals, 8,162 have CRP data, MDD / anxiety data, and genotyped data).

Measurement of CRP

Blood samples were separated into serum at the time of collection. Samples were stored at -70 degrees Celsius until time of measurement. A quantitative measurement of serum CRP (in mg/L) was obtained by enzyme-linked immunosorbent assay (ELISA), using a high-sensitivity sandwich assay.

Genotyping

A total of 9,394 individuals had genome-wide SNP data. Genotype data had been generated as part of a number of projects undertaken by the Genetic Epidemiology group at QIMRB. DNA samples were collected in accordance with standard protocols and submitted to different genotype centres using different SNP platforms (Illumina 317K, IlluminaHumanCNV370- Quadv3, and Illumina Human 610-Quad). SNPs were called using the Illumina BeadStudio software. A standard quality control procedure was applied to each project individually, prior to imputation (381). A detailed description of the quality control (QC) steps and procedure for detection of ancestry outliers is given elsewhere (382).

A set of 274,604 SNPs that were common to all of the genotyping chips were used for imputation, which was performed using the program MACH (383). The imputation process uses information on the haplotype structure in the human genome from the HapMap project (Release22 Build36) to impute non-genotyped SNPs in the sample. The imputed SNPs were screened further for Mendelian errors, minor allele frequency and missingness. Only SNPs with an imputation quality score (R^2) greater than 0.3 were retained, which resulted in a total of 2,380,486 SNPs.

Association between CRP, MDD and anxiety disorders

Individuals older than 70 years old (n=492) were removed from the analysis because the greater risk of physical comorbidities might confound the association of the mood disorders with CRP. Across the AX and NAG/IRPG studies, a total of 2,445 MDD cases and 5,786 MDD controls with CRP measures were identified (Table 6-1). A total of 1,783 individuals met the criteria for an anxiety disorder, and 7,061 individuals were classified as controls (Table 6-1). A total of 4,945 individuals did not meet the criteria for either disorder, 3,301 had either an anxiety disorder or depression, and 942 individuals met the criteria both for MDD and an anxiety disorder.

Table 6-1: Sample sizes for CRP phenotypic analyses

Phenotype	All			% Female	
	Families	Cases	Controls	Cases	Controls
CRP – sample size					
MDD	3184	2445	5786	63.3	53.2
Any Anxiety	3217	1783	7061	60.7	55.3
Social Phobia	3189	1373	6922	59.4	55.6
Panic Disorder	3190	359	7996	64.3	56.0
CSA	2695	1275	6008	73.3	51.3

Key: CRP = C-reactive protein; MDD = major depressive disorder

Statistical analysis:

CRP data were log-transformed to reduce skewness of the distribution. The relationship between log CRP levels and psychiatric disorders was evaluated by linear regression, with age at CRP measurement and sex included as covariates. We considered models where the psychiatric disorder cases used were 1) MDD, 2) anxiety disorders, 3) any disorder, or 4) both disorders. Only those not meeting the criteria for MDD or an anxiety disorder were considered as controls in models 3) and 4). Further models fitted BMI as a covariate. BMI was not measured in the AX study, but had been measured on the participants in previous studies; the BMI measurement taken closest to the time of the AX study was used (N =1,804). We also constructed linear regression models where log CRP levels in those with a history of childhood sexual abuse were compared to those with no history of childhood sexual abuse, with age (at time of CRP measurement), sex, and BMI as covariates.

Genetic Risk Profile Scoring

Genetic profile risk scores (GPRS) were estimated for each individual with genome-wide genotype data as the sum of the number of associated alleles (identified from the "discovery" sample) weighted by their estimated effect sizes. The discovery sample GWAS studies that identified associated alleles were the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium for CRP (368) and the Genetic Investigation of Anthropometric Traits (GIANT) Consortium (384) for BMI.

Results from the CHARGE meta-analysis of log CRP levels (368) were used to generate profile scores for all individuals who had been genotyped and who had either completed a telephone-based diagnostic interview or had been asked about childhood sexual abuse. The GIANT meta-analysis was conducted with QIMR samples removed so as to ensure no overlap between the discovery sample and the target sample in the present study. Sample sizes for the genetic profile scoring analyses are provided in the footnotes of Tables 6-3 and 6-4.

The profile scoring methodology has been described in detail elsewhere (333, 385). PLINK (344) was used first to perform LD-based clumping so as to make a quasi-independent SNP set. SNPs in LD with the most associated SNP in a 1MB region at $r^2 > 0.25$ were removed by the clumping algorithm. Then profile scoring was performed using PLINK. Genetic profile risk scores were generated for 5 sets of SNPs: SNPs with p-values of <0.001, <0.01, <0.1, <0.5, and all clumped SNPs respectively from the CRP and BMI meta-analyses. Linear regression was used to see how accurately the CRP and BMI profile scores could predict CRP and BMI respectively. Logistic regression was used for analysis of the case/control variables.

To aid in the interpretation of the results, one individual per family was selected for inclusion in the profile scoring analyses (n=2,394 individuals for CRP; n=2,396 individuals for BMI). For the MDD and anxiety analyses, cases were prioritised from families where there were cases and controls in order to increase statistical power. We subsequently conducted the profile scoring analyses with relatives included.

Initially, the CRP profile scores were used to predict CRP, MDD and anxiety disorders across the entire sample, and also to predict MDD and anxiety together as one case group. Profile scoring was also performed to test if the CRP profile scores predicted BMI.

Results

Association between CRP and MDD and Anxiety Disorders

The mean age at CRP measurement was 45.3 years (standard deviation (SD) 10.1 years). The mean BMI of 25.97 kg/m^2 (SD 4.87 kg/m^2) was slightly above the normal range of 20-25 for adults. Mean log CRP was 0.64 (SD 1.12). The mean log CRP for those with both MDD and a co-morbid anxiety disorder was higher than controls with neither disorder, however this difference in means was not statistically significant (p=0.13). The same pattern was seen in males and females although mean log CRP was higher in females (p =< 2.2x10^{-16}).

Consistent with another published study (386), mean log CRP was higher in those with a history of CSA in females (p=0.029, Table 6-2), however this difference was not significant after including BMI in the model (p=0.56). In males mean log CRP was higher in those without a history of CSA (p=0.11) (Table 6-2). When the total sample was divided into those with no history of CSA and those with a prior history of CSA, there was no significant difference in mean log CRP between MDD cases and controls (Table 6-2).

Association between MDD and CRP levels

The point estimates of log CRP levels were higher in MDD cases compared to controls (Table 6-2), although the difference was not significant (p=0.12). Upon inclusion of BMI in the model, the effect estimate and the statistical significance of the effect of MDD on log CRP levels were reduced, reflecting the highly significant effect of BMI on log CRP levels (β = 0.09, S.E = 0.002, p = < 2 x 10^{-16}). However, owing to missingness in the data, inclusion of BMI also reduced the number of MDD cases and controls to 2,433 cases and 5,755 controls, hence reducing power (Table 6-2).

Association between Anxiety Disorders and CRP levels

A nominally significant association between anxiety disorders and CRP was found (Table 6-2, p =0.012). The estimated effect and significance increased when including only controls who were negative for both anxiety disorders and MDD (Table 6-2, p =0.0047). However, this result barely survives correction for performing 10 tests (although this is conservative since owing to the high rate of comorbidity between CSA, anxiety and MDD, the statistical tests are correlated). Upon inclusion of BMI in the regression model, the estimate of the effect of having an anxiety disorder decreased, and statistical significance reduced, both when including those who did not have an anxiety disorder only, and when only including those who did not have either an anxiety disorder or MDD (Table 6-2).

A significant association was also found between higher log CRP levels and social phobia (p=0.0045), however this was no longer significant when BMI was included in the model (p=0.090, Table 6-2). This association was likely to be driving the association between CRP and anxiety disorders (in the combined anxiety disorders group), and when we excluded those individuals who met criteria for social phobia from the analysis, the association was no longer significant (p =0.099) – however excluding these individuals reduced the sample size to 515 cases (all 515 had BMI data).

Association between childhood sexual abuse and CRP levels

The association between log CRP levels and a history of CSA was not statistically significant (p=0.26 when controlling for age and sex, p=0.46 when also including BMI in the model – Table 6-2).

Table 6-2: Regression analyses for MDD, Anxiety disorders, or CSA explaining variation in log CRP

Sample	Number cases / controls[a]	β[a] (S.E.)	P-value[a]	Number cases / controls #	β (S.E.) #	P-value#
Regression of Log CRP on MDD vs No MDD						
Total sample	2445 / 5788	0.037(0.024)	0.12	2433 / 5755	0.015(0.022)	0.50
Regression of Log CRP on Anxiety vs No Anxiety						
Total sample	1783 / 7061	0.074(0.029)	0.012	1770 / 7027	0.052(0.027)	0.058
Regression of Log CRP on Anxiety vs No Anxiety or MDD						
Total sample	1783 / 4945	0.087(0.031)	0.0047	1770 / 4918	0.055(0.029)	0.055
Regression of Log CRP on Social phobia vs No Social phobia						
Total sample	1374 / 6924	0.083(0.029)	0.0045	1368 / 6885	0.046(0.027)	0.090
Regression of Log CRP on CSA vs No CSA						
Total sample	1275 / 6008	0.035(0.031)	0.26	1275 / 6001	0.021(0.029)	0.46
Males only	341 / 2924	-0.094(0.060)	0.11	341 / 2922	-0.008(0.057)	0.89
Females only	934 / 3084	0.094(0.043)	0.029	933 / 3077	0.023(0.039)	0.56
Regression of Log CRP on MDD vs No MDD in those reporting no CSA						
Total sample	1600 / 4262	0.051 (0.032)	0.11	1596 / 4257	0.035 (0.029)	0.24
Males only	667 / 2196	0.082 (0.046)	0.074	666 / 2195	0.060 (0.043)	0.16
Females only	933 / 2066	0.027 (0.045)	0.55	930 / 2062	0.015 (0.040)	0.71
Regression of Log CRP on MDD vs No MDD in those reporting CSA						
Total sample	580 / 666	0.042 (0.067)	0.54	579 / 666	0.004 (0.062)	0.95
Males only	144 / 188	0.073 (0.124)	0.56	144 / 188	-0.006 (0.122)	0.96
Females only	436 / 478	0.033 (0.080)	0.68	435 / 478	0.025 (0.071)	0.73

Key: a = regression includes age and sex as covariates; # = regression includes age, sex and BMI as covariates; β = regression coefficient for binary factors as listed; CSA = history of childhood sexual abuse; MDD = major depressive disorder; S.E = standard error

Genetic Profile Scoring

Results of the genetic profile scoring analyses that used the CRP GWAS results to predict CRP and also MDD and anxiety disorders in the QIMRB sample are presented in Table 6-3. CRP profile scores predicted CRP in the QIMRB sample (column 4), with up to 2.4% of the variance of CRP explained by this age, sex, and BMI adjusted model. The CRP profile scores did not predict MDD or anxiety case/control status (columns 5 - 7, Table 6-3). CRP profile scores did not predict BMI (results shown in Appendix, page 186).

Table 6-3: Log CRP genetic profile risk scores – prediction of CRP, MDD, Anxiety

CRP sample size = 2,637 individuals (1,246 males; 1,391 females)

P-value of SNPs in CRP discovery GWAS	N SNPs	Variance of CRP explained (R^2)	P-value for prediction of CRP	P-value for prediction of MDD	P-value for prediction of Anxiety	P-value for prediction of Any Disorder
P < 0.001	1,972	2.42	5.2×10^{-14}	0.44	0.98	0.88
P < 0.01	11,154	2.12	1.8×10^{-12}	0.77	0.73	0.85
P < 0.1	70,982	1.18	1.3×10^{-7}	0.92	0.10	0.36
P < 0.5	241,214	0.84	6.9×10^{-6}	0.78	0.18	0.41
All SNPs	361,282	0.79	1.4×10^{-5}	0.72	0.15	0.42

Key: Any Disorder = MDD or Anxiety or both; BMI = body mass index; CRP = C-reactive protein; MDD = major depressive disorder

MDD: number cases = 1,165 (487 males, 678 females); number controls = 1,182 (632 males, 550 females)

Any Anxiety: number cases = 978 (435 males, 543 females); number controls = 1,368 (704 males, 664 females)

Any Disorder: number cases = 1,752 (761 males, 991 females); number controls = 885 (485 males, 400 females)

BMI profile scores predicted BMI in the QIMRB sample, with up to 6.8% of the variance explained (Table 6-4). When BMI profile scores were used to predict MDD in the QIMRB sample, BMI did not predict MDD. Similarly, BMI SNPs did not significantly predict anxiety case/control status. Furthermore, BMI and CRP profile scores did not predict MDD or anxiety case / control status when relatives were included in the analyses (results not shown).

Table 6-4: BMI genetic profile risk scores – prediction of BMI, MDD, Anxiety;

BMI sample size = 3,235 individuals (1,424 males; 1,811 females)

P-value of SNPs in BMI discovery GWAS	N SNPs	Variance of BMI explained (R^2)	P-value for prediction of BMI	P-value for prediction of MDD	P-value for prediction of Anxiety	P-value for prediction of Any Disorder
P < 0.001	2966	6.54	1.3×10^{-7}	0.23	0.47	0.39
P < 0.01	10382	6.84	2.9×10^{-9}	0.34	0.41	0.26
P < 0.1	60778	6.47	2.9×10^{-7}	0.18	0.26	0.063
P < 0.5	233696	5.94	2.6×10^{-4}	0.22	0.79	0.26
All SNPs	370278	5.91	3.5×10^{-4}	0.16	0.70	0.19

Key: Any Disorder = MDD or Anxiety or both; BMI = body mass index; MDD = major depressive disorder

MDD: number cases = 1,596 (597 males, 999 females); number controls = 1,856 (831 males, 1,025 females)

Any Anxiety: number cases = 1,172 (505 males, 667 females); number controls = 1,616 (799 males, 817 females)

Any Disorder: number cases = 2,283 (915 males, 1,368 females); number controls = 952 (509 males, 443 females)

Discussion

CRP is an acute phase reactant, and higher levels of CRP have been associated with MDD (7, 271), anxiety disorders (33), and a history of early childhood adversity (28, 29). We set out to investigate the relationship between (i) CRP levels, MDD, and anxiety, and (ii) CRP levels and CSA, and to test whether those who carry more CRP increasing alleles, are at increased risk to develop depression and anxiety.

Consistent with previous studies we found some evidence of increased levels of CRP in those with MDD compared to controls, but this did not reach statistical significance (p = 0.12). Inclusion of BMI in the model reduced the significance of the association further, indicating that increased BMI in MDD cases may explain part of the increased levels of CRP.

Our results do not support a role for inflammation in the aetiology of anxiety disorders. Although CRP levels were significantly higher in those with any anxiety disorder compared to those who did not have either MDD or an anxiety disorder (p=0.0047), the association was reduced to non-significance after accounting for BMI. We specifically focused on the association between CRP and social phobia, as this was the anxiety disorder with the most cases. There was a significant association between CRP and social phobia (p=0.0045), but again after adjusting for BMI the association was no longer significant.

This study found that BMI appears to be important in the phenotypic relationship between CRP and MDD and anxiety. A prior study found the longitudinal association between Generalised Anxiety Disorder and later CRP levels was mediated by BMI (33). With the prevalence of obesity increasing globally (387), this mediation by BMI is important. Increased rates of obesity are associated with increased rates of cardiovascular disease, diabetes (388), and MDD (355). Furthermore, inflammation may contribute to increased risk of illnesses such as cardiovascular disease (359) and MDD (389).

This is one of the largest studies conducted of CRP with anxiety disorders to date and further supports the notion of shared aetiology between anxiety and MDD. In both men and women, the highest CRP levels were among those with comorbid anxiety and MDD. Although anxiety and MDD co-occur, BMI appears to mediate the relationship between these disorders and peripheral CRP levels.

In females, we found the highest CRP levels were in those with anxiety disorders and a history of CSA, however the difference compared to controls was not significant. Higher CRP levels in adults with a history of childhood adversity have been observed previously, however the difference was only statistically significant in those adults with current depression (28, 29). It is thought that childhood adversity is important in explaining the co-occurrence of depression and inflammation (28-30). Furthermore, an interaction effect has been observed between polygenic risk scores and childhood trauma in MDD (390). In males we found no evidence of a relationship between circulating CRP and report of childhood sexual abuse compared to males not reporting (p=0.11), however we note small numbers of males with this history and that men may be unlikely to admit to a history of sexual abuse in a phone interview.

We did not find significant evidence that individuals who carry more alleles that increase CRP levels or BMI are at greater risk of suffering from MDD or anxiety disorders. We could not find other studies that had used CRP genetic profile scores with the objective of predicting anxiety disorders. However, one previous study investigated whether BMI genetic risk scores predict obesity in patients with MDD (391). The authors found that genetic risk scores had a larger effect on BMI in depressed patients, with the largest effect in patients with severe MDD (391). In an earlier study, the same group used BMI genetic risk scores as instrumental variables in a Mendelian randomisation study (392). In this study, using weighted genetic risk scores or the *FTO* genotype as instrumental variables did not support the hypothesis that obesity increased the risk of MDD (392). The effect of the *FTO* gene on BMI has also been investigated in an earlier study examining the genetic mechanism underlying the reported association between BMI and MDD (393). The authors found the association between *FTO* and BMI to be specific to the group with MDD (and not the controls) (393). There appears to be a complex relationship between psychiatric disorders, CRP and BMI. Accounting for BMI in the present study reduced the significance of the association between CRP and MDD and anxiety.

Assessing measures of depression through a structured interview (even through a trained interviewer), is a limitation of this study. The use of telephone rather than face to face interviews may have resulted in participants providing different responses to some questions. In particular, it is unclear how telephone interviews would affect responses to questions of a sensitive nature such as exposure to childhood sexual abuse. It is possible that some participants may have felt less comfortable responding to these questions when asked over the telephone whereas others may have found it easier to disclose such experiences with the relative anonymity that the telephone interview provided.

Clinical samples may provide stronger evidence for the association between CRP and psychopathology. Sample sizes were small for some of the anxiety disorders, so individuals meeting criteria for any of the anxiety disorders were grouped together in the analysis. It was therefore not possible to compare the association with CRP across anxiety disorders. Further investigation of the role of inflammation in anxiety disorders is warranted in those disorders where there were small case numbers. A further limitation of the present study is that we correlate lifetime MDD and anxiety with current CRP, which does not give a picture of present levels of distress, and participants may be in remission and therefore have reduced levels of CRP.

CRP values have been reported to remain stable despite multiple freeze/thaw cycles (394). However another study found CRP values increased after long-term frozen storage, with values showing a high correlation between baseline (before storage) and after storage (395). In our study there was also some variation in the time of day that blood was collected, with most blood samples collected in the morning, and some collected in the afternoon or evening (to suit study participants). Furthermore, only one marker of inflammation was measured in the peripheral blood. Measuring CRP in the CSF may provide more sensitive results, however the invasiveness of this procedure would preclude this. Reliance on self-report BMI may also influence the results.

Conclusion

A point estimate of higher mean log CRP in the group that met criteria for MDD and a co-morbid anxiety disorder, compared to those with neither disorder was not statistically significant (p=0.13). The association between CRP and social phobia (p=0.0045), was not significant after adjusting for BMI (p=0.090). Other results were not significant. CRP

profile scores did not predict MDD or anxiety disorders in this large community sample. The complex relationship between CRP, BMI, MDD and anxiety disorders requires further study, but our results suggest that BMI is the key factor that mediates the reported relationship between circulating CRP and psychiatric disorders. Further phenotypic and genetic studies with greater power are needed; presentation of our results allows contribution to future meta-analyses.

CHAPTER 7: Thesis Discussion

The hypothesis of this thesis was that pro-inflammatory cytokines, inflammatory markers, and biomarkers altered in inflammatory states would be phenotypically and genetically associated with measures of depression in adolescents. This overarching hypothesis was developed from findings in the adult literature, where raised pro-inflammatory cytokines have been associated with Major Depressive Disorder (MDD) (6), anxiety disorders (16), and psychosis (15). Whilst the literature has expanded in recent years, systematic literature reviews (20, 301) have concluded further research is warranted, to improve the understanding of the role of cytokines and inflammatory markers in adolescent mental illness in relation to neurodevelopment, stress and maltreatment. Therefore, this chapter includes discussion of areas for further research, in the context of how this may lead to improved treatments for adolescent MDD. To provide context there is some discussion of similarities and differences in regard to inflammation between adolescents and adults with MDD.

MDD in children and adolescents appears to be associated with immune system dysregulation, including raised pro-inflammatory cytokines (161). More recently, a developmental model of psychoneuroimmunology has been proposed, which connects stress exposure in childhood to immune function and physical and mental illness in adulthood. This model links immune mechanisms from as early as the prenatal period to factors such as neurodevelopment and stress (396). Key to this model is the finding that a history of early childhood adversity has a significant role in the co-occurrence of MDD and inflammation throughout the life span, with this "biological embedding" seen as early as the adolescent years (28, 29).

Table 7-1 details the overall findings of this thesis. In summary, the findings with potential clinical implications are as follows: i) the relationship between cytokines and MDD may not be the same in adolescents as in adults, ii) cytokines able to be measured in plasma (tumour necrosis factor-receptor type 1 (TNFR1) and transforming growth factor-β1 (TGF-β1)) were found to be moderately heritable, and iii) the relationship between C-reactive protein (CRP) and MDD, and between CRP and anxiety disorders appears to be mediated by body mass index (BMI).

Table 7-1: Overall thesis findings

Thesis Chapter	Finding
Literature review (chapter 2)	Relationship between cytokines and MDD may not be the same in adolescents as that seen in adults and detailed investigation is warranted
Cytokines, other inflammatory markers, and other biomarkers of immune function in healthy adolescents (chapter 3, part 1)	Cytokines able to be measured in plasma (TNFR1 and TGF-β1) were found to be moderately heritable. Important limitations in the measurement of cytokines were encountered.
Iron (chapter 5)	The phenotypic and genetic correlations between measures of iron and depression were not different from zero. Depression measures in adolescents were significantly higher in those in the middle 10th percentile of transferrin saturation measures compared to those in the highest 10th percentile, which may imply a highly non-linear relationship between measures of blood iron and depression
C-reactive protein (chapter 6)	The relationship between (i) CRP and MDD, and (ii) CRP and anxiety disorders appears to be mediated by BMI

Cytokines in adolescent MDD

The systematic literature review (Chapter 2) found that depression in adolescents may not show the same immune changes that occur in adults. Specifically, results of plasma levels of tumour necrosis factor-α (TNF-α) in MDD have been inconsistent with different developmental stages. Plasma levels have been found to be significantly decreased in adolescents with suicidal ideation (191), yet in adults, regardless of suicidality, an association between elevated levels of circulating TNF-α and MDD has been observed in a meta-analysis (6).

Another conclusion of Chapter 2 was that interleukin-1β (IL-1β) levels have also been reported to differ in children and adults with MDD. In adults, elevated levels of IL-1β have

been reported (201), however in children with MDD normal levels of IL-1β were reported (183). The important potential confounder of stress was addressed in the study by Brambilla et al., who examined IL-1β and TNF-α levels in children with first episode MDD that was unlikely to have been preceded by stressful life events (183). This study also reported no difference in TNF-α levels between those with MDD and controls (183).

Cytokines in healthy adolescents

Studying the variation of cytokines in healthy adolescents provided useful information to guide future research of cytokines in clinical populations. By estimating the heritability of cytokines in healthy adolescents, we were able to determine which cytokines and other inflammatory markers could be measured, and which cytokines may have a genetic contribution to variation of circulating levels. We found a genetic contribution to variability of the cytokines TGF-β1 and TNFR1 in healthy adolescent twins. This is potentially of relevance in the aetiology of MDD in adolescents. In particular, the possibility of shared genetic factors between these cytokines and MDD could be investigated in future studies.

The negative correlation between pro-inflammatory cytokines and Vitamin D in adolescent twins (Chapter 3) was not significant. However, Grudet et al. found a negative correlation in a clinical sample of adults, and found that low levels of Vitamin D were associated with higher levels of the pro-inflammatory cytokines IL-6 and IL-1β in study participants with a psychiatric diagnosis (397). Understanding the role of Vitamin D deficiency in inflammation may be an important area for further study in both healthy and clinical samples (12, 25). It could be hypothesised that there will be a negative correlation found between Vitamin D and pro-inflammatory markers in MDD, anxiety disorders and psychosis. Study numbers included 27 participants with MDD in the study by Grudet et al. (397), so this could be tested in studies with larger numbers that use a longitudinal design (to explore the direction of association).

The pilot study conducted (Chapter 3) also highlighted the difficulties in measuring cytokines. The plasma cytokines measured as part of a multiplex did not generate meaningful results. The cytokines, other inflammatory markers, neurotrophins and antibodies measured in dried blood spots were sensitive to paper type and plate effects, often generating null reads for one or other paper. The problems associated with generation of high quality measures of circulating cytokines need to be addressed before

attempts can be made to measure circulating levels of cytokines in the clinical group of adolescents.

Cytokines in a clinical group of adolescents

As yet the levels of circulating cytokines have not been measured in the study in the clinical sample (Chapter 4), since the final sample size was too small to generate useful results. It is important that the cytokines can be measured accurately. Furthermore, combining the blood samples of the adolescents who participated in our study with another study will provide sufficient statistical power. Potential ways to progress the measurement of circulating cytokine levels in a clinical group of adolescents are discussed later in this chapter.

Although the cytokines have not yet been measured, this work was influential in changing research protocols at the Royal Brisbane and Women's Hospital (RBWH). Significant challenges were encountered in the collection of data, which included obtaining consent for study participation (prior to discharge) in those adolescents who had a brief admission to the Inpatient Unit. In view of these challenges in recruiting study participants, RBWH HRECs have now approved a process of consent where informed consent is obtained from parents over the telephone, and then later obtained from participants when they have capacity.

Iron

In addition to cytokines, other inflammatory markers, including measures of ferritin and transferrin, have been linked to MDD (9). Specifically, lower levels of serum iron and transferrin, and higher levels of serum ferritin have been found in MDD (9). These changes in iron measures may be seen in inflammatory states, however in the absence of inflammation, serum ferritin may be low if there is inadequate dietary intake of iron. In adolescents, depression measures were significantly higher in those in the middle 10th percentile of transferrin saturation measures compared to those in the highest 10th percentile of transferrin saturation measures (Chapter 5). As found in the existing literature examining the relationship between iron and depression (316), it may be that a phenotypic relationship is more likely to be observed at the lower range of iron measures. We did not measure circulating levels of haemoglobin, as we were hypothesising that

markers of inflammation rather than haemoglobin *per se* would be associated with MDD. We also do not have information on dietary intake of iron (or information on potential causes of iron loss) in our study, however using longitudinal measures of iron and depression could provide useful information.

To the best of our knowledge, there are no previous studies that had used genetic profile risk scores to investigate the relationship between iron measures and depression, so in keeping with our hypothesis that markers of inflammation would be associated with MDD, we used multiple approaches to explore evidence for a genetic relationship between these measures. The variation between individuals in iron measures is partly under genetic control, with highly significant SNPs in the gene *TF* (transferrin) (329) and SNPs affecting ferritin (330). After correction for multiple testing, genetic profile risk scores (GPRS) for the iron measures did not predict depression measures in the adolescent or adult cohort. GPRS for MDD showed no significant association with iron measures in adolescents or adults after correcting for multiple testing. So no strong evidence was found for a genetic relationship between iron and depression measures in adolescents or adults, despite the study having sufficient power to address the hypothesis.

C-Reactive Protein

BMI appears to mediate the reported relationship between circulating CRP levels and MDD and anxiety disorders. This is important in view of the increasing prevalence of obesity (387), and the high prevalence of MDD and anxiety disorders in the population, with the onset of many of these disorders often in childhood or adolescence.

Prior studies have found higher CRP levels in males with a history of MDD (compared to males with no history of MDD) (7, 32). However not all studies that have adjusted for BMI have found an association between elevated levels of CRP and MDD (145). Whilst a genetic relationship between CRP and MDD was not observed (Chapter 6), very few studies have used CRP genetic profile risk scores with the objective of predicting MDD or anxiety.

A previous study observed an association between higher CRP levels and Generalized Anxiety Disorder (GAD) (33). However that study had much larger numbers of participants. Whilst CRP did not predict anxiety status in our study, it is likely that the

sample size was too small (with the number of individuals who met criteria for any anxiety disorder besides social phobia being small).

With the expansion of the stress depression literature, there is more support for the hypothesis that early childhood adversity is associated with inflammation in later life (28, 29). However, we did not find a significant association between CRP levels and a history of childhood sexual abuse (CSA).

Figure 7-1 represents the relationships between inflammation and mental illness that were investigated during the thesis.

Figure 7-1: Schematic representation of links between inflammation and mental illness
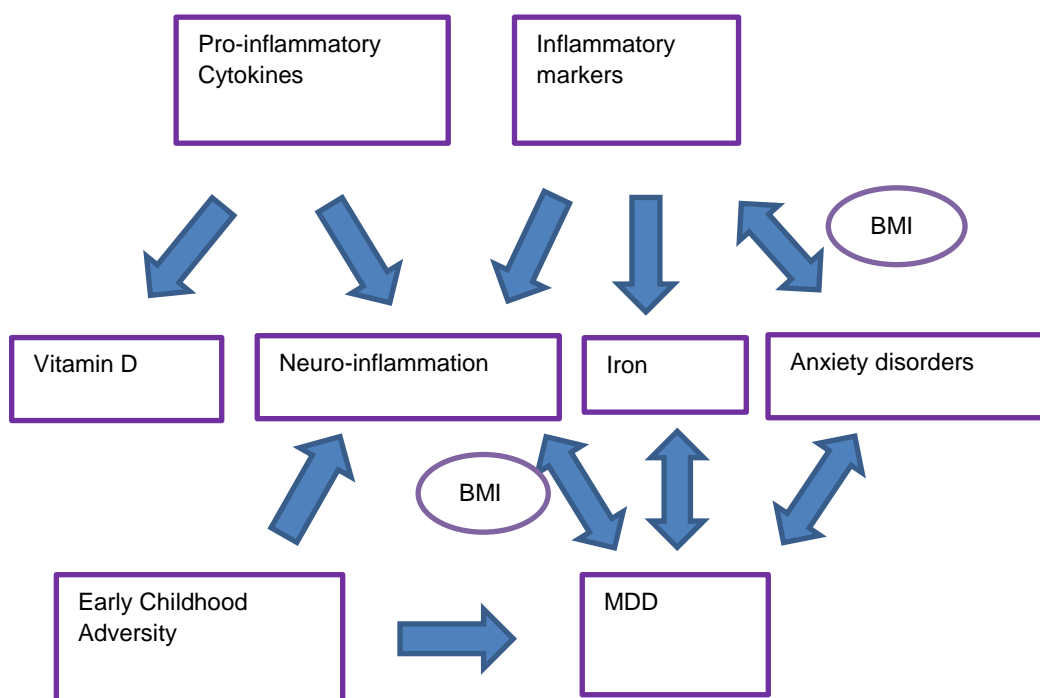
Thesis limitations and strengths

A limitation of this thesis has been the challenges in measuring cytokines and other inflammatory markers, particularly in healthy adolescents (who will have low levels of circulating cytokines). To address the limitation of assay sensitivity, high sensitivity ELISA single assays can be used. However, these assays generally cost more than a multiplex assay (295).

In Chapter 4, a limitation was the significant challenges that arose in attempting to collect data in a clinical sample. However, this resulted in changes to the consent process at the RBWH Mental Health Service (approved by the Human Research and Ethics Committees), which has recognised these challenges.

As a result of the challenges faced in collection of clinical data, the research reported in this thesis focused mostly on analysis of data from QIMRB studies of twins and their family members. A potential limitation of these data are that measures are cross-sectional measures (for example iron (Chapter 5) and CRP (Chapter 6) measures), so the direction of association is unable to be established. This is also a limitation of the literature, with most of the studies investigating cytokines or inflammatory markers in adolescents with MDD (or anxiety disorders) also using a cross-sectional design. Longitudinal designs generally require longer study durations. Repeated study measurements may cost more, but the extra data obtained from a longitudinal study can answer questions such as the direction of an association. Specifically, longitudinal studies can provide information on the temporal relationship between inflammation and mental illness, by addressing whether inflammation precedes or follows the development of MDD or anxiety disorders. This in turn can provide implications for treatment. Future study designs should aim to be longitudinal.

A strength of the study design used in this thesis is the large numbers of study participants in Chapters 5 and 6. A further strength is the quality of data collection employed in these large community studies of twins. This allowed for controlling of potential mediators such as body mass index. In Chapter 6, this also allowed analyses that stratified based on experience of childhood trauma. In the literature some published studies have not controlled for these potential mediators that influence inflammation, or taken into account a history of early childhood adversity.

Translation into clinical practice

The direction of association between inflammation and mental illness is of importance, as this has implications for treatment. In adults with MDD, there is evidence of a bidirectional association between inflammation and mood disorders, so inducing a pro-inflammatory state may induce mood symptoms (389). In children and adolescents, the direction of the association between inflammation and MDD is not yet established (301).

In MDD, a link has been observed between treatment response and levels of cytokines and inflammatory markers. Specifically, depressed patients who do not respond to selective serotonin re-uptake inhibitors have been found to have high levels of the pro-inflammatory cytokines TNF-α and IL-6 (216). A proof of concept study reported that adults with treatment resistant MDD were more likely to respond to treatment with a cytokine antagonist where cytokines were elevated at baseline (226).

With regard to anxiety disorders, the association between raised CRP and GAD observed in previous studies (33, 398) is important to note, in view of the link between early childhood adversity, persistent low grade inflammation and increased chance of developing mental and physical illness in adult life (29). However, the profile of inflammatory markers may not be the same in all anxiety disorders (399). Furthermore, in adults it has also been hypothesised that different pathways may be involved in the association between inflammation and anxiety (compared to those hypothesised to link inflammation and MDD) (33, 399, 400). Therefore, further understanding of the association between inflammation and anxiety disorders is needed prior to using interventions to target inflammation in these disorders.

Treatment implications

In adults with MDD, a small number of studies have investigated strategies to lower pro-inflammatory cytokines and inflammatory markers. These strategies have included exercise, omega-3 polyunsaturated fatty acids (omega-3 PUFAs), non-steroidal anti-inflammatory drugs (NSAIDs), selective serotonin re-uptake inhibitors (SSRI's) and cytokine antagonists.

Regular exercise and physical activity has been shown to reduce levels of IL-1β, TNF-α, IL-6 and CRP (96). A meta-analysis of adults with mood disorders indicated an antidepressant effect of omega-3 PUFAs (221). Adding the cyclooxygenase-2 inhibitor celecoxib to treatment with reboxetine or sertraline in adults with MDD has been found to induce an antidepressant response (229, 230). In adults with treatment resistant MDD and high baseline levels of CRP, infliximab (a cytokine antagonist) was found to reduce symptoms of depression (226).

Interventions that target inflammation successfully in adults may not have the same efficacy in adolescents, and only a small number of studies in this area have been conducted in children and adolescents. In children with a first episode of MDD, omega-3 PUFAs were found to improve depressive symptoms (222). One study which measured cytokines in adolescent females with MDD or anxiety disorders found lower levels of the pro-inflammatory cytokine IL-6 in those treated with SSRIs (164). The authors proposed this may be from anti-inflammatory properties of SSRIs (164). A recent study measured cytokines in children and adolescents before and after 8 weeks of treatment with the SSRI fluoxetine for depression and / or anxiety, and found levels of TNF-α, IL-6, and IL-1β were significantly higher in those who did not respond to treatment (273).

Raised pro-inflammatory cytokines and inflammatory markers are not specific to MDD. It is becoming more apparent that this also occurs in anxiety disorders and psychosis (15, 16). This is consistent with the non-specific symptoms (for example irritability) that can be seen across mental illnesses (17). Medications such as the newer atypical antipsychotics have been used to treat these non-specific symptoms in mental illnesses other than psychoses (401). In this way, it is becoming increasingly apparent that existing diagnostic categories do not represent discrete syndromes (298). Therefore, future studies that use the RDoC approach, examining domains such as these non-specific symptoms and inflammatory profiles, could potentially change diagnostic criteria and inform treatment interventions.

It is important to note that measures that reduce inflammation in MDD may have different efficacy in anxiety disorders or psychosis. Very few studies have investigated measures that target inflammation in anxiety disorders or psychosis, so this still needs to be established by conducting larger studies. One study noted an improvement of negative symptoms of schizophrenia (in individuals who had been diagnosed with schizophrenia in the preceding 2 years) when antipsychotic treatment (amisulpride) was augmented with celecoxib (402). However, to conduct these studies in adolescents, it would first need to be determined whether pro-inflammatory cytokines and other inflammatory markers are raised in those with treatment resistant illnesses.

The stage of intervention is also important. Specifically, the use of omega-3 PUFAs in adolescents and young adults assessed as at "ultra-high risk" of developing psychosis in the next 12 months has been found to reduce this risk (403). However, no evidence has

been found by meta-analysis of an improvement in psychotic symptoms when omega-3 PUFAs are used as an augmentation to treatment for schizophrenia (404).

Therefore, measures that reduce inflammation in a particular mental illness may not have the same efficacy in reducing inflammation in the presence of other mental illnesses. Similarly, interventions that target inflammation could potentially be associated with different clinical outcomes at different stages of illness.

Future directions for research

As a result of my thesis research I have the following recommendations for future research:

1) An affordable assay that can successfully measure cytokines in low concentrations is important. This was a major limitation of this thesis. Whilst high sensitivity ELISA single assays can be used, these assays generally cost more (295). The multiplex assay allows measurement of the concentrations of several inflammatory markers simultaneously in small amounts of sample (243). Therefore, improving the ability of the multiplex assay to accurately measure cytokines and inflammatory markers in low concentrations is a potential area for further research.

Considering less invasive ways to measure cytokines and inflammatory markers is also important, particularly in children and adolescents. Inflammatory markers have been measured in saliva in adolescents (405). Serum levels of CRP have been found to correlate with saliva levels of CRP in adolescents expected to have high levels of inflammation, however the study sample size was small (405). Therefore, future studies with larger sample sizes could explore this further.

2) Further exploration of the relationship, in particular the direction of the association, between cytokines and other inflammatory markers in depression in adolescents. These studies should be longitudinal, as longitudinal designs are essential to understanding childhood mental illness (406), and the direction of association between inflammation and mental illness in adolescents. However, strategies would also need to be implemented (as discussed further in recommendation number 7) to improve recruitment of adolescents.

At present, elevated levels of circulating pro-inflammatory cytokines and inflammatory markers do not appear to be specific to MDD, anxiety disorders, or psychosis. So in adults, schizophrenia is also associated with immune system dysregulation, however whether this is a biomarker of illness (rather than causal) is not yet established (15, 407). However, there are very few studies of cytokines in adolescents with anxiety or psychotic disorders. So further investigation of the relationship between inflammatory markers and anxiety disorders, and between inflammatory markers and psychotic disorders in adolescents is required, as these disorders in this age group may not have the same immune changes reported in adults. As there is considerable co-morbidity between anxiety disorders, depression and symptoms of psychosis in youth (297), combining samples from individuals with each of these disorders could increase study power.

In adolescents, studying each of these disorders could include measurement of circulating cytokines and inflammatory markers in the acute stage and again in the recovery stage of illness. A study hypothesis in this clinical group could be that lower levels of circulating cytokines and inflammatory markers would be found in the recovery stage of illness (compared to the acute stage).

3) Further study to develop additional treatment options of adolescent MDD and other mental illnesses seen in adolescence, particularly in those individuals who have associated raised pro-inflammatory markers. MDD is a heterogeneous disorder, and in adults different MDD subtypes have been associated with different inflammatory marker profiles (408). Measurement of circulating cytokines and inflammatory markers in the acute stage and again after allowing an adequate trial of treatment may identify a subgroup of individuals in whom inflammatory markers remain elevated. For this subgroup, this may lead to trials of interventions that target inflammation (such as exercise and / or omega-3 PUFAs) as an augmentation of treatment for adolescent MDD. If these interventions were to be trialled, cytokines and inflammatory markers could be measured prior to augmenting treatment, with repeat measurement of cytokine levels, inflammatory markers, and clinical outcome after several months of intervention (e.g. regular exercise).

4) Examining the genetic relationship between cytokines and mental illness (including whether there is a shared liability to immune system dysregulation and mental illness) in adolescents could increase our understanding and also have implications for treatment of mental illness in this age group. For example, if some variants of the TNF-α gene are

more common in individuals with MDD (found by GWAS), an important question to ask would be whether these individuals also have higher levels of TNF-α that do not change with treatment of the depressive episode.

5) The relationship between pro-inflammatory cytokines and Vitamin D in healthy adolescent twins (Chapter 3) requires further investigation, particularly as Vitamin D is easier (and cheaper) to measure than cytokines. Vitamin D has been found to decrease pro-inflammatory cytokines (such as IL-1β, TNF-α and IL-6), and increase anti-inflammatory cytokines (such as IL-4 and IL-10) (409, 410). To explore this further, the relationship between Vitamin D and cytokines associated with MDD (such as TNF-α and IL-6) could be investigated in a clinical group of adolescents. It may be that a non-linear relationship is found, where those who have Vitamin D levels at the extreme ends of the range have increased rates of MDD. This pattern has been reported previously for Vitamin D and schizophrenia, with neonates who had either low or high levels of Vitamin D observed to have an increased risk of schizophrenia later in life (249).

If lower levels of Vitamin D are associated with MDD in adolescents, Vitamin D supplementation (and assessing clinical outcome of MDD) could be considered. It could also be of benefit to see if increased levels of Vitamin D (with supplementation) decrease pro-inflammatory cytokines.

6) Examining the relationship between externalizing or internalizing behaviours and inflammation in childhood and adolescence. Non-specific symptoms or behaviours of some mental illnesses have been associated with inflammation. Specifically, externalizing behaviours in children have been associated with raised pro-inflammatory markers (411). However, very few studies have examined this. It is possible that inflammation in childhood is a pathway through which behaviour problems early in life may increase risk for chronic diseases in adulthood (411). Therefore examining this relationship further, with a longitudinal study design and in combination with the study suggested above (in recommendation number 2), could increase study power and provide useful information in a developmental psychoneuroimmunology context.

7) Implementing strategies to increase success of data collection in child and adolescent mental health clinics. This may be more likely to succeed if less invasive ways to measure cytokines and inflammatory markers can be further developed in this age group. These

strategies will also be more likely to succeed if clinicians working in the clinics are also actively involved in the research projects.

Conclusion

An improved understanding of the role of cytokines and other inflammatory markers in depression in adolescents needs to be extended to understanding the role of these biomarkers in other mental illnesses seen in adolescents. This could lead to improved treatment of mental illness in adolescents, particularly in the subgroup of individuals who have persistent raised levels of pro-inflammatory cytokines or inflammatory markers.

References

1. Irwin M, Miller A. Depressive disorders and immunity:20 years of progress and discovery. . Brain, Behavior, and Immunity. 2007;21:374-83.
2. Miller AH, Maletic V, Raison CL. Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression Biological Psychiatry. 2009;65(9):732-41.
3. Licinio J, Wong ML. Pathways and mechanisms for cytokine signaling of the central nervous system. Journal of Clinical Investigation. 1997;100(12):2941-7.
4. Khairova RA, Machado-Vieira R, Du J, Manji HK. A potential role for pro-inflammatory cytokines in regulating synaptic plasticity in major depressive disorder. International Journal of Neuropsychopharmacology. 2009;12(4):561-78.
5. Wahl SM. Transforming Growth-Factor-Beta (TGF-Beta) in inflammation - a cause and a cure. Journal of Clinical Immunology. 1992;12(2):61-74.
6. Dowlati Y, Herrman N, Swardfager W, Liu H, Sham L, Reim EK, et al. A Meta-Analysis of Cytokines in Major Depression. Biological Psychiatry. 2010;67:446-57.
7. Howren MB, Lamkin DM, Suls J. Associations of depression with C-reactive protein, IL-1, and IL-6: A meta-analysis. Psychosomatic Medicine. 2009;71(2):171-86.
8. Feelders RA, Vreugdenhil G, Eggermont AMM, Kuiper-Kramer PA, van Eijk HG, Swaak AJG. Regulation of iron metabolism in the acute-phase response: interferon gamma and tumour necrosis factor alpha induce hypoferraemia, ferritin production and a decrease in circulating transferrin receptors in cancer patients. European Journal of Clinical Investigation. 1998;28(7):520-7.
9. Maes M, VandeVyvere J, Vandoolaeghe E, Bril T, Demedts P, Wauters A, et al. Alterations in iron metabolism and the erythron in major depression: Further evidence for a chronic inflammatory process. Journal of Affective Disorders. 1996;40(1-2):23-33.
10. Postolache TT, Cook TB. Is latent infection with Toxoplasma gondii a risk factor for suicidal behavior? Expert Review of Anti-Infective Therapy. 2013;11(4):339-42.
11. Ludvigsson JF, Reutfors J, Osby U, Ekbom A, Montgomery SM. Coeliac disease and risk of mood disorders - A general population-based cohort study. Journal of Affective Disorders. 2007;99(1-3):117-26.
12. Yin K, Agrawal DK. Vitamin D and inflammatory diseases. Journal of Inflammation Research. 2014;7:69-87.
13. Tolppanen A-M, Sayers A, Fraser WD, Lewis G, Zammit S, Lawlor DA. The association of serum 25-hydroxyvitamin D3 and D2 with depressive symptoms in childhood - a prospective cohort study. Journal of Child Psychology and Psychiatry. 2012;53(7):757-66.
14. Guest PC, Guest FL, Martins-de Souza D. Making Sense of Blood-Based Proteomics and Metabolomics in Psychiatric Research. International Journal of Neuropsychopharmacology. 2016;19(6).
15. Miller BJ, Buckley P, Seabolt W, Mellor A, Kirkpatrick B. Meta-Analysis of Cytokine Alterations in Schizophrenia: Clinical Status and Antipsychotic Effects. Biological Psychiatry. 2011;70(7):663-71.
16. Hoge EA, Brandstetter K, Moshier S, Pollack MH, Wong KK, Simon NM. Broad Spectrum of Cytokine Abnormalities in Panic Disorder and Post-traumatic Stress Disorder. Depression and Anxiety. 2009;26(5):447-55.
17. Garber J, Weersing VR. Comorbidity of Anxiety and Depression in Youth: Implications for Treatment and Prevention. Clinical Psychology Science and Practice. 2010;17:293-306.
18. Kapur S, Phillips AG, Insel TR. Why has it taken so long for biological psychiatry to develop clinical tests and what to do about it? Molecular Psychiatry. 2012;17(12):1174-9.
19. Perlis RH. Translating biomarkers to clinical practice. Molecular Psychiatry. 2011;16(11):1076-87.

20.   Mills NT, Scott JG, Wray NR, Cohen-Woods S, Baune BT. Research Review: The role of cytokines in depression in adolescents: a systematic review. The Journal of Child Psychology and Psychiatry. 2013;54(8):816-35.
21.   Palm S, Postler E, Hinrichsen H, Maier H, Zabel P, Kirch W. Twenty-four-hour analysis of lymphocyte subpopulations and cytokines in healthy subjects. Chronobiology International. 1996;13(6):423-34.
22.   Noble JE, Wang L, Cerasoli E, Knight AE, Porter RA, Gray E, et al. An international comparability study to determine the sources of uncertainty associated with a non-competitive sandwich fluorescent ELISA. Clinical Chemistry and Laboratory Medicine. 2008;46:1033-45.
23.   Carruthers VB, Suzuki Y. Effects of Toxoplasma gondii infection on the brain. Schizophrenia Bulletin. 2007;33(3):745-51.
24.   Kalaydjian AE, Eaton W, Cascella N, Fasano A. The gluten connection: the association between schizophrenia and celiac disease. Acta Psychiatrica Scandinavica. 2006;113(2):82-90.
25.   Mills NT, Wright MJ, Henders AK, Eyles DW, Baune BT, McGrath JJ, et al. Heritability of Transforming Growth Factor-beta 1 and Tumor Necrosis Factor-Receptor Type 1 Expression and Vitamin D Levels in Healthy Adolescent Twins. Twin Research and Human Genetics. 2015;18(1):28-35.
26.   Hartwell KJ, Moran-Santa Maria MM, Twal WO, Shaftman S, DeSantis SM, McRae-Clark AL, et al. Association of elevated cytokines with childhood adversity in a sample of healthy adults. Journal of Psychiatric Research. 2013;47(5):604-10.
27.   Lu SJ, Peng HJ, Wang LF, Vasisha S, Zhang Y, Gao WJ, et al. Elevated specific peripheral cytokines found in major depressive disorder patients with childhood trauma exposure: A cytokine antibody array analysis. Comprehensive Psychiatry. 2013;54(7):953-61.
28.   Danese A, Caspi A, Williams B, Ambler A, Sugden K, Mika J, et al. Biological embedding of stress through inflammation processes in childhood. Molecular Psychiatry. 2011;16:244-6.
29.   Danese A, Moffitt TE, Pariante CM, Ambler A, Poulton R, Caspi A. Elevated Inflammation Levels in Depressed Adults With a History of Childhood Maltreatment. Archives of General Psychiatry. 2008;65(4):409-16.
30.   Miller GE, Cole SW. Clustering of Depression and Inflammation in Adolescents Previously Exposed to Childhood Adversity. Biological Psychiatry. 2012;72:34-40.
31.   Slopen N, Kubzansky LD, McLaughlin KA, Koenen KC. Childhood adversity and inflammatory processes in youth: A prospective study. Psychoneuroendocrinology. 2013;38(2):188-200.
32.   Copeland WE, Shanahan L, Worthman C, Angold A, Costello EJ. Cumulative Depression Episodes Predict Later C-Reactive Protein Levels: A Prospective Analysis. Biological Psychiatry. 2012;71:15-21.
33.   Copeland WE, Shanahan L, Worthman C, Angold A, Costello EJ. Generalized anxiety and C-reactive protein levels: a prospective, longitudinal analysis. Psychological Medicine. 2012;42(12):2641-50.
34.   McGorry P, Keshavan M, Goldstone S, Amminger P, Allott K, Berk M, et al. Biomarkers and clinical staging in psychiatry. World Psychiatry. 2014;13(3):211-23.
35.   Verduijn J, Milaneschi Y, Schoevers RA, van Hemert A, Beekman ATF, Penninx B. Pathophysiology of major depressive disorder: mechanisms involved in etiology are not associated with clinical progression. Translational Psychiatry. 2015;5, e649.
36.   Moussavi S, Chatterji S, Verdes E, Tandon A, Patel V, Ustun B. Depression, chronic diseases, and decrements in health: results from the World Health Surveys. Lancet. 2007;370(9590):851-8.
37.   Andrade L, Caraveo-Anduaga J, Berglund P, Bijl R, De Graaf R, Volleburgh W, et al. The epidemiology of major depressive episodes: results from the International Consortium of Psychiatric Epidemiology (ICPE) Surveys. International Journal of Methods in Psychiatric Research. 2003;12(1):3-21.
38.   Ustun TB, Chatterji S. Global burden of depressive disorders and future projections. Dawson A, Tylee A, editors. London: BMJ Books; 2001.

39.   Birmaher B, Ryan ND, Williamson DE, Brent DA, Kaufman J, Dahl RE, et al. Childhood and adolescent depression: a review of the past ten years. Part I. Journal American Academy of Child and Adolescent Psychiatry. 1996;35:1427-39.

40.   Weissman M, Wolk S, Goldstein R, Moreau D, Adams P, Greenwald S, et al. Depressed adolescents grown up. Journal of the American Medical Association. 1999;281:1707-13.

41.   Dunn V, Goodyer IM. Longitudinal investigation into childhood- and adolescence-onset depression: psychiatric outcome in early adulthood. British Journal of Psychiatry. 2006;188:216-22.

42.   American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders (4th ed., text revision). . Washington, DC: Author; 2000.

43.   American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders (5th ed.). Arlington, VA: American Psychiatric Publishing; 2013.

44.   Kaufman J, Martin A, King RA, Charney D. Are child-, adolescent-, and adult-onset depression one and the same disorder? Biological Psychiatry. 2001;49(12):980-1001.

45.   Kovacs M. Presentation and course of major depressive disorder during childhood and later years of the life span. Journal of the American Academy of Child and Adolescent Psychiatry. 1996;35(6):705-15.

46.   Zisook S, Rush A, Albala A, Alpert J, Balasubramani G, Fava M, et al. Factors that differentiate early vs. later onset of major depression disorder. Psychiatry Research. 2004;129(2):127-40.

47.   McDermott B, Baigent M, Chanen A, Fraser L, Graetz B, Hayman N, et al. Clinical practice guidelines: depression in adolescents and young adults. Melbourne: *beyondblue:* the national depression initiative; 2010.

48.   Hill J, Pickles A, Rollinson L, Davies R, Byatt M. Juvenile- *versus* adult-onset depression: multiple differences imply different pathways. Psychological Medicine. 2004;34:1483-93.

49.   Jaffee SR, Moffitt TE, Caspi A, Fombonne E, Poulton R, Martin J. Differences in Early Childhood Risk Factors for Juvenile-Onset and Adult-Onset Depression. Archives of General Psychiatry. 2002;58:215-22.

50.   Goodyer I. Emmanuel Miller Lecture: Early onset depressions - meanings, mechanisms and processes. The Journal of Child Psychology and Psychiatry. 2008;49(12):1239-56.

51.   McGuffin P, Katz R, Watkins S, Rutherford J. A Hospital-Based Twin Register of the Heritability of DSM-IV Unipolar Depression. Archives of General Psychiatry. 1996;53:129-36.

52.   Nanni V, Uher R, Danese A. Childhood Maltreatment Predicts Unfavorable Course of Illness and Treatment Outcome in Depression: A Meta-Analysis. American Journal of Psychiatry. 2012;169:141-51.

53.   Danese A, Pariante CM, Caspi A, Taylor A, Poulton R. Childhood maltreatment predicts adult inflammation in a life-course study. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(4):1319-24.

54.   Caspi A, Sugden K, E. MT, Taylor A, Craig IW, Harrington H, et al. Influence of Life Stress on Depression: Moderation by a Polymorphism in the 5-HTT Gene. Science. 2003;301(5631):386-9.

55.   Risch N, Herrell R, Lehner T, Liang KY, Eaves L, Hoh J, et al. Interaction Between the Serotonin Transporter Gene (5-HTTLPR), Stressful Life Events, and Risk of Depression: A Meta-analysis. Jama-Journal of the American Medical Association. 2009;301(23):2462-71.

56.   Karg K, Burmeister M, Shedden K, Sen S. The Serotonin Transporter Promoter Variant (5-HTTLPR), Stress, and Depression Meta-analysis Revisited: Evidence of Genetic Moderation. Archives of General Psychiatry. 2011;68(5):444-54.

57.   Uher R, Caspi A, Houts R, Sugden K, Williams B, Poulton R, et al. Serotonin transporter gene moderates childhood maltreatment's effects on persistent but not single-episode depression: Replications and implications for resolving inconsistent results. Journal of Affective Disorders. 2011;135:56-65.

58.   McGuffin P, Alsabban S, Uher R. The truth about genetic variation in the serotonin transporter gene and response to stress and medication. The British Journal of Psychiatry. 2011;198:424-7.

59. Dantzer R, O'Connor J, Freund G, Johnson R, Kelley K. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature Reviews Neuroscience. 2008;9(1):46-57.

60. Baune BT, Dannlowski U, Domschke K, Janssen DGA, Jordan MA, Ohrmann P, et al. The Interleukin 1 Beta (IL 1B) Gene Is Associated with Failure to Achieve Remission and Impaired Emotion Processing in Major Depression. Biological Psychiatry. 2010;67(6):543-9.

61. Baune BT, Konrad C, Grotegerd D, Suslow T, Ohrmann P, Bauer J, et al. Tumor Necrosis Factor Gene Variation Predicts Hippocampus Volume in Healthy Individuals. Biological Psychiatry. 2012;72(8):655-62.

62. Bufalino C, Hepgul N, Aguglia E, Pariante CM. The role of immune genes in the association between depression and inflammation: A review of recent clinical studies. Brain Behavior and Immunity. 2013;31:31-47.

63. Szelenyi J, Vizi ES. The catecholamine-cytokine balance - Interaction between the brain and the immune system. Annals of the New York Academy of Sciences. 2007;1113:311-24.

64. Janssen DGA, Caniato RN, Verster JC, Baune BT. A psychoneuroimmunological review on cytokines involved in antidepressant treatment response. Human Psychopharmacology-Clinical and Experimental. 2010;25(3):201-15.

65. Song C. The effect of thymectomy and IL-1 on memory: Implications for the relationship between immunity and depression. Brain, Behavior, and Immunity. 2002;16:557-68.

66. McKittrick C, Magarinos A, Blanchard D, Blanchard R, McEwen B, Saki R. Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. Synapse. 2000;36:85-94.

67. Benes FM. Myelination of Cortical-Hippocampal Relays During Late Adolescence. Schizophrenia Bulletin. 1989;15(4):585-93.

68. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, Ioannidis JPA, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. Bmj-British Medical Journal. 2009;339.

69. Haroon E, Raison CL, Miller AH. Psychoneuroimmunology Meets Neuropsychopharmacology: Translational Implications of the Impact of Inflammation on Behavior. Neuropsychopharmacology 2012;37(1):137-62.

70. Capuron L, Miller AH. Immune system to brain signaling: Neuropsychopharmacological implications. Pharmacology & Therapeutics. 2011;130(2):226-38.

71. Dantzer R. Cytokine, Sickness Behavior, and Depression. Immunology and Allergy Clinics of North America. 2009;29(2):247-64.

72. Loftis JM, Huckans M, Morasco BJ. Neuroimmune mechanisms of cytokine-induced depression: Current theories and novel treatment strategies. Neurobiology of Disease. 2010;37(3):519-33.

73. Sharpley CF, Agnew LL. Cytokines and depression: findings, issues, and treatment implications. Reviews in the Neurosciences. 2011;22(3):295-302.

74. Maes M, Yirmiya R, Noraberg J, Brene S, Hibbeln J, Perini G, et al. The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. Metabolic Brain Disease. 2009;24(1):27-53.

75. Ransohoff RM, Benveniste EN, editors. Cytokines and the CNS. New York: Taylor & Francis Group; 2006.

76. Rothwell N, Loddick S, editors. Immune and Inflammatory Responses in the Nervous System. New York: Oxford University Press; 2002.

77. Rothermundt M, Arolt V, Fenker J, Gutbrodt H, Peters M, Kirchner H. Different immune patterns in melancholic and non-melancholic major depression. European Archives of Psychiatry and Clinical Neuroscience. 2001;251(2):90-7.

78. De Craen A, Posthuma D, Remarque E, Van den Biggelaar A, Westendorp R. Heritability estimates of innate immunity: an extended twin study. Genes and Immunity. 2005;6:167-70.

79. Worns MA, Victor A, Galle PR, Hohler T. Genetic and environmental contributions to plasma C-reactive protein and interleukin-6 levels - a study in twins. Genes and Immunity. 2006;7:600-5.

80.   Sas AA, Jamshidi Y, Zheng D, Wu T, Korf J, Alizadeh BZ, et al. The age-dependency of genetic and environmental influences on serum cytokine levels: A twin study. Cytokine. 2012;60(1):108-13.

81.   Raggi P, Su S, Karohl C, Veledar E, Rojas-Campos E, Vaccarino V. Heritability of Renal Function and Inflammatory Markers in Adult Male Twins. American Journal of Nephrology. 2010;32:317-23.

82.   Jun TY, Pae CU, Hoon H, Chae JH, Bahk WM, Kim KS, et al. Possible association between G308A tumour necrosis factor-alpha gene polymorphism and major depressive disorder in the Korean population. Psychiatric Genetics. 2003;13:179-81.

83.   Rosa A, Peralta V, Papiol S, Cuesta MJ, Serrano F, Martinez-Larrea A, et al. Interleukin-1beta (IL-1beta) gene and increased risk for the depressive symptom-dimension in schizophrenia spectrum disorders. Americal Journal of Medical Genetics Part B, Neuropsychiatric Genetics. 2004;124B:10-4.

84.   Fertuzinhos SMM, Oliveira JRM, Nishimura AL, Pontual D, Carvalho DR, Sougey EB, et al. Analysis of IL-1 alpha, IL-1 beta, and IL-RA polymorphisms in dysthymia. Journal of Molecular Neuroscience. 2004;22(3):251-5.

85.   Smith RS. The Macrophage Theory of Depression. Medical Hypotheses. 1991;35(4):298-306.

86.   Einvik G, Vistnes M, Hrubos-Strom H, Randby A, Namtvedt SK, Nordhus IH, et al. Circulating cytokine concentrations are not associated with major depressive disorder in a community-based cohort. General Hospital Psychiatry. 2012;34:262-7.

87.   Steptoe A, Kunz-Ebrecht SR, Owen N. Lack of association between depressive symptoms and markers of immune and vascular inflammation in middle-aged men and women. Psychological Medicine. 2003;33(4):667-74.

88.   Gimeno D, Kivimaki M, Brunner E, Elovainio M, De Vogli R, Steptoe A, et al. Associations of C-reactive protein and interleukin-6 with cognitive symptoms of depression: 12-year follow-up of the Whitehall II study. Psychological Medicine. 2009;39(3):413-23.

89.   Baune BT, Smith E, Reppermund S, Air T, Samaras K, Lux O, et al. Inflammatory biomarkers predict depressive, but not anxiety symptoms during aging: The prospective Sydney Memory and Aging Study. Psychoneuroendocrinology. 2012;37(9):1521-30.

90.   Udina M, Castellvi P, Moreno-Espana J, Navines R, Valdes M, Forns X, et al. Interferon-Induced Depression in Chronic Hepatitis C: A Systematic Review and Meta-Analysis. Journal of Clinical Psychiatry. 2012;73(8):1128-38.

91.   Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia. 2007;55(5):453-62.

92.   Kent S, Bluthe RM, Kelley KW, Dantzer R. Sickness behavior as a new target for drug development. Trends in Pharmacological Sciences. 1992;13(1):24-8.

93.   Merali Z, Brennan K, Brau P, Anisman H. Dissociating anorexia and anhedonia elicited by interleukin-1 beta: antidepressant and gender effects on responding for "free chow" and "earned" sucrose intake. Psychopharmacology (Berl). 2003;165:413-8.

94.   Yirmiya R, Weidenfeld J, Pollak Y, Morag M, Morag A, Avitsur R, et al. Cytokines, "depression due to a general medical condition," and antidepressant drugs. In: Dantzer R, Wollman EE, Yirmiya R, editors. Cytokines, Stress, and Depression. Advances in Experimental Medicine and Biology. 1999; 461:. p. 283-316.

95.   Frenois F, Moreau M, O'Connor J, Lawson M, Micon C, Lestage J, et al. Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. Psychoneuroendocrinology. 2007;32(5):516-31.

96.   Eyre H, Baune BT. Neuroimmunological effects of physical exercise in depression. Brain Behavior and Immunity. 2012;26(2):251-66.

97.   Eyre H, Baune BT. Neuroplastic changes in depression: A role for the immune system. Psychoneuroendocrinology. 2012;37:1397-416.

98.	Komaki G, Arimura A, Koves K. Effect of intravenous injection of IL-1 beta on PGE2 levels in several brain areas as determined by microdialysis. American Journal of Physiology. 1992;262(2):E246-E51.

99.	Breder CD, Dinarello CA, Saper CB. Interleukin-1 immunoreactive innervation of the human hypothalamus. Science. 1988;240:321-4.

100.	Ericsson A, Kovacs KJ, Sawchenko PE. A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons. . Journal of Neuroscience. 1994;14(2):897-913.

101.	Cao CY, Matsumura K, Yamagata K, Watanabe Y. Involvement of cyclooxygenase-2 in LPS-induced fever and regulation of its mRNA by LPS in the rat brain. American Journal of Physiology-Regulatory Integrative and Comparative Physiology. 1997;272(6):R1712-R25.

102.	Fabry Z, Fitzsimmons KM, Herlein JA, Moninger TO, Dobbs MB, Hart MN. Production of the cytokines interleukin 1 and 6 by murine brain microvessel endothelium and smooth muscle pericytes. Journal of Neuroimmunology. 1993;47(1):23-34.

103.	Kilbourn RG, Belloni P. Endothelial Cell Production of Nitrogen Oxides in Response to Interferon gamma in Combination With Tumor Necrosis Factor, Interleukin-1, or Endotoxin. Journal of the National Cancer Institute. 1990;82:772-6.

104.	Banks WA, Kastin AJ, Durham DA. Bidirectional transport of interleukin-1 alpha across the blood-brain-barrier. . Brain Research Bulletin. 1989;23(6):433-7.

105.	Watkins LR, Wiertelak EP, Goehler LE, Mooneyheiberger K, Martinez J, Furness L, et al. Neurocircuitry of Illness-Induced Hyperalgesia Brain Research. 1994;639(2):283-99.

106.	Bluthe RM, Walter V, Parnet P, Laye S, Lestage J, Verrier D, et al. Lipopolysaccharide induces sickness behavior in rats by a vagal mediated mechanism. . Comptes Rendus De L Academie Des Sciences Serie III-Sciences De La Vie-Life Sciences. 1994;317(6):499-503.

107.	D'Mello C, Le T, Swain MG. Cerebral Microglia Recruit Monocytes into the Brain in Response to Tumor Necrosis Factor alpha Signaling during Peripheral Organ Inflammation. Journal of Neuroscience. 2009;29(7):2089-102.

108.	Kronfol Z, Remick DG. Cytokines and the Brain: Implications for Clinical Psychiatry. American Journal of Psychiatry. 2000;157(5):683-94.

109.	Connor TJ, Leonard BE. Depression, stress and immunological activation: the role of cytokines in depressive disorders. Life Sciences. 1998;62:583-606.

110.	Carlezon Jr. WA, Nestler EJ. Elevated levels of GluR1 in the midbrain: a trigger for sensitization to drugs of abuse? Trends in Neurosciences. 2002;25:610-5.

111.	Du J, Gray NA, Falke CA, Chen W, Yuan P, Szabo ST, et al. Modulation of synaptic plasticity by antimanic agents: the role of AMPA glutamate receptor subunit 1 synaptic expression. Journal of Neuroscience. 2004;24(29):6578-89.

112.	Du J, Suzuki K, Wei Y, Wang Y, Blumenthal R, Chen Z, et al. The anticonvulsants lamotrigine, riluzole, and valproate differentially regulate AMPA receptor membrane localization: relationship to clinical effects in mood disorders. Neuropsychopharmacology. 2007;32(4):793-802.

113.	Du J, Creson TK, Wu LJ, Ren M, Gray NA, Falke C, et al. The role of hippocampal GluR1 and GluR2 receptors in manic-like behavior. Journal of Neuroscience. 2008;28(1):68-79.

114.	Kendell SF, Krystal JH, Sanacora G. GABA and glutamate systems as therapeutic targets in depression and mood disorders. Expert Opinion on Therapeutic Targets. 2005;9:153-68.

115.	Malenka RC. The long-term potential of LTP. Nature Reviews Neuroscience. 2003;4(11):923-6.

116.	Sun X, Zhao Y, Wolf ME. Dopamine receptor stimulation modulates AMPA receptor synaptic insertion in prefrontal cortex neurons. Journal of Neuroscience. 2005;25:7342-51.

117.	Wolf ME, Sun X, Mangiavacchi S, Chao SZ. Psychomotor stimulants and neuronal plasticity. Neuropharmacology. 2004;47(Suppl. 1):61-79.

118.	Ruhe HG, Mason NS, Schene AH. Mood is indirectly related to serotonin, norepinephrine, and dopamine levels in humans: a meta-analysis of monoamine depletion studies. Molecular Psychiatry. 2007;12(4):331-59.

119.	Maes M, Wauters A, Verkerk R, Demedts P, Neels H, Van Gastel A, et al. Lower serum L-tryptophan availability in depression as a marker of a more generalized disorder in protein metabolism. Neuropsychopharmacology. 1996;15(3):243-51.

120.	Maes M, Jacobs M-P, Suy E, Minner B, Leclercq C, Christiaens F, et al. Suppressant effects of dexamethasone on the availability of plasma L-tryptophan and tyrosine in healthy controls and in depressed patients. Acta Psychiatrica Scandinavica. 1990;81:19-23.

121.	Maes M, Meltzer HY, Scharpe S, Bosmans E, Suy E, Demeester I, et al. Relationships between lower plasma L-tryptophan levels and immune-inflammatory variables in depression. Psychiatry Research. 1993;49(2):151-65.

122.	Babcock TA, Carlin JM. Transcriptional activation of indoleamine dioxygenase by interleukin 1 and tumor necrosis factor alpha in interferon-treated epithelial cells. Cytokine. 2000;12(6):588-94.

123.	Goldstein LE, Leopold MC, Huang X, Atwood CS, Saunders AJ, Hartshorn M, et al. 3-Hydroxykynurenine and 3-hydroxyanthranilic acid generate hydrogen peroxide and promote alpha-crystallin cross-linking by metal ion reduction. Biochemistry. 2000;39:7266-75.

124.	Schwarcz R, Whetsell WJ, Mangano R. Quinolinic acid: An endogenous metabolite that produces axon-sparing lesions in rat brain. Science. 1983;219:316-8.

125.	Deinzer R, Granrath N, Stuhl H, Twork L, Idel H, Waschul B, et al. Acute stress effects on local IL-1beta responses to pathogens in a human in vivo model. Brain Behavior and Immunity. 2004;18(5):458-67.

126.	Goebel MU, Mills PJ, Irwin MR, Ziegler MG. Interleukin-6 and tumor necrosis factor-alpha production after acute psychological stress, exercise, and infused isoproterenol: differential effects and pathways. Psychosomatic Medicine. 2000;62(4):591-8.

127.	Madrigal JL, Hurtado O, Moro MA, Lizasoain I, Lorenzo P, Castrillo A, et al. The increase in TNF-alpha levels is implicated in NF-kappaB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. Neuropsychopharmacology. 2002;26(2):155-63.

128.	O'Connor KA, Johnson JD, Hansen MK, Wieseler Frank JL, Maksimova E, Watkins LR, et al. Peripheral and central proinflammatory cytokine response to a severe acute stressor. Brain Research. 2003;991(1-2):123-32.

129.	Capuron L, Raison CL, Musselman DL, Lawson DH, Nemeroff CB, Miller AH. Association of Exaggerated HPA Axis Response to the Initial Injection of Interferon-Alpha With Development of Depression During Interferon-Alpha Therapy. American Journal of Psychiatry. 2003;160(7):1342-5.

130.	Cavanagh J, Mathias C. Inflammation and its relevance to psychiatry. Advances in Psychiatric Treatment. 2008;14:248-55.

131.	Szczepankiewicz A, Leszczynska-Rodziewicz A, Pawlak J, Rajewska-Rager A, Dmitrzak-Weglarz M, Wilkosc M, et al. Glucocorticoid receptor polymorphism is associated with major depression and predominance of depression in the course of bipolar disorder. Journal of Affective Disorders. 2011;134:138-44.

132.	van West D, Van Den Eede F, Del-Favero J, Souery D, Norrback KF, Van Duijn C, et al. Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. Neuropsychopharmacology. 2006;31(3):620-7.

133.	Mill J, Wigg K, Burcescu I, Vetro A, Kiss E, Kapornai K, et al. Mutation Screen and Association Analysis of the Glucocorticoid Receptor Gene (*NR3C1*) in Childhood-Onset Mood Disorders (COMD). American Journal of Medical Genetics Part B: Neuropsychiatric Genetics. 2009;150B:866-73.

134.	Ripke S, Wray NR, Lewis CM, Hamilton SP, Weissman MM, Breen G, et al. A mega-analysis of genome-wide association studies for major depressive disorder. Molecular Psychiatry. 2013;18(4):497-511.

135.	Bet PM, Penninx BWJH, Bochdanovits Z, Uitterlinden AG, Beekman ATF, van Schoor NM, et al. Glucocorticoid Receptor Gene Polymorphisms and Childhood Adversity Are Associated With Depression: New Evidence for a Gene-Environment Interaction. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics. 2008;150B(5):660-9.

136.   Meaney M, Szyf M. Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. Dialogues in Clinical Neuroscience. 2005;7(2):103-23.

137.   de Rooij SR, Costello PM, Veenendaal MV, Lillycrop KA, Gluckman PD, Hanson MA, et al. Associations between DNA methylation of a glucocorticoid receptor promoter and acute stress responses in a large healthy adult population are largely explained by lifestyle and educational differences. Psychoneuroendocrinology. 2012;37(6):782-8.

138.   Solomon MB, Furay AR, Jones K, Packard AEB, Packard BA, Wulsin AC, et al. Deletion Of Forebrain Glucocorticoid Receptors Impairs Neuroendocrine Stress Responses And Induces Depression-like Behavior In Males But Not Females. Neuroscience. 2012;203:135-43.

139.   Koo JW, Duman RS. IL-1 beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(2):751-6.

140.   Palucha A, Pilc A. Metabotropic glutamate receptor ligands as possible anxiolytic and antidepressant drugs. Pharmacology & Therapeutics. 2007;115(1):116-47.

141.   Pittenger C, Sanacora G, Krystal JH. The NMDA Receptor as a Therapeutic Target in Major Depressive Disorder. CNS & Neurological Disorders - Drug Targets. 2007;6(2):101-15.

142.   McNally L, Bhagwagar Z, Hannestad J. Inflammation, Glutamate, and Glia in Depression: *A Literature Review*. CNS Spectrums. 2008;13(6):501-10.

143.   Miller GE, Chen E. Harsh Family Climate in Early Life Presages the Emergence of a Proinflammatory Phenotype in Adolescence. Psychological Science. 2010;21(6):848-56.

144.   Caserta MT, Wyman PA, Wang H, Moynihan J, O'Connor TG. Associations among depression, perceived self-efficacy, and immune function and health in preadolescent children. Development and Psychopathology. 2011;23:1139-47.

145.   Chaiton M, O'Loughlin J, Karp I, Lambert M. Depressive Symptoms and C-Reactive Protein Are Not Associated in a Population-Based Sample of Adolescents. International Journal of Behavioral Medicine. 2010;17(3):216-22.

146.   Hood KK, Lawrence JM, Anderson A, Bell R, Dabalea D, Daniels S, et al. Metabolic and Inflammatory Links to Depression in Youth With Diabetes. Diabetes Care. 2012;35:2443-6.

147.   Misener VL, Gomez L, Wigg KG, Luca P, King N, Kiss E, et al. Cytokine Genes *TNF, IL1A, IL1B, IL6, IL1RN* and *IL10,* and Childhood-Onset Mood Disorders. Neuropsychobiology. 2008;58:71-80.

148.   Pandey GN, Dwivedi Y, Rizavi HS, Ren X, Pandey SC, Pesold C, et al. Higher expression of serotonin 5-HT(2A) receptors in the postmortem brains of teenage suicide victims. American Journal of Psychiatry. 2002;159(3):419-29.

149.   Fagundes CP, Glaser R, Kiecolt-Glaser JK. Stressful early life experiences and immune dysregulation across the lifespan. Brain, Behavior, and Immunity. 2013;27:8-12.

150.   Taylor SE, Lehman BJ, Kiefe CI, Seeman TE. Relationship of Early Life Stress and Psychological Functioning to Adult C-Reactive Protein in the Coronary Artery Risk Development in Young Adults Study. Biological Psychiatry. 2006;60:819-24.

151.   Dixon D, Meng H, Goldberg R, Schneiderman N, Delamater A. Stress and body mass index each contributes independently to tumor necrosis factor-alpha production in prepubescent latino children. Journal of Pediatric Nursing. 2009;24(5):378-88.

152.   Wilson CJ, Finch CE, Cohen HJ. Cytokines and cognition - The case for a head-to-toe inflammatory paradigm. Journal of the American Geriatrics Society. 2002;50(12):2041-56.

153.   Peng YP, Qiu YH, Lu JH, Wang HJ. Interleukin-6 protects cultured cerebellar granule neurons against glutamate-induced neurotoxicity. Neuroscience Letters. 2005;374(3):192-6.

154.   Godbout JP, Johnson RW. Interleukin-6 in the aging brain. Journal of Neuroimmunology. 2004;147(1-2):141-4.

155.   Morales I, Farias G, Maccioni RB. Neuroimmunomodulation in the pathogenesis of Alzheimer's disease. Neuroimmunomodulation. 2010;17:202-4.

156.   Baune BT, Konrad C, Grotegerd D, Suslow T, Birosova E, Ohrmann P, et al. Interleukin-6 gene (IL-6): a possible role in brain morphology in the healthy adult brain. Journal of Neuroinflammation. 2012;9.

157. Talge N, Neal C, Glover V. Antenatal maternal stress and long-term effects on child neurodevelopment: How and why? Journal of Child Psychology and Psychiatry. 2007;48:245-61.

158. Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. Nature Neuroscience. 2004;7(8):847-54.

159. McCrory E, De Brito SA, Viding E. Research Review: The neurobiology and genetics of maltreatment and adversity. The Journal of Child Psychology and Psychiatry. 2010;51(10):1079-95.

160. Fisher HL, Cohen-Woods S, Hosang GM, Uher R, Powell-Smith G, Keers R, et al. Stressful life events and the serotonin transporter gene (*5-HTT*) in recurrent clinical depression. Journal of Affective Disorders. 2012;136:189-93.

161. Gabbay V, Klein RG, Alonso CM, Babb JS, Nishawala M, De Jesus G, et al. Immune system dysregulation in adolescent major depressive disorder. Journal of Affective Disorders. 2009;115(1-2):177-82.

162. Gabbay V, Klein RG, Katz Y, Mendoza S, Guttman LE, Alonso CM, et al. The possible role of the kynurenine pathway in adolescent depression with melancholic features. Journal of Child Psychology and Psychiatry. 2010;51(8):935-43.

163. Quinones MP, Williamson DE, Livi CB, Olvera R, Walss-Bass C. Role of the Immunomodulatory Cytokines Interferon Alpha (IFNalpha)-2 and Interleukin (IL)-10 in the Pathogenesis of Anxiety and Depression Symptoms in Adolescents: Effect of familial Risk and Stress. Biological Psychiatry. 2012;71(8):215S-6S.

164. Blom EH, Lekander M, Ingvar M, Asberg M, Mobarrez F, Serlachius E. Pro-inflammatory cytokines are elevated in adolescent females with emotional disorders not treated with SSRIs. Journal of Affective Disorders. 2012;136(3):716-23.

165. Bao A-M, Hestiantoro A, Van Someron EJW, Swaab DF, Zhou J-N. Colocalization of corticotropin-releasing hormone and oestrogen receptor-alpha in the paraventricular nucleus of the hypothalamus in mood disorders. Brain 2005;128:1301-13.

166. Angold A, Costello E, Worthman C. Puberty and depression: the roles of age, pubertal status and pubertal timing. Psychological Medicine. 1998;28:51-61.

167. Schwarz E, Schafer C, Bode J, Bode C. Influence of the menstrual cycle on the LPS-induced cytokine response of monocytes. Cytokine. 1999;12:413-6.

168. Verthelyi D. Sex hormones as immunomodulators in health and disease. International Immunopharmacology. 2001;1:983-93.

169. O'Brien SM, Fitzgerald P, Scully P, Landers AM, Scott LV, Dinan TG. Impact of Gender and Menstrual Cycle Phase on Plasma Cytokine Concentrations. Neuroimmunomodulation. 2007;14:84-90.

170. Kronfol Z, Nair M, Goodson J, Goel K, Haskett R, Schwartz S. Natural killer cell activity in depressive illness: A preliminary report. Biological Psychiatry. 1989;26(7):753-6.

171. Nerozzi D, Santoni A, Bersani G, Magnani A, Bressan A, Pasini A, et al. Reduced natural killer cell activity in major depression: Neuroendocrine implications. Psychoneuroendocrinology. 1989;14(4):295-301.

172. Irwin M, Patterson T, Smith TL, Caldwell C, Brown SA, Gillin JC, et al. Reduction of Immune Function in Life Stress and Depression. Biological Psychiatry. 1990;27(1):22-30.

173. Schleifer SJ, Keller SE, Meyerson AT, Raskin MJ, Davis KL, Stein M. Lymphocyte Function in Major Depressive Disorder. Archives of General Psychiatry. 1984;41(5):484-6.

174. Bartlett JA, Schleifer SJ, Demetrikopoulos MK, Keller SE. Immune differences in children with and without depression. Biological Psychiatry. 1995;38(11):771-4.

175. Birmaher B, Rabin BS, Garcia MR, Jain U, Whiteside TL, Williamson DE, et al. Cellular immunity in depressed, conduct disorder, and normal adolescents: role of adverse life events. Journal American Academy of Child and Adolescent Psychiatry. 1994;33(5):671-8.

176. Schleifer S, Bartlett J, Keller S, Eckholdt H, Shiflett S, Delaney B. Immunity in adolescents with major depression. Journal American Academy of Child and Adolescent Psychiatry. 2002;41:1054-60.

177. Shain BN, Kronfol Z, Naylor M, Goel K, Evans T, Schaefer S. Natural killer cell activity in adolescents with major depression. Biological Psychiatry. 1991;29(5):481-4.

178.   Targum SD, Clarkson LL, Magac-Harris K, Marshall LE, Skwerer RG. Measurement of cortisol and lymphocyte subpopulations in depressed and conduct-disordered adolescents. Journal of Affective Disorders. 1990;18:91-6.

179.   Endicott J, Spitzer RL. Diagnostic Interview - Schedule for Affective-Disorders and Schizophrenia. Archives of General Psychiatry. 1978;35(7):837-44.

180.   Shaffer D, Schwabstone M, Fisher P, Cohen P, Piacentini J, Davies M, et al. The Diagnostic Interview Schedule for Children Revised Version (DISC-R) .1. Preparation, Field Testing, Interrater Reliability, and Acceptability. Journal of the American Academy of Child and Adolescent Psychiatry. 1993;32(3):643-50.

181.   Kovacs M. The Children's Depression Inventory. New York: Multi-Health Systems. 1992.

182.   Kuo H-K, Yen C-J, Chang C-H, Kuo C-K, Chen J-H, Sorond F. Relation of C-reactive protein to stroke, cognitive disorders, and depression in the general population: systematic review and meta-analysis. Lancet Neurology. 2005;4(6):371-80.

183.   Brambilla F, Monteleone P, Maj M. Interleukin-1 beta and tumor necrosis factor-alpha in children with major depressive disorder or dysthymia. Journal of Affective Disorders. 2004;78(3):273-7.

184.   Tuglu C, Kara S, Caliyurt O, Vardar E, Abay E. Increased serum tumor necrosis factor-alpha levels and treatment response in major depressive disorder. Psychopharmacology (Berl). 2003;170:429-33.

185.   Leo R, Di Lorenzo G, Tesauro M, Razzini C, Forleo GB, Chiricolo G, et al. Association between enhanced soluble CD40 ligand and proinflammatory and prothrombotic states in major depressive disorder:Pilot observations on the effects of selective serotonin reuptake inhibitor therapy. Journal of Clinical Psychiatry. 2006;67(11):1760-6.

186.   Pavon L, Sandoval-Lopez G, Hernandez ME, Loria F, Estrada I, Perez M, et al. Th2 cytokine response in Major Depressive Disorder patients before treatment. Journal of Neuroimmunology. 2006;172(1-2):156-65.

187.   Kim YK, Na KS, Shin KH, Jung HY, Choi SH, Kim JB. Cytokine imbalance in the pathophysiology of major depressive disorder. Progress in Neuro-Psychopharmacology & Biological Psychiatry. 2007;31:1044-53.

188.   Mendlovic S, Mozes E, Eilat E, Doron A, Lereya J, Zakuth V, et al. Immune activation in non-treated suicidal major depression. Immunology Letters. 1999;67:105-8.

189.   Kim YK, Lee SW, Kim SH, Shim SH, Han SW, Choi SH, et al. Differences in cytokines between non-suicidal patients and suicidal patients in major depression. Progress in Neuro-Psychopharmacology & Biological Psychiatry. 2008;32:356-61.

190.   Janelidze S, Mattei D, Westrin A, Traskman-Bendz L, Brundin L. Cytokine levels in the blood may distinguish suicide attempters from depressed patients. Brain, Behavior, and Immunity. 2011;25:335-9.

191.   Gabbay V, Klein RG, Guttman LE, Babb JS, Alonso CM, Nishawala M, et al. A Preliminary Study of Cytokines in Suicidal and Nonsuicidal Adolescents with Major Depression. Journal of Child and Adolescent Psychopharmacology. 2009;19(4):423-30.

192.   Mann JJ, Arango V, Marzuk PM, Theccanat S, Reis DJ. Evidence for the 5-HT hypothesis of suicide. A review of post-mortem studies. British Journal of Psychiatry. 1989;155:7-14.

193.   Tonelli LH, Stiller J, Rujescu D, Giegling I, Schneider B, Maurer K, et al. Elevated cytokine expression in the orbitofrontal cortex of victims of suicide. Acta Psychiatrica Scandinavica. 2008;117(3):198-206.

194.   Capuron L, Miller AH. Cytokines and Psychopathology: Lessons from Interferon-alpha. Biological Psychiatry. 2004;56:819-24.

195.   Arango V, Underwood MD, Mann JJ. Postmortem findings in suicide victims - Implications for in vivo imaging studies. Annals of the New York Academy of Sciences. 1997;836:269-87.

196.   Kamata M, Higuchi H, Yoshimoto M, Yoshida K, Shimizu T. Effect of single intracerebroventricular injection of alpha-interferon on monoamine concentrations in the rat brain. European Neuropsychopharmacology. 2000;10(2):129-32.

197.   Pandey GN, Rizavi HS, Ren X, Fareed J, Hoppensteadt DA, Roberts RC, et al. Proinflammatory cytokines in the prefrontal cortex of teenage suicide victims. Journal of Psychiatric Research. 2012;46:57-63.

198.   Chambers WJ, Puigantich J, Hirsch M, Paez P, Ambrosini PJ, Tabrizi MA, et al. The Assessment of Affective-Disorders in Children and Adolescents by Semistructured Interview - Test-Retest Reliability of the Schedule for Affective-Disorders and Schizophrenia for School-Age Children, Present Episode Version. Archives of General Psychiatry. 1985;42(7):696-702.

199.   Kaufman J, Birmaher B, Brent D, Rao U, Flynn C, Moreci P, et al. Schedule for Affective Disorders and Schizophrenia for School-Age Children Present and Lifetime version (K-SADS-PL): Initial reliabilil and validity data. Journal of the American Academy of Child and Adolescent Psychiatry. 1997;36(7):980-8.

200.   Goodman R, Ford T, Richards H, Gatward R, Meltzer H. The Development and Well-Being Assessment: Description and initial validation of an integrated assessment of child and adolescent psychopathology. Journal of Child Psychology and Psychiatry and Allied Disciplines. 2000;41(5):645-55.

201.   Maes M, Bosmans E, Suy E, Vandervorst C, Dejonckheere C, Raus J. Depression-related disturbances in mitogen-induced lymphocyte responses and interleukin-1beta and soluble interleukin-2 receptor production. Acta Psychiatrica Scandinavica. 1991;84:379-86.

202.   Dantzer R. Depression and Inflammation: An Intricate Relationship. Biological Psychiatry. 2012;71:4-5.

203.   Adrian C, Hammen C. Stress Exposure and Stress Generation in Children of Depressed Mothers. Journal of Consulting and Clinical Psychology. 1993;61(2):354-9.

204.   Ford DE, Erlinger TP. Depression and C-reactive protein in US adults - Data from the Third National Health and Nutrition Examination Survey. Archives of Internal Medicine. 2004;164(9):1010-4.

205.   Angold A, Costello EJ. The Child and Adolescent Psychiatric Assessment (CAPA). Journal of the American Academy of Child and Adolescent Psychiatry. 2000;39(1):39-48.

206.   Angold A, Cox A, Prendergast M, Rutter M, Simonoff E, Costello EJ, et al. The Young Adult Psychiatric Assessment (YAPA). Durham, NC: Duke University Medical Center1999.

207.   First MB, Spitzer RL, Gibbon M, Williams JBW. Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Non-Patient Edition (SCID-I/NP). New York: Biometrics Research, New York State Psychiatric Institute. 2002.

208.   Radloff LS. The CES-D scale: A self-report depression scale for research in the general population. Applied Psychological Measurement. 1977;1(3):385-401.

209.   Bengtsson B, Zhu J, Thorell L, Olsson T, Link H, Walinder J. Effects of zimeldine and its metabolites, clomipramine, imipramine, and maprotiline in experimental allergic neuritis in Lewis rats. Journal of Neuroimmunology. 1992;39(1-2):109-22.

210.   Xia Z, DePierre JW, Nassberger L. Tricyclic antidepressants inhibit IL-6, IL-1 beta and TNF-alpha release in human blood monocytes and IL-2 and interferon-gamma in T cells. Immunopharmacology. 1996;34:27-37.

211.   Kubera M, Kenis G, Bosmans E, Zieba A, Dudek D, Nowak G, et al. Plasma levels of interleukin-6, interleukin-10, and interleukin-1 receptor antagonist in depression: comparison between the acute state and after remission. Polish Journal of Pharmacology. 2000;52:237-41.

212.   Sutcigil L, Oktenli C, Musabak U, Bozkurt A, Cansever A, Uzun O, et al. Pro- and anti-inflammatory cytokine balance in major depression: effect of sertraline therapy. Clinical and Developmental Immunology. 2007;26438.

213.   Taler M, Gil-Ad I, Lomnitski L, Korov I, Baharav E, Bar M, et al. Immunomodulatory effect of selective serotonin reuptake inhibitors (SSRIs) on human T lymphocyte function and gene expression. European Neuropsychopharmacology. 2007;17(12):774-80.

214.   Sluzewska A, Sobieska M, Rybakowski J. Changes in acute-phase proteins during lithium potentiation of antidepressants in refractory depression. Neuropsychobiology. 1997;35:123-7.

215.   Maes M, Bosmans E, De Jongh R, Kenis G, Vandoolaeghe E, Neels H. Increased serum IL-6 and IL-1 receptor antagonist concentrations in major depression and treatment resistant depression. Cytokine. 1997;9:853-8.

216.   O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. Journal of Psychiatric Research. 2007;41:326-31.

217.   Fischer CP. Interleukin-6 in acute exercise and training: what is the biological relevance? Exercise Immunology Review. 2006;12:6-33.

218.   Pedersen BK. Edward F Adolph distinguished lecture: muscle as an endocrine organ: IL-6 and other myokines. Journal of Applied Physiology. 2009;107(4):1006-14.

219.   Steensberg A, Fischer CP, Keller C, Moller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. American Journal of Physiology-Endocrinology and Metabolism. 2003;285(2):E433-E7.

220.   Funk JA, Gohlke J, Kraft AD, McPherson CA, Collins JB, Harry GJ. Voluntary exercise protects hippocampal neurons from trimethyltin injury: Possible role of interleukin-6 to modulate tumor necrosis factor receptor-mediated neurotoxicity. Brain Behavior and Immunity. 2011;25(6):1063-77.

221.   Lin PY, Su KP. A meta-analytic review of double-blind, placebo-controlled trials of antidepressant efficacy of omega-3 fatty acids. Journal of Clinical Psychiatry. 2007;68(7):1056-61.

222.   Nemets H, Nemets B, Apter A, Bracha Z, Belmaker RH. Omega-3 treatment of childhood depression: A controlled, double-blind pilot study. American Journal of Psychiatry. 2006;163(6):1098-100.

223.   Krishnan R, Cella D, Leonardi C, Papp K, Gottlieb A, Dunn M, et al. Effects of etanercept therapy on fatigue and symptoms of depression in subjects treated for moderate to severe plaque psoriasis for up to 96 weeks. British Journal of Dermatology. 2007;157:1275-7.

224.   Tyring S, Gottlieb A, Papp K, Gordon K, Leonardi C, Wang A, et al. Etanercept and clinical outcomes, fatigue and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet. 2006;367:29-35.

225.   Dantzer R, editor. Mechanisms of the behavioral effects of cytokines. New York: Kluwer Academic/ Plenum Publishers; 1999.

226.   Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF, et al. A Randomized Controlled Trial of the Tumor Necrosis Factor Antagonist Infliximab for Treatment-Resistant Depression. Jama Psychiatry. 2013;70(1):31-41.

227.   Yirmiya R, Pollak Y, Morag M, Reichenberg A, Barak O, Avitsur R, et al. Illness, cytokines, and depression. Annals of the New York Academy of Sciences. 2000;917:478-87.

228.   Mendlewicz J, Kriwin P, Oswald P, Souery D, Alboni S, Brunello N. Shortened onset of action of antidepressants in major depression using acetylsalicylic acid augmentation: A pilot open-label study. International Clinical Psychopharmacology. 2006;21(4):227-31.

229.   Muller N, Schwarz MJ, Dehning S, Douhe A, Cerovecki A, Goldstein-Muller B, et al. The cyclooxygenase-2 inhibitor celecoxib has therapeutic effects in major depression: results of a double-blind, randomized, placebo controlled, add-on pilot study to reboxetine. Molecular Psychiatry. 2006;11(7):680-4.

230.   Abbasi S-H, Hosseini F, Modabbernia A, Ashrafi M, Akhondzadeh S. Effect of celecoxib add-on treatment on symptoms and serum IL-6 concentrations in patients with major depressive disorder: Randomized double-blind placebo-controlled study. Journal of Affective Disorders. 2012;141:308-14.

231.   Maes M. Targeting cyclooxygenase-2 in depression is not a viable therapeutic approach and may even aggravate the pathophysiology underpinning depression. Metabolic Brain Disease. 2012;27(4):405-13.

232.   Arias I, Sorlozano A, Villegas E, de Dios Luna J, McKenney K, Cervilla J, et al. Infectious agents associated with schizophrenia: A meta-analysis. Schizophrenia Research. 2012;136(1-3):128-36.

233.   Kendler KS. What psychiatric genetics has taught us about the nature of psychiatric illness and what is left to learn. Molecular Psychiatry. 2013;18(10):1058-66.

234.   Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: Review and meta-analysis. American Journal of Psychiatry. 2000;157(10):1552-62.

235.   Glowinski AL, Madden PAF, Bucholz KK, Lynskey MT, Heath AC. Genetic epidemiology of self-reported lifetime DSM-IV major depressive disorder in a population-based twin sample of female adolescents. Journal of Child Psychology and Psychiatry and Allied Disciplines. 2003;44(7):988-96.

236.   Schwab SG, Wildenauer DB. Genetics of psychiatric disorders in the GWAS era: an update on schizophrenia. European Archives of Psychiatry and Clinical Neuroscience. 2013;263:S147-S54.

237.   Wajant H, Pfizenmaier K, Scheurich P. Review: Tumor necrosis factor signaling. Cell Death and Differentiation. 2003;10:45-65.

238.   Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Frontiers in Bioscience. 2002;7:D793-D807.

239.   Benros ME, Mortensen PB, Eaton WW. Autoimmune diseases and infections as risk factors for schizophrenia. Annals of the New York Academy of Sciences. 2012;1262:56-66.

240.   Ripke S, Neale BM, Corvin A, Walters JTR, Farh KH, Holmans PA, et al. Biological insights from 108 schizophrenia-associated genetic loci. Nature. 2014;511(7510):421-7.

241.   Posthuma D, Meulenbelt I, de Craen AJM, de Geus EJC, Slagboom PE, Boomsma DI, et al. Human cytokine response to ex vivo amyloid-beta stimulation is mediated by genetic factors. Twin Research and Human Genetics. 2005;8(2):132-7.

242.   Stein CM, Guwatudde D, Nakakeeto M, Peters P, Elston RC, Tiwari HK, et al. Heritability analysis of Cytokines as intermediate phenotypes of tuberculosis. Journal of Infectious Diseases. 2003;187(11):1679-85.

243.   Skogstrand K. Multiplex assays of inflammatory markers, a description of methods and discussion of precautions - Our experience through the last ten years. Methods. 2012;56(2):204-12.

244.   Holick MF. Vitamin D deficiency. New England Journal of Medicine. 2007;357(3):266-81.

245.   Cantorna MT. Vitamin D, multiple sclerosis and inflammatory bowel disease. Archives of Biochemistry and Biophysics. 2012;523(1):103-6.

246.   Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin D-3 receptor in the immune system. Archives of Biochemistry and Biophysics. 2000;374(2):334-8.

247.   Bruce D, Yu SH, Ooi JH, Cantorna MT. Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling. International Immunology. 2011;23(8):519-28.

248.   Hoang MT, DeFina LF, Willis BL, Leonard DS, Weiner MF, Brown ES. Association Between Low Serum 25-Hydroxyvitamin D and Depression in a Large Sample of Healthy Adults: The Cooper Center Longitudinal Study. Mayo Clinic Proceedings. 2011;86(11):1050-5.

249.   McGrath JJ, Eyles DW, Pedersen CB, Anderson C, Ko P, Burne TH, et al. Neonatal Vitamin D Status and Risk of Schizophrenia: A Population-Based Case-Control Study. Archives of General Psychiatry. 2010;67(9):889-94.

250.   Hunter D, De Lange M, Snieder H, MacGregor AJ, Swaminathan R, Thakker RV, et al. Genetic contribution to bone metabolism, calcium excretion, and vitamin D and parathyroid hormone regulation. Journal of Bone and Mineral Research. 2001;16(2):371-8.

251.   Wjst M, Altmueller J, Braig C, Bahnweg M, Andre E. A genome-wide linkage scan for 25-OH-D-3 and 1,25-(OH)(2)-D-3 serum levels in asthma families. Journal of Steroid Biochemistry and Molecular Biology. 2007;103(3-5):799-802.

252.   Karohl C, Su SY, Kumari M, Tangpricha V, Veledar E, Vaccarino V, et al. Heritability and seasonal variability of vitamin D concentrations in male twins. American Journal of Clinical Nutrition. 2010;92(6):1393-8.

253.   Snellman G, Melhus H, Gedeborg R, Olofsson S, Wolk A, Pedersen NL, et al. Seasonal Genetic Influence on Serum 25-Hydroxyvitamin D Levels: A Twin Study. Plos One. 2009;4(11).

254.   Arguelles LM, Langman CB, Ariza AJ, Ali FN, Dilley K, Price H, et al. Heritability and Environmental Factors Affecting Vitamin D Status in Rural Chinese Adolescent Twins. Journal of Clinical Endocrinology & Metabolism. 2009;94(9):3273-81.

255.   Wright MJ, Martin NG. Brisbane Adolescent Twin Study: Outline of study methods and research projects. Australian Journal of Psychology. 2004;56(2):65-78.

256.   Skogstrand K, Ekelund CK, Thorsen P, Vogel I, Jacobsson B, Norgaard-Pedersen B, et al. Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples. Journal of Immunological Methods. 2008;336(1):78-84.
257.   Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel DE, Sorensen LC, Hougaard DM. Simultaneous Measurement of 25 Inflammatory Markers and Neurotrophins in Neonatal Dried Blood Spots by Immunoassay with xMAP Technology. Clinical Chemistry. 2005;51(10):1854-66.
258.   Yolken RH, Torrey EF, Lieberman JA, Yang S, Dickerson FB. Serological evidence of exposure to Herpes Simplex Virus type 1 is associated with cognitive deficits in the CATIE schizophrenia sample. Schizophrenia Research. 2011;128(1-3):61-5.
259.   Eyles D, Anderson C, Ko P, Jones A, Thomas A, Burne T, et al. A sensitive LC/MS/MS assay of 25OH vitamin D-3 and 25OH vitamin D-2 in dried blood spots. Clinica Chimica Acta. 2009;403(1-2):145-51.
260.   Kvaskoff D, Ko P, Simila HA, Eyles DW. Distribution of 25-hydroxyvitamin D-3 in dried blood spots and implications for its quantitation by tandem mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences. 2012;901:47-52.
261.   Barnett AG, Dobson AJ. Analysing Seasonal Health Data. Analysing Seasonal Health Data. Statistics for Biology and Health2010.
262.   Neale M, Boker S, Xie G, Maes H. Mx: Statistical Modeling. Seventh Edition ed. VCU, Richmond2006.
263.   Paxton GA, Teale GR, Nowson CA, Mason RS, McGrath JJ, Thompson MJ, et al. Vitamin D and health in pregnancy, infants, children and adolescents in Australia and New Zealand: a position statement. Medical Journal of Australia. 2013;198(3):142-3.
264.   Ripke S, Sanders AR, Kendler KS, Levinson DF, Sklar P, Holmans PA, et al. Genome-wide association study identifies five new schizophrenia loci. Nature Genetics. 2011;43(10):969-76.
265.   Shiina T, Hosomichi K, Inoko H, Kulski JK. The HLA genomic loci map: expression, interaction, diversity and disease. Journal of Human Genetics. 2009;54(1):15-39.
266.   Lehner T. The Genes in the Major Histocompatibility Complex as Risk Factors for Schizophrenia: De Omnibus Dubitandum. Biological Psychiatry. 2012;72(8):615-6.
267.   Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D-3 and IL-2 Combine to Inhibit T Cell Production of Inflammatory Cytokines and Promote Development of Regulatory T Cells Expressing CTLA-4 and FoxP3. Journal of Immunology. 2009;183(9):5458-67.
268.   Ozfirat Z, Chowdhury TA. Vitamin D deficiency and type 2 diabetes. Postgraduate Medical Journal. 2010;86(1011):18-25.
269.   McAfoose J, Koerner H, Baune BT. The effects of TNF deficiency on age-related cognitive performance. Psychoneuroendocrinology. 2009;34(4):615-9.
270.   Flislak R, Jaroszewicz J, Lapinski TW, Flislak I, Rogalska M, Prokopowicz D. Plasma transforming growth factor beta(1), metalloproteinase-1 and tissue inhibitor of metalloproteinases-1 in acute viral hepatitis type B. Regulatory Peptides. 2005;131(1-3):54-8.
271.   Valkanova V, Ebmeier KP, Allan CL. CRP, IL-6 and depression: A systematic review and meta-analysis of longitudinal studies. Journal of Affective Disorders. 2013;150(3):736-44.
272.   Bocchio-Chiavetto L, Bagnardi V, Zanardini R, Molteni R, Nielsen MG, Placentino A, et al. Serum and plasma BDNF levels in major depression: A replication study and meta-analyses. World Journal of Biological Psychiatry. 2010;11(6):763-73.
273.   Amitai M, Taler M, Carmel M, Michaelovsky E, Eilat T, Yablonski M, et al. The Relationship Between Plasma Cytokine Levels and Response to Selective Serotonin Reuptake Inhibitor Treatment in Children and Adolescents with Depression and / or Anxiety Disorders. Journal of Child and Adolescent Psychopharmacology. 2016;26.
274.   Huang EJ, Reichardt LF. Neurotrophins: Roles in neuronal development and function. Annual Review of Neuroscience. 2001;24:677-736.
275.   Linker RA, Gold R, Luhder F. Function of Neurotrophic Factors Beyond the Nervous System: Inflammation and Autoimmune Demyelination. Critical Reviews in Immunology. 2009;29(1):43-68.

276.	Pallavi P, Sagar R, Mehta M, Sharma S, Subramanium A, Shamshi F, et al. Serum neurotrophic factors in adolescent depression: Gender difference and correlation with clinical severity. Journal of Affective Disorders. 2013;150(2):415-23.

277.	Otsuki K, Uchida S, Watanuki T, Wakabayashi Y, Fujimoto M, Matsubara T, et al. Altered expression of neurotrophic factors in patients with major depression. Journal of Psychiatric Research. 2008;42(14):1145-53.

278.	Dalman C, Allebeck P, Gunnell D, Harrison G, Kristensson K, Lewis G, et al. Infections in the CNS during childhood and the risk of subsequent psychotic illness: A cohort study of more than one million Swedish subjects. American Journal of Psychiatry. 2008;165(1):59-65.

279.	Groer MW, Yolken RH, Xiao JC, Beckstead JW, Fuchs D, Mohapatra SS, et al. Prenatal depression and anxiety in Toxoplasma gondii-positive women. American Journal of Obstetrics and Gynecology. 2011;204(5).

280.	Yolken RH, Dickerson FB, Torrey EF. Toxoplasma and schizophrenia. Parasite Immunology. 2009;31(11):706-15.

281.	Schwarcz R, Hunter CA. Toxoplasma gondii and schizophrenia: Linkage through astrocyte-derived kynurenic acid? Schizophrenia Bulletin. 2007;33(3):652-3.

282.	da Silva RC, Langoni H. Toxoplasma gondii: host-parasite interaction and behavior manipulation. Parasitology Research. 2009;105(4):893-8.

283.	Petitto JM, McCarthy DB, Rinker CM, Huang Z, Getty T. Modulation of behavioral and neurochemical measures of forebrain dopamine function in mice by species-specific interleukin-2. Journal of Neuroimmunology. 1997;73(1-2):183-90.

284.	Flegr J. Effects of Toxoplasma on human behavior. Schizophrenia Bulletin. 2007;33(3):757-60.

285.	Jackson JR, Eaton WW, Cascella NG, Fasano A, Kelly DL. Neurologic and Psychiatric Manifestations of Celiac Disease and Gluten Sensitivity. Psychiatric Quarterly. 2012;83(1):91-102.

286.	Eaton WW, Mortensen PB, Agerbo E, Byrne M, Mors O, Ewald H. Coeliac disease and schizophrenia: population based case control study with linkage of Danish national registers. British Medical Journal. 2004;328(7437):438-9.

287.	Okusaga O, Yolken RH, Langenberg P, Sleemi A, Kelly DL, Vaswani D, et al. Elevated gliadin antibody levels in individuals with schizophrenia. World Journal of Biological Psychiatry. 2013;14(7):509-15.

288.	Karlsson H, Blomstrom A, Wicks S, Yang S, Yolken RH, Dalman C. Maternal Antibodies to Dietary Antigens and Risk for Nonaffective Psychosis in Offspring. American Journal of Psychiatry. 2012;169(6):625-32.

289.	Rubicz R, Leach CT, Kraig E, Dhurandhar NV, Duggirala R, Blangero J, et al. Genetic Factors Influence Serological Measures of Common Infections. Human Heredity. 2011;72(2):133-41.

290.	Powell JE, Henders AK, McRae AF, Caracella A, Smith S, Wright MJ, et al. The Brisbane Systems Genetics Study: Genetical Genomics Meets Complex Trait Genetics. Plos One. 2012;7(4):e35430.

291.	Mortensen PB, Norgaard-Pedersen B, Waltoft BL, Sorensen TL, Hougaard D, Torrey EF, et al. Toxoplasma gondii as a risk factor for early-onset schizophrenia: analysis of filter paper blood samples obtained at birth. Biological psychiatry. 2007;61(5):688-93.

292.	Powell JE, Henders AK, McRae AF, Kim J, Hemani G, Martin NG, et al. Congruence of Additive and Non-Additive Effects on Gene Expression Estimated from Pedigree and SNP Data. Plos Genetics. 2013;9(5):e1003502.

293.	Gottschalk P, Dunn J. Analytical Biochemistry. 2005;343:54-65.

294.	Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP. Multiplex bead array assays for detection of soluble cytokines: Comparisons of sensitivity and quantitative values among kits from multiple manufacturers. Cytometry Part B-Clinical Cytometry. 2004;61B(1):35-9.

295.	Leng SX, McElhaney JE, Walston JD, Xie DX, Fedarko NS, Kuchel GA. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. Journals of Gerontology Series a-Biological Sciences and Medical Sciences. 2008;63(8):879-84.

296.   Slade T, Johnston A, Browne MAO, Andrews G, Whiteford H. 2007 National Survey of Mental Health and Wellbeing: methods and key findings. Australian and New Zealand Journal of Psychiatry. 2009;43(7):594-605.

297.   Wigman JTW, van Nierop M, Vollebergh WAM, Lieb R, Beesdo-Baum K, Wittchen HU, et al. Evidence That Psychotic Symptoms Are Prevalent in Disorders of Anxiety and Depression, Impacting on Illness Onset, Risk, and Severity - Implications for Diagnosis and Ultra-High Risk Research. Schizophrenia Bulletin. 2012;38(2):247-57.

298.   McGorry PD, Purcell R, Hickie IB, Yung AR, Pantelis C, Jackson HJ. Clinical staging: a heuristic model for psychiatry and youth mental health. Medical Journal of Australia. 2007;187(7):S40-S2.

299.   Insel TR. The NIMH Research Domain Criteria (RDoC) Project: Precision Medicine for Psychiatry. American Journal of Psychiatry. 2014;171(4):395-7.

300.   Casey BJ, Craddock N, Cuthbert BN, Hyman SE, Lee FS, Ressler KJ. DSM-5 and RDoC: progress in psychiatry research? Nature Reviews Neuroscience. 2013;14(11):810-4.

301.   Kim JW, Szigethy EM, Melhem NM, Saghafi EM, Brent DA. Inflammatory Markers and the Pathogenesis of Pediatric Depression and Suicide: A Systematic Review of the Literature. Journal of Clinical Psychiatry. 2014;75(11):1242-53.

302.   Sullivan PF, Fan C, Perou CM. Evaluating the Comparability of Gene Expression in Blood and Brain. American Journal of Medical Genetics Part B (Neuropsychiatric Genetics). 2006;141B:261-8.

303.   Glatt SJ, Everall IP, Kremen WS, Corbeil J, Sasik R, Khanlou N, et al. Comparative gene expression analysis of blood and brain provides concurrent validation of *SELENBP1* up-regulation in schizophrenia. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(43):15533-8.

304.   Dalgaard P. Power and the computation of sample size. Introductory Statistics with R, Second Edition. Statistics and Computing Series2008. p. 155-62.

305.   Hickie IB, Davenport TA, Hadzi-Pavlovic D, Koschera A, Naismith SL, Scott EM, et al. Development of a simple screening tool for common mental disorders in general practice. Medical Journal of Australia. 2001;175 S10-S7.

306.   Eysenck SBG. Junior Eysenck Personality Inventory. EdITS/ Educational and Industrial Testing Service. San Diego, CA.1972.

307.   Foa EB, Johnson KM, Feeny NC, Treadwell KR. The Child PTSD Symptom Scale: A Preliminary Examination of its Psychometric Properties. Journal of Clinical Child Psychology. 2001;30(3):376-84.

308.   McFarlane AC, McKenzie DP, Van Hooff M, Browne D. Somatic and psychological dimensions of screening for psychiatric morbidity: A community validation of the SPHERE Questionnaire. Journal of Psychosomatic Research. 2008;65:337-45.

309.   Hansell NK, Wright MJ, Medland SE, Davenport TA, Wray NR, Martin NG, et al. Genetic co-morbidity between neuroticism, anxiety/depression and somatic distress in a population sample of adolescent and young adult twins. Psychological Medicine. 2012;42:1249-60.

310.   Eysenck HJ, Eysenck SBG, editors. Manual for the Eysenck Personality Questionnaire (Adult and Junior) San Diego, CA: Digits; 1975.

311.   Williams DG. Effects of Psychoticism, Extraversion, and Neuroticism in Current Mood - A Statistical Review of 6 Studies. Personality and Individual Differences. 1990;11(6):615-30.

312.   Caruso JC, Edwards S. Reliability generalization of the Junior Eysenck Personality Questionnaire. Personality and Individual Differences. 2001;31(2001):173-84.

313.   Gillespie NA, Evans DE, Wright MM, Martin NG. Genetic simplex modeling of Eysenck's dimensions of personality in a sample of young Australian twins. Twin Research. 2004;7(6):637-48.

314.   Lustig SL, Botelho C, Lynch L, Nelson SV, Eichelberger WJ, Vaughan BL. Implementing a randomized clinical trial on a pediatric psychiatric inpatient unit at a children's hospital: the case of clonidine for post-traumatic stress. General Hospital Psychiatry. 2002;24(6):422-9.

315.   Furimsky I, Cheung AH, Dewa CS, Zipursky RB. Strategies to enhance patient recruitment and retention in research involving patients with a first episode of mental illness. Contemporary Clinical Trials. 2008;29(6):862-6.

316.   Beard JL, Hendricks MK, Perez EM, Murray-Kolb LE, Berg A, Vernon-Feagans L, et al. Maternal iron deficiency anemia affects postpartum emotions and cognition. Journal of Nutrition. 2005;135(2):267-72.

317.   Yi S, Nanri A, Poudel-Tandukar K, Nonaka D, Matsushita Y, Hori A, et al. Association between serum ferritin concentrations and depressive symptoms in Japanese municipal employees. Psychiatry Research. 2011;189(3):368-72.

318.   Shariatpanaahi MV, Shariatpanaahi ZV, Moshtaaghi M, Shahbaazi SH, Abadi A. The relationship between depression and serum ferritin level. European Journal of Clinical Nutrition. 2007;61(4):532-5.

319.   Hunt JR, Penland JG. Iron status and depression in premenopausal women: An MMPI study. Behavioral Medicine. 1999;25(2):62-8.

320.   Khedr E, Hamed SA, Elbeih E, El-Shereef H, Ahmad Y, Ahmed S. Iron states and cognitive abilities in young adults: neuropsychological and neurophysiological assessment. European Archives of Psychiatry and Clinical Neuroscience. 2008;258(8):489-96.

321.   Kassebaum NJ, Jasrasaria R, Naghavi M, Wulf SK, Johns N, Lozano R, et al. A systematic analysis of global anemia burden from 1990 to 2010. Blood. 2014;123(5):615-24.

322.   Pasricha SRS, Flecknoe-Brown SC, Allen KJ, Gibson PR, McMahon LP, Olynyk JK, et al. Diagnosis and management of iron deficiency anaemia: a clinical update. Medical Journal of Australia. 2010;193(9):525-32.

323.   UNICEF, WHO. Report of the UNICEF / WHO Regional Consultation: Prevention and Control of Iron Deficiency Anaemia in Women and Children. Geneva, Switzerland: 1999.

324.   Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, et al. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet. 2013;382(9904):1575-86.

325.   Baune BT, Neuhauser H, Ellert U, Berger K. The role of the inflammatory markers ferritin, transferrin and fibrinogen in the relationship between major depression and cardiovascular disorders - The German Health Interview and Examination Survey. Acta Psychiatrica Scandinavica. 2010;121:135-42.

326.   Njajou OT, Alizadeh BZ, Aulchenko Y, Zillikens MC, Pols HAP, Oostra BA, et al. Heritability of Serum Iron, Ferritin and Transferrin Saturation in a Genetically Isolated Population, the Erasmus Rucphen Family (ERF) Study. Human Heredity. 2006;61:222-8.

327.   Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, et al. Effects of *HFE* C282Y and H63D Polymorphisms and Polygenic Background on Iron Stores in a Large Community Sample of Twins. American Journal of Human Genetics. 2000;66(4):1246-58.

328.   Traglia M, Sala C, Masciullo C, Cverhova V, Lori F, Pistis G, et al. Heritability and Demographic Analyses in the Large Isolated Population of Val Borbera Suggest Advantages in Mapping Complex Traits Genes. Plos One. 2009;4(10):e7554.

329.   Benyamin B, McRae AE, Zhu G, Gordon S, Henders AK, Palotie A, et al. Variants in TF and HFE Explain similar to 40% of Genetic Variation in Serum-Transferrin Levels. American Journal of Human Genetics. 2009;84(1):60-5.

330.   Benyamin B, Esko T, Ried JS, Radhakrishnan A, Vermeulen SH, Traglia M, et al. Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis. Nature Communications. 2014;5.

331.   Visscher PM, Brown MA, McCarthy MI, Yang J. Five Years of GWAS Discovery. American Journal of Human Genetics. 2012;90(1):7-24.

332.   Hiraki LT, Major JM, Chen C, Cornelis MC, Hunter DJ, Rimm EB, et al. Exploring the Genetic Architecture of Circulating 25-Hydroxyvitamin D. Genetic Epidemiology. 2013;37(1):92-8.

333.   Wray NR, Lee SH, Mehta D, Vinkhuyzen AAE, Dudbridge F, Middeldorp CM. Research Review: Polygenic methods and their application to psychiatric traits. Journal of Child Psychology and Psychiatry. 2014;55(10):1068-87.

334.   Hickie I, Hadzi-Pavlovic D, Scott E, Davenport T, Koschera A, Naismith S. SPHERE: A National Depression Project. Australasian Psychiatry. 1998;6(5):248-50.

335.   Neale MC, Eaves LJ, Kendler KS. The power of the classical twin study to resolve variation in threshold traits. Behavior Genetics. 1994;24:239-58.

336.   Crichton RR, Charloteaux-Wauters M. Iron transport and storage. European Journal of Biochemistry. 1987;164(3):485-506.

337.   Bedford A, Deary IJ. The Delusions-Symptoms-States Inventory (DSSI): Construction, applications and structural analyses. Personality and Individual Differences. 1999;26(3):397-424.

338.   Derogatis LR, Rickels K, Rock AF. SCL-90 and MMPI - step in validation of a new self-report scale. British Journal of Psychiatry. 1976;128(Mar):280-9.

339.   Visscher PM. Power of the classical twin design revisited. Twin Research. 2004;7(5):505-12.

340.   Visscher PM, Gordon S, Neale MC. Power of the classical twin design revisited: II detection of common environmental variance. Twin Research and Human Genetics. 2008;11(1):48-54.

341.   McLaren CE, Garner CP, Constantine CC, McLachlan S, Vulpe C, Snively BM, et al. Genome-Wide Association Study Identifies Genetic Loci Associated with Iron Deficiency. PLoS ONE. 2011;6(3): e17390.

342.   Tanaka T, Roy CN, Yao W, Matteini A, Semba RD, Arking D, et al. A genome-wide association analysis of serum iron concentrations. Blood. 2010;115:94-6.

343.   Purcell S, Cherny SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics. 2003;19(1):149-50.

344.   Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. American Journal of Human Genetics. 2007;81(3):559-75.

345.   Bulik-Sullivan BK, Loh PR, Finucane HK, Ripke S, Yang J, Patterson N, et al. LD Score regression distinguishes confounding from polygenicity in genome-wide association studies. Nature Genetics. 2015;47(3):291-5.

346.   Bulik-Sullivan B, Finucane HK, Anttila V, Gusev A, Day FR, Loh PR, et al. An Atlas of Genetic Correlations across Human Diseases and Traits. Nature Genetics. 2015;47(11):1236-41.

347.   Firkin F, Rush B. Interpretation of biochemical tests for iron deficiency: diagnostic difficulties related to limitations of individual tests. Australian Prescriber. 1997;20:74-6.

348.   Fairweather-Tait SJ, Guile GR, Valdes AM, Wawer AA, Hurst R, Skinner J, et al. The Contribution of Diet and Genotype to Iron Status in Women: A Classical Twin Study. Plos One. 2013;8(12).

349.   Black S, Kushner I, Samols D. C-reactive protein. Journal of Biological Chemistry. 2004;279(47):48487-90.

350.   Greenfield JR, Samaras K, Jenkins AB, Kelly PJ, Spector TD, Gallimore JR, et al. Obesity is an important determinant of baseline serum C-reactive protein concentration in monozygotic twins, independent of genetic influences. Circulation. 2004;109(24):3022-8.

351.   Mendall MA, Patel P, Ballam L, Strachan D, Northfield TC. C Reactive protein and its relation to cardiovascular risk factors: A population based cross sectional study. British Medical Journal. 1996;312(7038):1061-5.

352.   Frohlich M, Imhof A, Berg G, Hutchinson WL, Pepys MB, Boeing H, et al. Association between C-reactive protein and features of the metabolic syndrome - A population-based study. Diabetes Care. 2000;23(12):1835-9.

353.   Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. Jama-Journal of the American Medical Association. 1999;282(22):2131-5.

354.   Khaodhiar L, Ling PR, Blackburn GL, Bistrian BR. Serum levels of interleukin-6 and C-reactive protein correlate with body mass index across the broad range of obesity. Journal of Parenteral and Enteral Nutrition. 2004;28(6):410-5.

355.   Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx B, et al. Overweight, Obesity, and Depression A Systematic Review and Meta-analysis of Longitudinal Studies. Archives of General Psychiatry. 2010;67(3):220-9.

356.   Tiemeier H, Hofman A, van Tuijl HR, Kiliaan AJ, Meijer J, Breteler MMB. Inflammatory proteins and depression in the elderly. Epidemiology. 2003;14(1):103-7.

357.   Baumeister D, Akhtar R, Ciufolini S, Pariante C, Mondelli V. Childhood trauma and adulthood inflammation: a meta-analysis of peripheral C-reactive protein, interleukin-6 and tumour necrosis factor-a Molecular Psychiatry. 2016;21(5):642-9.
358.   Miller G, Chen E, Cole SW. Health Psychology: Developing Biologically Plausible Models Linking the Social World and Physical Health. Annual Review of Psychology. 2009;60:501-24.
359.   Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, et al. Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. British Medical Journal. 2000;321(7255):199-204.
360.   Hettema JM. What is the genetic relationship between anxiety and depression? American Journal of Medical Genetics Part C-Seminars in Medical Genetics. 2008;148C(2):140-6.
361.   Retterstol L, Eikvar L, Berg K. A twin study of C-Reactive Protein compared to other risk factors for coronary heart disease. Atherosclerosis. 2003;169(2):279-82.
362.   MacGregor AJ, Gallimore JR, Spector TD, Pepys MB. Genetic effects on baseline values of C-reactive protein and serum amyloid A protein: A comparison of monozygotic and dizygotic twins. Clinical Chemistry. 2004;50(1):130-4.
363.   Sas AA, Rijsdijk FV, Ormel J, Snieder H, Riese H. The Relationship Between Neuroticism and Inflammatory Markers: A Twin Study. Twin Research and Human Genetics. 2014;17(3):177-82.
364.   Maes HHM, Neale MC, Eaves LJ. Genetic and environmental factors in relative body weight and human adiposity. Behavior Genetics. 1997;27(4):325-51.
365.   Stunkard AJ, Foch TT, Hrubec Z. A Twin Study of Human Obesity. Jama-Journal of the American Medical Association. 1986;256(1):51-4.
366.   Halder I, Marsland AL, Cheong J, Muldoon MF, Ferrell RE, Manuck SB. Polymorphisms in the CRP gene moderate an association between depressive symptoms and circulating levels of C-reactive protein. Brain Behavior and Immunity. 2010;24(1):160-7.
367.   Cai N, Bigdeli TB, Kretzschmar W, Li YH, Liang JQ, Song L, et al. Sparse whole-genome sequencing identifies two loci for major depressive disorder. Nature. 2015;523(7562):588-91.
368.   Dehghan A, Dupuis J, Barbalic M, Bis JC, Eiriksdottir G, Lu C, et al. Meta-Analysis of Genome-Wide Association Studies in > 80 000 Subjects Identifies Multiple Loci for C-Reactive Protein Levels. Circulation. 2011;123(7):731-8.
369.   Sheehan NA, Didelez V, Burton PR, Tobin MD. Mendelian randomisation and causal inference in observational epidemiology. Plos Medicine. 2008;5(8):1205-10.
370.   Timpson NJ, Nordestgaard BG, Harbord RM, Zacho J, Frayling TM, Tybjaerg-Hansen A, et al. C-reactive protein levels and body mass index: elucidating direction of causation through reciprocal Mendelian randomization. International Journal of Obesity. 2011;35(2):300-8.
371.   Kirk KM, Birley AJ, Statham DJ, Haddon B, Lake RI, Andrews JG, et al. Anxiety and depression in twin and sib pairs extremely discordant and concordant for neuroticism: prodromus to a linkage study. Twin Research. 2000;3(4):299-309.
372.   Hansell NK, Agrawal A, Whitfield JB, Morley KI, Zhu G, Lind PA, et al. Long-term stability and heritability of telephone interview measures of alcohol consumption and dependence. Twin Research and Human Genetics. 2008;11(3):287-305.
373.   Nelson EC, Lynskey MT, Heath AC, Madden PAF, Martin NG. A Family Study of Adult Twins with and without a History of Childhood Abuse: Stability of Retrospective Reports of Maltreatment and Associated Family Measures. Twin Research and Human Genetics. 2010;13(2):121-30.
374.   Mosing MA, Gordon SD, Medland SE, Statham DJ, Nelson EC, Heath AC, et al. Genetic and environmental influences on the co-morbidity between depression, panic disorder, agoraphobia, and social phobia: a twin study. Depression and Anxiety. 2009;26(11):1004-11.
375.   Nelson EC, Heath AC, Madden PAF, Cooper ML, Dinwiddie SH, Bucholz KK, et al. Association between self-reported childhood sexual abuse and adverse psychosocial outcomes - Results from a twin study. Archives of General Psychiatry. 2002;59(2):139-45.
376.   Nelson EC, Heath AC, Lynskey MT, Bucholz KK, Madden PAF, Statham DJ, et al. Childhood sexual abuse and risks for licit and illicit drug-related outcomes: a twin study. Psychological Medicine. 2006;36(10):1473-83.

377.   Heath AC, Howells W, Kirk KM, Madden PAF, Bucholz KK, Nelson EC, et al. Predictors of Non-Response to a Questionnaire Survey of a Volunteer Twin Panel: Findings from the Australian 1989 Twin Cohort. Twin Research and Human Genetics. 2001;4(2):73-80.

378.   Fergusson DM, Lynskey MT, Horwood LJ. Childhood sexual abuse and psychiatric disorder in young adulthood .1. Prevalence of sexual abuse and factors associated with sexual abuse. Journal of the American Academy of Child and Adolescent Psychiatry. 1996;35(10):1355-64.

379.   Fergusson DM, Lynskey MT. Physical punishment/maltreatment during childhood and adjustment in young adulthood. Child Abuse & Neglect. 1997;21(7):617-30.

380.   Fergusson DM, Horwood LJ, Shannon FT, Lawton JM. The Christchurch Child Development Study: a review of epidemiological findings. Paediatric and Perinatal Epidemiology. 1989;3:302-25.

381.   Byrne EM, Raheja UK, Stephens SH, Heath AC, Madden PAF, Vaswani D, et al. Seasonality Shows Evidence for Polygenic Architecture and Genetic Correlation With Schizophrenia and Bipolar Disorder. Journal of Clinical Psychiatry. 2015;76(2):128-34.

382.   Medland SE, Nyholt DR, Painter JN, McEvoy BP, McRae AF, Zhu G, et al. Common Variants in the Trichohyalin Gene Are Associated with Straight Hair in Europeans. American Journal of Human Genetics. 2009;85(5):750-5.

383.   Li Y, Willer C, Sanna S, Abecasis G. Genotype Imputation. Annual Review of Genomics and Human Genetics. 2009;10:387-406.

384.   Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nature Genetics. 2010;42(11):937-48.

385.   Purcell SM, Wray NR, Stone JL, Visscher PM, O'Donovan MC, Sullivan PF, et al. Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. Nature. 2009;460(7256):748-52.

386.   Bertone-Johnson ER, Whitcomb BW, Missmer SA, Karlson EW, Rich-Edwards JW. Inflammation and Early-Life Abuse in Women. American Journal of Preventive Medicine. 2012;43(6):611-20.

387.   Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet. 2011;377(9765):557-67.

388.   Wang YC, McPherson K, Marsh T, Gortmaker SL, Brown M. Obesity 2 Health and economic burden of the projected obesity trends in the USA and the UK. Lancet. 2011;378(9793):815-25.

389.   Rosenblat JD, Cha DS, Mansur RB, McIntyre RS. Inflamed moods: A review of the interactions between inflammation and mood disorders. Progress in Neuro-Psychopharmacology & Biological Psychiatry. 2014;53:23-34.

390.   Peyrot WJ, Milaneschi Y, Abdellaoui A, Sullivan PF, Hottenga JJ, Boomsma DI, et al. Effect of polygenic risk scores on depression in childhood trauma. British Journal of Psychiatry. 2014;205(2):113-9.

391.   Hung CF, Breen G, Czamara D, Corre T, Wolf C, Kloiber S, et al. A genetic risk score combining 32 SNPs is associated with body mass index and improves obesity prediction in people with major depressive disorder. Bmc Medicine. 2015;13.

392.   Hung CF, Rivera M, Craddock N, Owen MJ, Gill M, Korszun A, et al. Relationship between obesity and the risk of clinically significant depression: Mendelian randomisation study. British Journal of Psychiatry. 2014;205(1):24-8.

393.   Rivera M, Cohen-Woods S, Kapur K, Breen G, Ng MY, Butler AW, et al. Depressive disorder moderates the effect of the FTO gene on body mass index. Molecular Psychiatry. 2012;17(6):604-11.

394.   Macy EM, Hayes TE, Tracy RP. Variability in the measurement of C-reactive protein in healthy subjects: Implications for reference intervals and epidemiological applications. Clinical Chemistry. 1997;43(1):52-8.

395.	Ishikawa S, Kayaba K, Gotoh T, Nakamura Y, Kario K, Ito Y, et al. Comparison of C-reactive protein levels between serum and plasma samples on long-term frozen storage after a 13.8 year interval: The JMS cohort study. Journal of Epidemiology. 2007;17(4):120-4.

396.	O'Connor TG, Moynihan JA, Caserta MT. Annual Research Review: The neuroinflammation hypothesis for stress and psychopathology in children - developmental psychoneuroimmunology. Journal of Child Psychology and Psychiatry. 2014;55(6):615-31.

397.	Grudet C, Malm J, Westrin A, Brundin L. Suicidal patients are deficient in vitamin D, associated with a pro-inflammatory status in the blood. Psychoneuroendocrinology. 2014;50:210-9.

398.	Bankier B, Barajas J, Martinez-Rumayor A, Januzzi JL. Association between C-reactive protein and generalized anxiety disorder in stable coronary heart disease patients. European Heart Journal. 2008;29(18):2212-7.

399.	Vogelzangs N, Beekman ATF, de Jonge P, Penninx B. Anxiety disorders and inflammation in a large adult cohort. Translational Psychiatry. 2013;3:e249.

400.	Moffitt TE, Caspi A, Harrington H, Milne BJ, Melchior M, Goldberg D, et al. Generalized anxiety disorder and depression: childhood risk factors in a birth cohort followed to age 32. Psychological Medicine. 2007;37(3):441-52.

401.	Vitiello B, Correll C, van Zwieten-Boot B, Zuddas A, Parellada M, Arango C. Antipsychotics in children and adolescents: Increasing use, evidence for efficacy and safety concerns. European Neuropsychopharmacology. 2009;19(9):629-35.

402.	Muller N, Krause D, Dehning S, Musil R, Schennach-Wolff R, Obermeier M, et al. Celecoxib treatment in an early stage of schizophrenia: Results of a randomized, double-blind, placebo-controlled trial of celecoxib augmentation of amisulpride treatment. Schizophrenia Research. 2010;121(1-3):118-24.

403.	Amminger GP, Schafer MR, Papageorgiou K, Klier CM, Cotton SM, Harrigan SM, et al. Long-Chain omega-3 Fatty Acids for Indicated Prevention of Psychotic Disorders A Randomized, Placebo-Controlled Trial. Archives of General Psychiatry. 2010;67(2):146-54.

404.	Fusar-Poli P, Berger G. Eicosapentaenoic Acid Interventions in Schizophrenia Meta-Analysis of Randomized, Placebo-Controlled Studies. Journal of Clinical Psychopharmacology. 2012;32(2):179-85.

405.	Byrne ML, O'Brien-Simpson NM, Reynolds EC, Walsh KA, Laughton K, Waloszek JM, et al. Acute phase protein and cytokine levels in serum and saliva: A comparison of detectable levels and correlations in a depressed and healthy adolescent sample. Brain Behavior and Immunity. 2013;34:164-75.

406.	Sonuga-Barke EJS. Editorial: Developmental foundations of mental health and disorder - moving beyond 'Towards...'. Journal of Child Psychology and Psychiatry. 2014;55(6):529-31.

407.	Miller BJ, Gassama B, Sebastian D, Buckley P, Mellor A. Meta-Analysis of Lymphocytes in Schizophrenia: Clinical Status and Antipsychotic Effects. Biological Psychiatry. 2013;73(10):993-9.

408.	Lamers F, Vogelzangs N, Merikangas KR, de Jonge P, Beekman ATF, Penninx BWJH. Evidence for a differential role of HPA-axis function, inflammation and metabolic syndrome in melancholic versus atypical depression. Molecular Psychiatry. 2013;18(6):692-9.

409.	Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. Current Opinion in Pharmacology. 2010;10(4):482-96.

410.	Zhang Y, Leung DYM, Richers BN, Liu YS, Remigio LK, Riches DW, et al. Vitamin D Inhibits Monocyte/Macrophage Proinflammatory Cytokine Production by Targeting MAPK Phosphatase-1. Journal of Immunology. 2012;188(5):2127-35.

411.	Slopen N, Kubzansky LD, Koenen KC. Internalizing and externalizing behaviors predict elevated inflammatory markers in childhood. Psychoneuroendocrinology. 2013;38(12):2854-62.
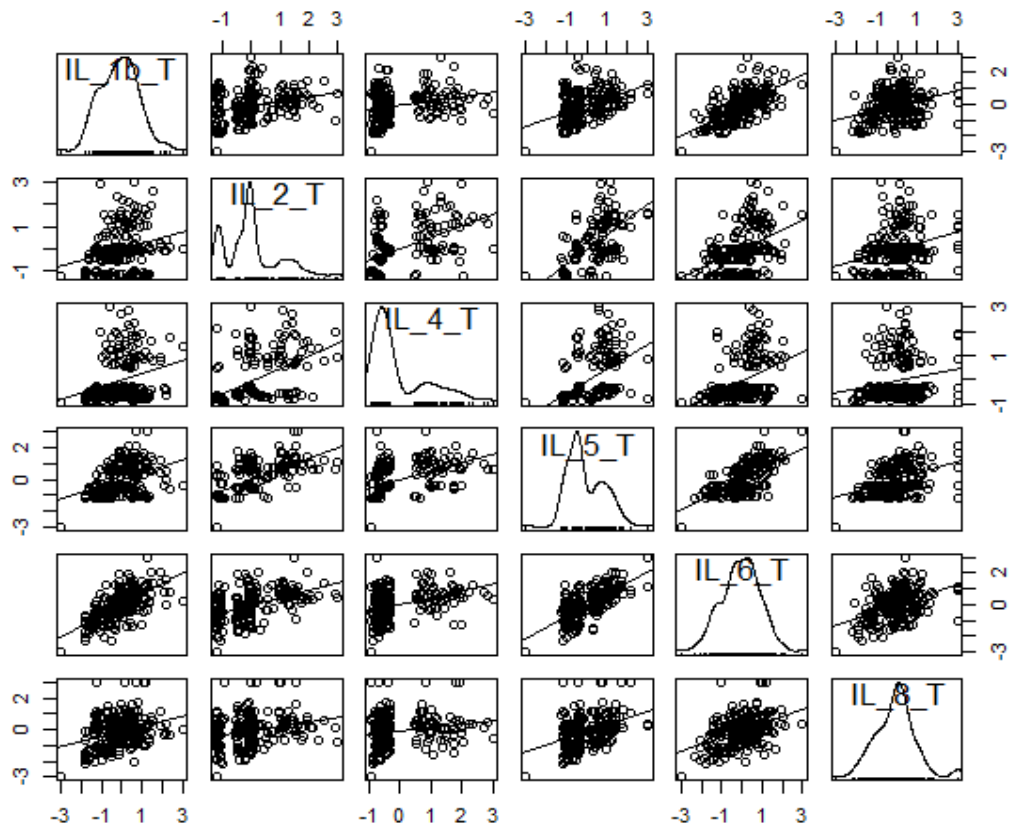
Appendices

Figure 8-1: Distribution and correlations of cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 (measured in DBS)
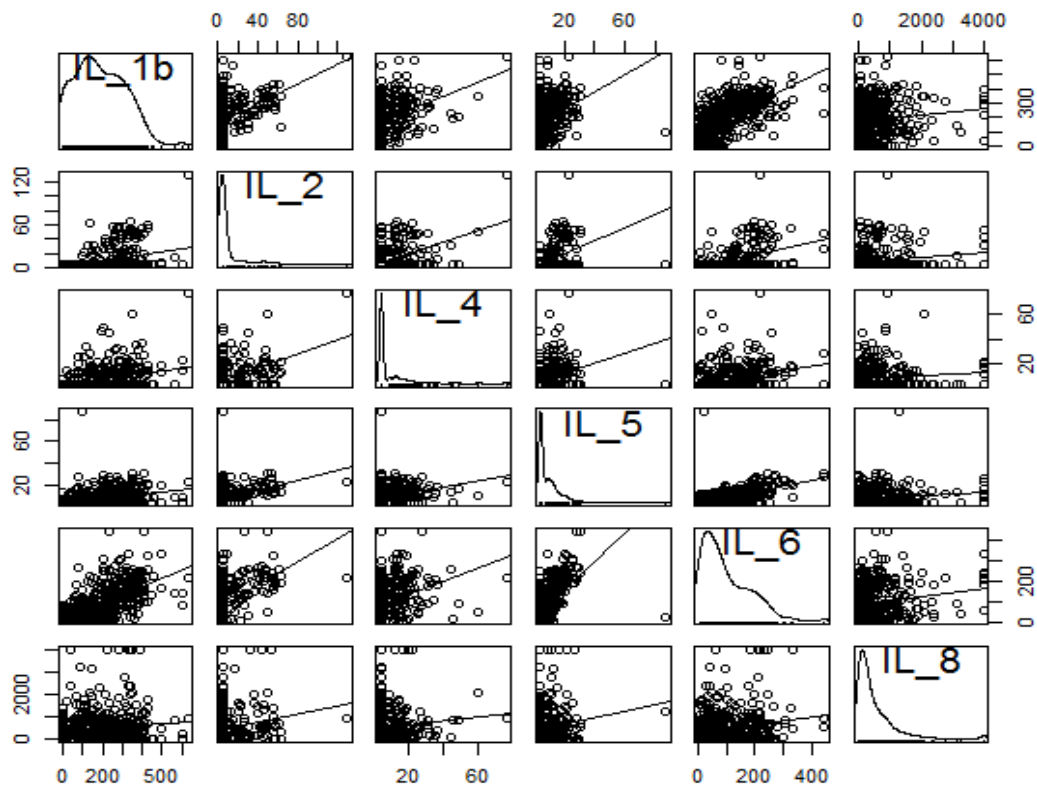
Key: IL=interleukin

Note: diagonal shows distribution of each cytokine; off-diagonals show correlations between a pair of cytokines (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokines IL-5 and IL-6).
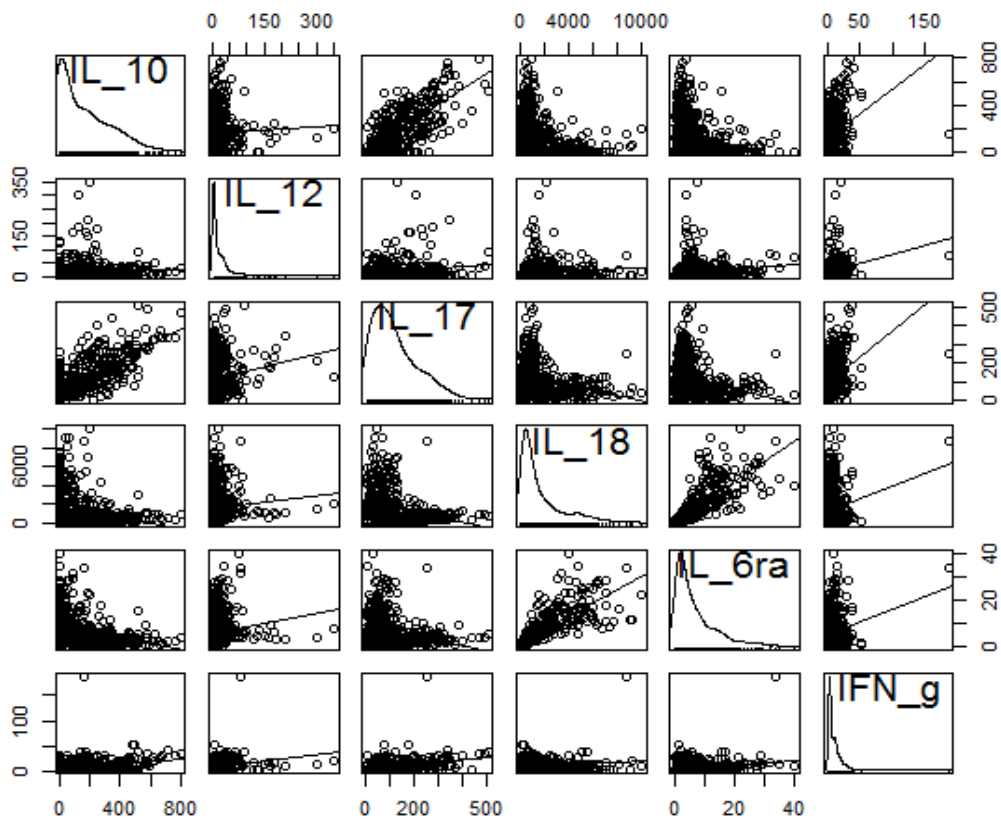
Figure 8-2: Distribution and correlation of cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 after regression of within-paper rank normalised measures on sex and plate (measured in DBS)

Key: IL=interleukin

Note: diagonal shows distribution of each cytokine; off-diagonals show correlations between a pair of cytokines (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokines IL-5 and IL-6).

Figure 8-3: Distribution and correlation of cytokines IL-10, IL-12, IL-17, IL-18, IL-6ra, and IFN-γ (measured in DBS)

Key: IFN=interferon; IL=interleukin

Note: diagonal shows distribution of each cytokine; off-diagonals show correlations between a pair of cytokines (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokines IL-18 and IL-6ra).

Figure 8-4: Distribution and correlation of cytokines IL-10, IL-12, IL-17, IL-18, IL-6ra, and IFN-γ after regression of within-paper rank normalised measures on sex and plate (measured in DBS)
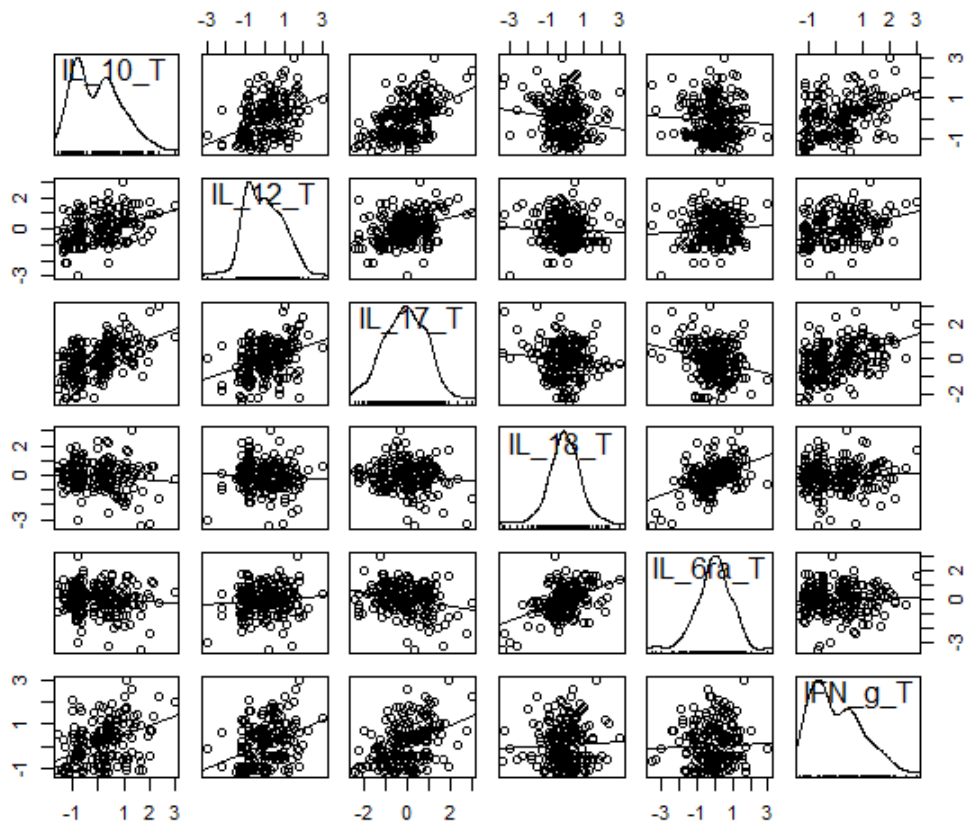
Key: IFN=interferon; IL=interleukin

Note: diagonal shows distribution of each cytokine; off-diagonals show correlations between a pair of cytokines (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokines IL-18 and IL-6ra).
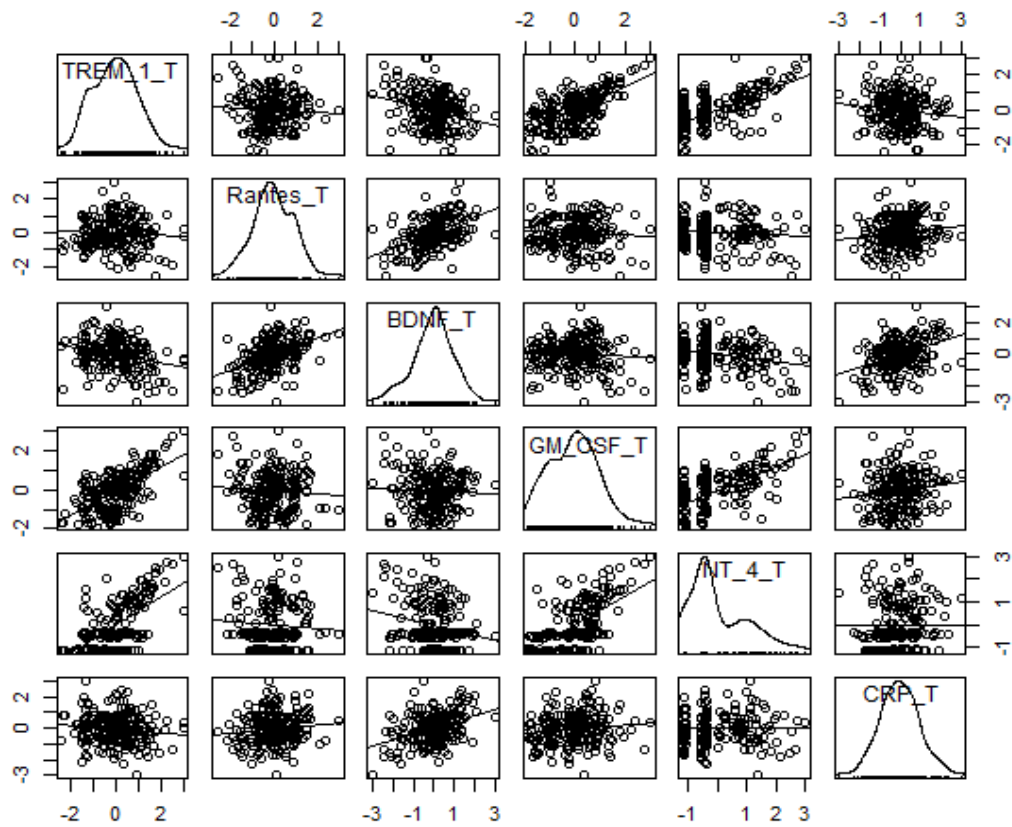
Figure 8-5: Distribution and correlation of TREM-1, RANTES, BDNF, GM-CSF, NT-4 and CRP (measured in DBS)
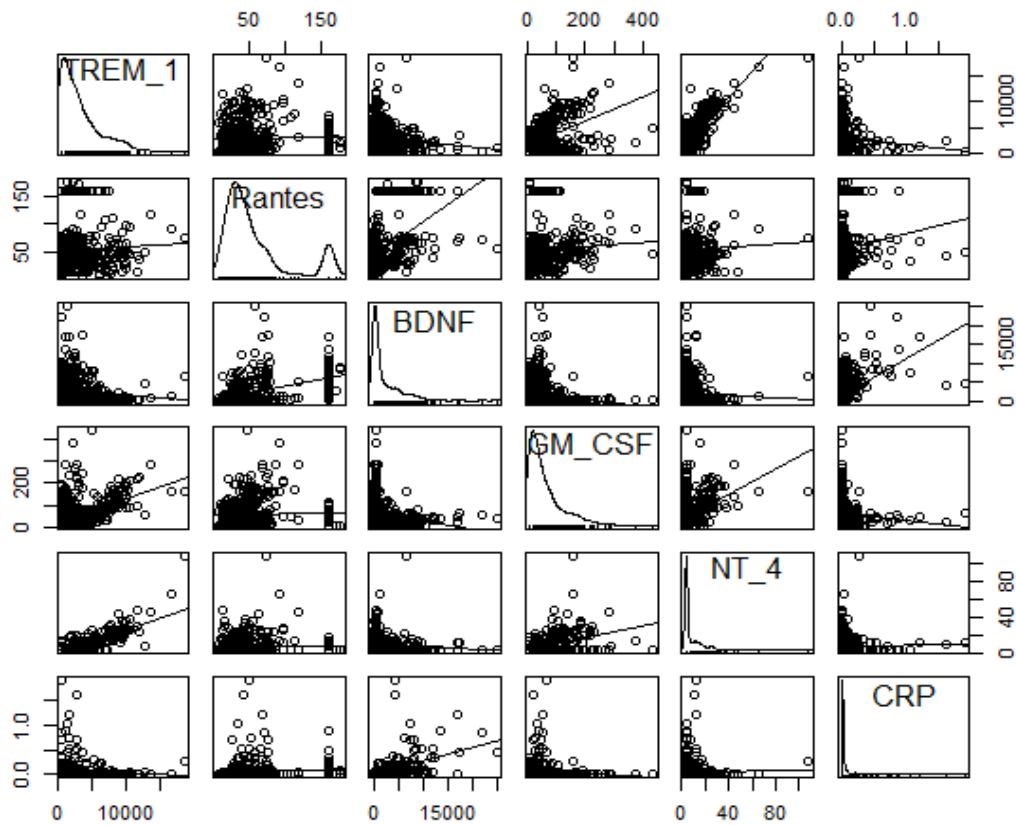
Key: BDNF=brain-derived neurotrophic factor; CRP=C-reactive protein; GM-CSF=Granulocyte Macrophage-Colony Stimulating Factor; NT-4=neurotrophin-4; RANTES= Regulated and Normal T-cell Expressed and Secreted; TREM-1=Triggering Receptor Expressed on Myeloid cells-1

Note: diagonal shows distribution of each cytokine, inflammatory marker, and neurotrophin; off-diagonals show correlations between a pair (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokine GM-CSF and the neurotrophin NT-4).
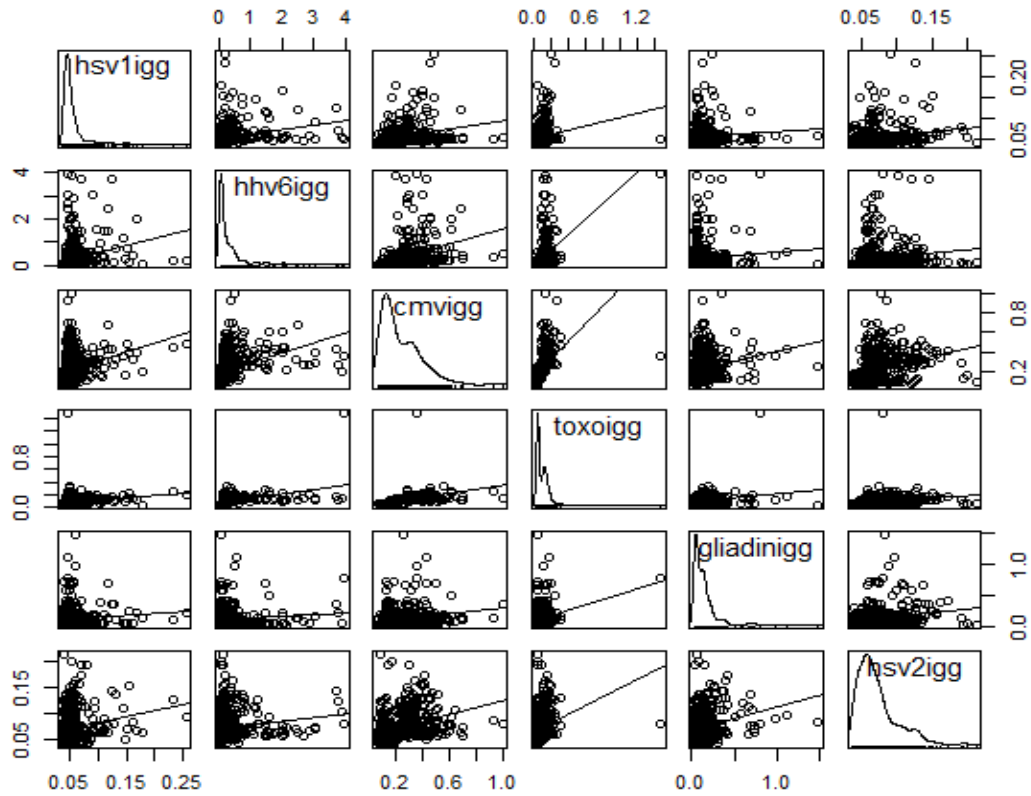
Figure 8-6: Distribution and correlation of TREM-1, RANTES, BDNF, GM-CSF, NT-4 and CRP after regression of within-paper rank normalised measures on sex and plate (measured in DBS)

Key: BDNF=brain-derived neurotrophic factor; CRP=C-reactive protein; GM-CSF=Granulocyte Macrophage-Colony Stimulating Factor; NT-4=neurotrophin-4; RANTES= Regulated and Normal T-cell Expressed and Secreted; TREM-1=Triggering Receptor Expressed on Myeloid cells-1

Note: diagonal shows distribution of each cytokine, inflammatory marker, and neurotrophin; off-diagonals show correlations between a pair (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the cytokine GM-CSF and the neurotrophin NT-4).
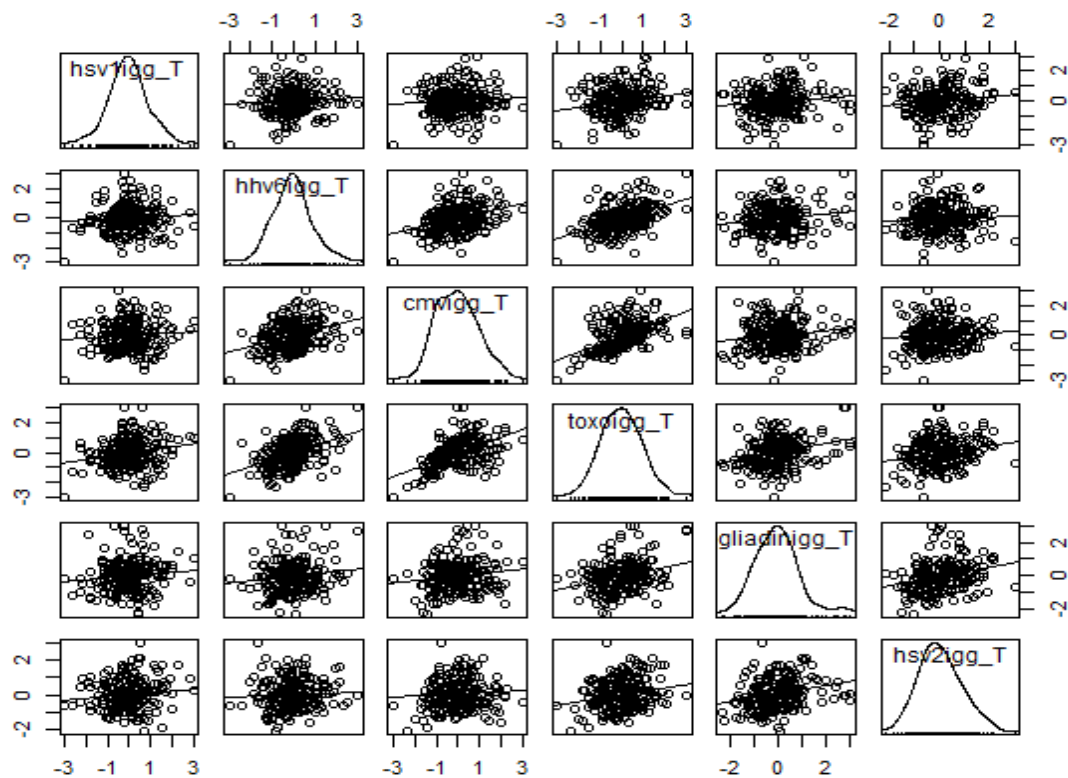
Figure 8-7: Distribution and correlation of antibodies hsv1igg, hhv6igg, cmvigg, toxoigg, gliadinigg, and hsv2igg (measured in DBS)

Key: cmv=cytomegalovirus; hhv6igg=Human Herpes Virus 6; hsv=Herpes Simplex Virus; igg=immunoglobulin; toxo=toxoplasmosis

Note: diagonal shows distribution of each antibody; off-diagonals show correlations between a pair of antibodies (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the toxoplasmosis and gliadin antibodies)

Figure 8-8: Distribution and correlation of hsv1igg, hhv6igg, cmvigg, toxoigg, gliadinigg, & hsv2igg after regression of within-paper rank normalised measures on sex and plate (measured in DBS)

Key: cmv=cytomegalovirus; hhv6igg=Human Herpes Virus 6; hsv=Herpes Simplex Virus; igg=immunoglobulin; toxo=toxoplasmosis

Note: diagonal shows distribution of each antibody; off-diagonals show correlations between a pair of antibodies (for example, the plot on the intersection of the 4th row and 5th column is a plot showing the correlation between the toxoplasmosis and gliadin antibodies)

Table 8-1: Log CRP genetic profile risk scores – prediction of BMI

P-value of SNPs in CRP discovery GWAS	N SNPs	Variance of BMI explained (R^2)	P-value for prediction of BMI
P < 0.001	1,972	0	0.52
P < 0.01	11,154	0.04	0.18
P < 0.1	70,982	0	0.62
P < 0.5	241,214	0.03	0.21
All SNPs	361,282	0.04	0.18

Key: BMI = body mass index; CRP = C-reactive protein