IDENTIFYING BASELINE PREDICTORS OF RELAPSE AND STRATIFYING IMMUNE COMPOSITION IN MAJOR DEPRESSIVE DISORDER

IDENTIFYING BASELINE PREDICTORS OF RELAPSE AND STRATIFYING IMMUNE COMPOSITION IN MAJOR DEPRESSIVE DISORDER

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ABSTRACT

A major challenge in the treatment of major depressive disorder (MDD) is relapse, which is defined as the return of depressive symptoms during a period of remission (Bockting et al., 2015). Relapse rates in MDD are high, with approximately 50% of individuals relapsing following treatment of their first depressive episode (Moriarty et al., 2020), therefore early intervention to prevent relapse is crucial. Evidence suggests that immune dysregulation may be linked to longitudinal changes in depressive severity (Bell et al., 2017; Chu et al., 2019; Khandaker et al., 2014). However, it is currently unknown whether inflammation can predict future relapse in MDD. The objective of this project was to identify potential immune predictors of relapse in participants that responded to a treatment or a combination of treatments for MDD. A secondary objective was to investigate immune composition in efforts to stratify MDD individuals into more homogenous groups and further explore these groups in relation to clinical symptoms. This project is part of the Wellness Monitoring for Major Depressive Disorder longitudinal study (NCT02934334) of responders to antidepressant treatment conducted at 6 clinical sites across Canada. Montgomery Asberg Depression Rating Scale (MADRS) scores were used to assess depression severity and to categorize participants into ultrastable, unstable, and relapse groups. Plasma immune profiles were generated using the LEGENDplex Human Th Cytokine Panel immunoassay. Principal Component Analysis and Kruskal-Wallis tests of individual immune cytokines did not show differences between ultrastable, unstable, or relapse groups. Principal Component Analysis did reveal two cytokine clusters. Hierarchical Clustering analysis identified

three distinct immune biotypes characterized by differing levels of Th cytokines and validated the presence of the cytokine clusters. Neither of these outcomes was predictive of relapse in this cohort. Our findings have shown that immune composition may serve as an important factor in parsing heterogeneity that is observed in this disorder through identification of distinct immune biotypes and highly interconnected cytokine subnetworks in major depression. The potential for immune biotypes for optimizing treatment regimens and relapse prevention necessitates further investigation and replication.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BD	Bipolar Disorder
BMI	Body mass index
CV	Coefficient of Variance
CRP	C-reactive protein
GAD	Generalized Anxiety Disorder
GAD7	Generalized Anxiety Disorder 7-Item Scale
HAM-D	Hamilton Depressing Rating Scale
IL	Interleukin
IFN-ɣ	Interferon-gamma
MADRS	Montgomery Asperg Depression Rating Scale
HC	Hierarchical clustering
MCP-1	Monocyte Chemoattractant Protein-1
MDD	Major Depressive Disorder
MDE	Major Depressive Episode
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
QC	Quality control
QIDS-SR	Quick Inventory of Depressive Symptomology (QIDS-SR)
RA	Rheumatoid arthritis
SHAPS	Snaith-Hamilton Pleasure Scale

SNP	Single nucleotide polymorphism
SOP	Standard Operating Procedure
SSRI	Selective serotonin reuptake inhibitor
STD	Standard
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
Th22	T helper type 22
TNF-α	Tumor necrosis factor-alpha
UND	Undetected

DECLARATION OF ACADEMIC ACHIEVEMENT

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1.0 INTRODUCTION

1.1 Relapse in Major Depressive Disorder

Major depressive disorder (MDD) is a heterogenous, complex mood disorder defined in the DSM-5 that is characterized by discrete major depressive episodes (MDE) of at least two weeks that are marked by clear changes in affect and cognition (American Psychiatric Association, 2014). In addition, for an MDD diagnosis, five or more of the following symptoms need to be present within the same two week period and represent a change from previous functioning, including; depressed mood, diminished interest or pleasure (anhedonia), significant increases or decreases in weight, sleep disturbances (including insomnia or hypersomnia), psychomotor disturbances, fatigue, feelings of worthlessness or excessive guilt, diminished concentration or decisiveness, or recurrent thoughts of death with or without suicidal ideation or intent (American Psychiatric Association, 2014). MDD is a debilitating illness, causing significant reductions in quality of life and productivity, making it one of the leading causes of disability worldwide (Culpepper et al., 2015). Given that MDD is highly recurrent in nature, relapse, which is marked as the return of depressive symptoms during a period of remission (Bockting et al., 2015), is a significant barrier in the treatment of the disorder. Relapse rates in MDD are high, with approximately 50% of individuals relapsing following treatment of their first MDE (Moriarty et al., 2020). Thus, interventions targeted toward the prevention of relapse and subsequent maintenance of remission are essential.

1.2 Peripheral and Central Immune Communication in Major Depression

Cytokines are a type of glycoprotein responsible for orchestrating the immune response. Pro-inflammatory cytokines, such as interleukin (IL)-6, IL-1B, interferon-gamma (IFN-ɣ), and tumor necrosis factor-alpha (TNF-α), are responsible for upregulation of the inflammatory

response (Borish & Steinke, 2003; Zhang & An, 2007). On the other hand, anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, are responsible for controlling and modulating the pro-inflammatory response (Zhang & An, 2007). These cytokines can act upon specific pathways and mechanisms to initiate and perpetuate illness course in depression. Either via direct transport (Gutierrez et al., 1993) or by interacting with circumventricular organs (Herkenham et al., 1998; Quan et al., 1999), blood-brain barrier cells (Herkenham et al., 1998; Quan et al., 1999), and peripheral nerves such as the vagus nerve (Maier et al., 1998), cytokines in the periphery are able to propagate signals into the brain parenchyma and influence pathways that are implicated in the development of depression. Specifically, animal studies have demonstrated cytokine-induced behavioural changes, termed "sickness behaviour", that are consistent with key features of major depression in humans. Using a two-hit model of depression (chronic stress + induced acute inflammation) in rats, researchers found that increased serum levels of IFN-ɣ in the periphery were associated with anhedonic-like behaviours (Géa et al., 2019). In a different study, researchers administered an intraperitoneal injection of IL-1B which resulted in decreased locomotor activity post-treatment in rats (Munshi et al., 2019). These findings support the bidirectional communication between the peripheral and central immune systems during psychiatric illness.

1.3 Inflammation in Major Depressive Disorder

A host of literature provides sufficient evidence to support the idea that there is a significant immune component in MDD, including a variety of clinical studies, that suggests MDD is an inflammatory-driven illness. Specifically, a significant portion of the evidence supporting the link between inflammation and MDD fall under three primary observations: 1) MDD is associated with increased levels of inflammatory markers, even in the absence of other

clinically significant illnesses, 2) medical illnesses associated with peripheral inflammation show increased prevalence of MDD, and 3) patients who have received cytokine therapy have demonstrated increased risk of developing MDD (Krishnadas et al., 2011).

Clinical studies (Piletz et al., 2009; Simon et al., 2008) and meta-analyses (Dowlati et al., 2010; Liu et al., 2012) have all supported the observation that depressed individuals demonstrate altered levels of inflammatory markers in the blood relative to healthy controls, most common of which include increased levels of pro-inflammatory cytokines IL-1B, IL-6 and TNF-α. In addition, other studies have also reported increased levels of anti-inflammatory cytokines in MDD participants, such as IL-4, and IL-10, which may be released in response to increased inflammatory states (Hernández et al., 2008). Studies of this nature support the idea that depressed individuals demonstrate increased levels of both pro- and anti-inflammatory cytokines, indicating an overall increased inflammatory state in this population.

Illnesses with a significant inflammatory component, including rheumatoid arthritis (RA), have been associated with increased occurrence of depressive symptoms. Serum levels of IL-1β, IL-6, and IL-17, in addition to depression scores, were found to be higher in individuals with RA relative to healthy controls (Li et al., 2019). Illness severity was also associated with depression symptoms in the RA group (Li et al., 2019). In addition, MDD is a common psychiatric diagnosis in individuals with autoimmune illnesses. Rates of MDD have been estimated to be between 13-17% in individuals with inflammatory conditions such psoriasis, inflammatory bowel disease, and RA (Krishnadas et al., 2011). These findings suggest that peripheral inflammation plays a putative role in the development of MDD.

Various immunotherapies utilize cytokines, including IFN-α and IL-2, to treat medical illnesses such as cancer or chronic hepatitis C, which in turn have been associated with an

increased occurrence of MDD in this population. A study by Capuron and colleagues discovered that when cancer patients were treated with IL-2 or IL-2+IFN-α, increases in depressive symptoms were positively associated with increases in IL-10 in the serum, demonstrating an inflammatory response (Capuron et al., 2001). Further, a study by Bonaccorso and colleagues demonstrated that IFN-a-induced increases in serum cytokines, including IL-6, was positively correlated with increases in depressive symptoms in individuals undergoing cytokine therapy for chronic hepatitis C (Bonaccorso et al., 2001). The findings from these studies show that individuals undergoing cytokine therapy demonstrate activation of cytokine networks which are associated with an increased risk of developing depressive symptoms.

Additionally, there is evidence supporting a causal relationship between peripheral immune activation and disease course in MDD. In the study by Dahl and colleagues, it was found that levels of plasma cytokines were elevated during ongoing depression in the MDD group (Dahl et al., 2014). Following 12-weeks of antidepressant treatment, the levels of plasma cytokines significantly decreased in the MDD group and did not differ relative to healthy controls, accompanied by an associated decrease in the severity of depressive symptoms (Dahl et al., 2014). In summary, the studies outlined in this section support the idea that MDD is an inflammatory-driven illness, where peripheral inflammation may play a role in perpetuating the disease course of MDD.

1.4 The Role of T-Helper Cytokines and the Adaptive Immune System in Major Depression

Pro-inflammatory and anti-inflammatory cytokines are produced primarily by different subsets of T helper cells, which are a type of T cell that play an essential role in adaptive immunity. IL-2 and IFN-ɣ are produced primarily by the T helper type 1 (Th1) subset; IL-4, IL-5, IL-9, IL-10, and IL-13 are produced primarily by the T helper type 2 (Th2) subset, IL-17 is

produced primarily by the T helper type 17 (Th17) subset, and IL-22 is produced primarily by the T helper type 22 (Th22) subset (Carboni et al., 2019; Martino et al., 2012; A. Myint et al., 2005; A.-M. Myint et al., 2005; Raphael et al., 2015; Schwarz et al., 2001).

A host of clinical studies highly support the role of Th cytokines in the development and maintenance of MDD. Specifically, the pro/anti-inflammatory Th1/Th2 cytokine imbalance has been reported in psychiatric illness, including MDD (Schwarz et al., 2001). A study by Myint and others explored the Th1/Th2 imbalance by measuring the IFN-ɣ/IL-4 ratio in plasma and found that MDD participants demonstrated higher Th1/Th2 ratio when compared to healthy controls at baseline (A.-M. Myint et al., 2005). In addition, the Th1/Th2 ratio was significantly reduced following 8 weeks of anti-depressant treatment in the depressed group (A.-M. Myint et al., 2005). In a different study, researchers investigated the Th1/Th2 cytokine imbalance through IL-2 and IFN-ɣ/IL-4 and IL-13 and found that the MDD group had higher serum levels of Th1 and lower levels of Th2 cytokines compared to healthy controls (Pavón et al., 2006). Overall, these findings indicate a dynamic imbalance between Th1 and Th2 cytokines in major depression.

IL-10 is a predominant anti-inflammatory cytokine produced by Th2 cells that plays an essential role in regulating the inflammatory response and dysregulation of IL-10 is associated with psychiatric illness, namely major depression. Various clinical studies have observed that altered levels of IL-10 in the blood may be associated with depressive severity and disease onset (Anjum et al., 2020; Gazal et al., 2015). Serum levels of IL-10 in medication-free MDD participants were shown to be significantly increased relative to healthy controls and was positively associated with depression severity, as measured by the Hamilton Depression Rating Scale (HAM-D) (Anjum et al., 2020). IL-10 is elevated in response to an immune challenge,

which may be caused by imbalances between Th1 and Th2 cytokines in major depression (Anjum et al., 2020). In addition, higher peripheral levels of IL-10 have been associated with later disease-onset relative to those with earlier disease onset and healthy controls (Gazal et al., 2015). This finding may reflect how the pro/anti-inflammatory imbalance plays a significant role in the disease course in major depression (Gazal et al., 2015). Overall, these findings suggest a putative role of Th cytokines in pathogenesis and development of MDD.

1.5 Link between Inflammation and Relapse in Major Depressive Disorder

Regarding the role of peripheral immune activation in relapse specifically, several studies have found that baseline inflammation is linked to future changes in depressive severity. Given that relapse is defined as the return of depressive symptoms, it is plausible that baseline immune composition may serve as a predictor of relapse. To determine whether inflammation precedes depression, studies have investigated baseline immune concentrations and disease course on a longitudinal basis. One study by Khandaker and colleagues examined peripheral blood levels of two inflammatory markers (IL-6 and C-reactive protein (CRP)) at 9-years of age and psychiatric assessments at 18-years of age in 4,500 participants (Khandaker et al., 2014). The study concluded that higher levels of IL-6 during childhood was associated with an increased risk of developing depression later in life (Khandaker et al., 2014). Another study by Bell and others examined systemic inflammation, as measured by serum levels of CRP, and depressive symptoms found that participants with higher than normal levels of CRP (>3mg/l) during early timepoints (2004 and 2008) were significantly more likely to express depressive symptoms in 2012 relative to those who presented with normal levels of CRP during early timepoints (Bell et al., 2017). In a similar study, Chu and colleagues explored whether baseline levels of serum IL-6 and CRP during childhood (9-years of age) were associated with depressive symptoms in early

adulthood (19-years of age) (Chu et al., 2019). Indeed, baseline levels of IL-6 was associated with depressive symptoms at follow-up, including measurements of mood, fatigue, and sleep disturbances (Chu et al., 2019).

In addition to observations that inflammation precedes depression and predicts future changes in depressive symptoms, various studies have linked baseline inflammation to depressive relapse specifically. Bond and colleagues investigated the associated between peripheral inflammation, as measured by serum levels of TNF-α, IFN-ɣ, monocyte chemoattractant protein-1 (MCP-1), IL-1α, IL-2, IL-6, IL-8, IL-3 and IL-10, and depressive relapse in bipolar I disorder (BD) (Bond et al., 2016). Linear regression showed that baseline levels of IL-1α and MCP-1 were able to predict depressive relapse 12-months post-cytokine measurement (Bond et al., 2016). The researchers concluded that inflammation plays an important role in depressive illness course, specifically as a predictor of depressive relapse (Bond et al., 2016). Freemen and others also investigated baseline inflammation, as measured by plasma levels of CRP and IL-6, and the association with future depressive relapse in women undergoing infertility treatment with histories of MDD or BD (Freeman et al., 2018). It was found that levels of CRP were associated with depressive relapse at a 6-month follow-up (Freeman et al., 2018). These studies do provide some evidence for the ability of baseline peripheral inflammation to predict depressive relapse, although it is important to highlight the fact that these studies were not conducted using populations with MDD exclusively (Bond et al., 2016; Freeman et al., 2018). In addition to the use of a population undergoing subsequent infertility treatment, the need to explore baseline peripheral inflammation as a predictor of relapse in MDD exclusively is required.

1.6 Clinical Correlates of Inflammation in Major Depressive Disorder

While investigating the role of peripheral inflammation as a predictor of relapse in MDD, it is important to take into consideration clinical variables that may play a putative role in this relationship. Of particular interest are clinical measures of anhedonia and anxiety, given numerous studies strongly linking these features to inflammation and subsequent illness course. Anhedonia is a key feature of MDD, being one of the symptoms that is necessary to determine the presence of a MDE, and thus an important indicator of relapse (American Psychiatric Association, 2014). Anhedonia has been documented as a clinical correlate of inflammation in psychiatric illnesses. Freed and colleagues explored the association between peripheral inflammation and anhedonia in a psychiatric cohort (Freed et al., 2019). Relative to healthy controls, numerous pro- and anti-inflammatory cytokines, including but not limited to IL-1α, IL-2, IL-4, IL-6, IL-9, IL-10 and IL-17A, were positively associated with anhedonia, as measured by the Snaith-Hamilton Pleasure Scale (SHAPS) (Freed et al., 2019). Several other clinical studies have also linked inflammation in major depressive disorder with measures of anhedonia (Bekhbat et al., 2022; Dunjic-Kostic et al., 2013; Rush et al., 2016).

Anxiety is another important clinical feature in major depressive disorder, as it is well documented as a common comorbidity that is associated with a more severe disease course (Wang et al., 2022; Zimmerman et al., 2000). Anxiety has been reported as being associated with increased inflammation in the context of MDD. In a recent study by Wang and others, MDD comorbid with generalized anxiety disorder (GAD) were explored in relation to inflammation as measured by an inflammatory index (levels of three serum inflammatory markers) (Wang et al., 2022). MDD participants with comorbid GAD demonstrated higher inflammation relative to the non-comorbid group (Wang et al., 2022).

Anhedonia and anxiety are important clinical correlates of inflammation MDD that should be considered when exploring the relationship between baseline immune activation and depressive relapse. Further, these clinical measurements may be able to characterize different groups of MDD individuals, such as a high anxiety or high anhedonia.

1.7 Stratification of Major Depressive Disorder in Psychiatric Research

Identifying subtypes of MDD is an emerging area of research in psychiatric illness. Efforts up until this point have primarily focused on forming subtypes based on clinical features, including symptom presentation, illness severity, and disease course (Rush, 2007). However, these subtypes (melancholic, anxious, and atypical subtypes) have further been tested in terms of predicting treatment response and were found to be of little value (Arnow et al., 2015; Rush et al., 2008). Additionally, when these subtypes were explored in a new cohort, significant overlap between the groups was observed (Arnow et al., 2015). As such, recent efforts have focused on identifying data-driven subtypes utilizing a host of biological data, with the overarching goal being to identify more homogenous subtypes within MDD. A variety of different biomarkers have been explored, ranging from neuroimaging to genetic correlates. Data from resting state functional magnetic resonance imaging (rs-fMRI) and clustering analyses have been used to divide patients with depression into different subtypes, or 'biotypes' (Chen et al., 2023; Fatt et al., 2023; Liang et al., 2020). These studies have been able to identify biotypes of depression based on altered functional connectivity within clinically significant brain regions in MDD, such as the default mode network (Chen et al., 2023; Fatt et al., 2023; Liang et al., 2020). It is also important to note that these subtypes have been associated with different treatment outcomes, including remission and response rates (Fatt et al., 2023). Liang and others explored white matter abnormalities in MDD using hierarchical clustering analysis and identified three subtypes with

anatomically-associated neurocognitive deficits (Liang et al., 2019). A study by Yu and colleagues utilized single-nucleotide polymorphism (SNP)-genotyping data from MDD individuals and healthy controls and identified a latent subtype in the Mexican-American MDD group, marked by increased genetic substrates of MDD and increased anxiety (Yu et al., 2017). In addition, Nguyen and colleagues identified 16 subtypes of MDD, also using SNP-genotyping data, that were distinct in their SNP-heritability, and found positive genetic correlations with significant clinical features, such as body mass index (BMI) (Nguyen et al., 2022). The above studies provide evidence for the existence of biotypes in MDD and their association with clinical features and treatment outcomes. However, there have been no studies to the best of our knowledge that utilize immunological subtyping.

1.8 Project Rationale, Objectives and Hypothesis

MDD patients often do not obtain medical attention until after relapse has occurred. As a result, immune predictors that may be present prior to relapse are not well understood and require further investigation. In addition, studies directly investigating the relationship between baseline peripheral inflammation and relapse in MDD are scarce. Identification of immune predictors could allow for prediction of relapse at an individual level, which allows for treatment regimens to be tailored towards management and prevention of relapse in MDD.

Therefore, the primary objective of this project was to investigate baseline clinical and molecular data to identify predictors of relapse. A secondary objective was to investigate immune composition in efforts to stratify MDD individuals into more homogenous groups and explore these groupings in relation to clinical correlates of inflammation. To study this, immune profile data was generated using baseline plasma samples from all study participants and was analyzed in combination with a host of clinical data, including measures of anxiety and

anhedonia. It was hypothesized that was that baseline immune composition would be able to predict future relapse in participants with MDD. Specifically, baseline levels of plasma cytokines would predict relapse in participants belonging to the relapse group but not for those belonging to the unstable or ultrastable groups. Formulation of this hypothesis is based on the observation previously documented in the literature that baseline inflammation has been associated with future changes in MDD disease course, including severity of depressive symptoms and occurrence of the disorder (Bell et al., 2017; Chu et al., 2019; Khandaker et al., 2014). Further, studies have provided evidence for baseline immune activation predicting future depressive relapse in other psychiatric illnesses (Bond et al., 2016; Freeman et al., 2018). Anxiety and anhedonia are important correlates of inflammation in the context of MDD that may help in stratifying the disorder, therefore are taken into consideration throughout my project (Bekhbat et al., 2022; Dunjic-Kostic et al., 2013; Freed et al., 2019).

My project extends the work identifying predictors of depressive relapse and stratifying MDD by utilizing data-driven analyses of baseline cytokine and clinical data obtained from participants enrolled in the Wellness Monitoring for Major Depressive Disorder study. This project utilized a homogenous, stable population at baseline who were later classified into three relapse status groups (ultrastable, unstable, or relapse) according to clinically validated relapse criterion.

2.0 METHODS

2.1 The Wellness Monitoring for Major Depressive Disorder Study

The Wellness Monitoring for Major Depressive Disorder study involved a follow-up of responders to anti-depressant treatment at 6 clinical sites across Canada (National Library of

Medicine [NLM], NCT02934334). The study included a screening phase of up to 2 weeks and an observation phase for up to 2 years with study visits every 8 weeks. After inclusion and exclusion criterion were applied, a total of 95 participants (56 males (61.1%), 37 females (38.9%)) enrolled in the study. The study was observational in nature, as such no study-related treatments or interventions were performed. Participants continued their antidepressant regimen as prescribed by their physician. Participants were grouped into three medication regimen groups: no antidepressants, monotherapy (participant was on one antidepressant medication throughout the duration of the study), or combination-therapy (participant was on more than one antidepressant medication at any point in the study). At baseline and every 8 weeks following, participants were assessed in person for presence and severity of clinical symptoms, in addition to providing blood samples. Participants provided additional clinical data through weekly self-reports using a study-specific smartphone (LogPad®). Blood samples were collected from the 6 clinical sites across Canada and banked at the Douglas Research Institute in Montreal. Aliquots of plasma were shipped to St. Joseph's Healthcare and stored at -80 °C until processing. In this project, plasma samples and clinical data collected at the baseline clinical visit was used.

2.1.1. Classification of Relapse

Montgomery Asberg Depression Rating Scale (MADRS) scores were used to assess presence and severity of clinical symptoms (Montgomery & Asberg, 1979). In addition, symptom profiles were reviewed to identify additional relapses. Using the MADRS score, participants were categorized into ultrastable, unstable, and relapse groups. Ultrastable participants were those who maintained a MADRS score of less than 14 throughout the study and were not considered to be in relapse. Unstable participants were those whose MADRS score was above the inclusion criteria of 14 but were below the protocol defined criterion for relapse of

22. Relapse participants were those whose MADRS score exceeded 22 for at least 2 weeks during the study and were verified to be in relapse with an additional visit, and those identified after clinical review of the symptom profile. Protocol relapses (MADRS >22) were clinically validated. At the beginning of the study, all participants had a MADRS score less than 14, as outlined in the inclusion criteria (National Library of Medicine [NLM], NCT02934334). Categorization into ultrastable, unstable, or relapse groups was dependent on changes in participants' MADRS scores from baseline throughout the duration of the study. Ultrastable, unstable, and relapse groups were comprised of 27 (28.4%), 29 (30.5%), and 39 (41.1%) participants, respectively.

2.1.2. Clinical Measures

Clinical data on anxiety, depressive severity, and anhedonia were selected as measures of interest. The Generalized Anxiety Disorder 7-item (GAD7) is a 7-item self-report completed to assess presence and severity of anxiety disorder (Spitzer et al., 2006). The Quick Inventory of Depressive Symptomology Self-Report (QIDS-SR) is a 16-item self-report that assesses severity of depressive symptomology (Rush et al., 2003). The Snaith-Hamilton Pleasure Scale (SHAPS) is a 14-item self-report completed by participants to assess hedonic capacity (Snaith et al., 1995).

2.2 LEGENDplex™ Immunoassay

Immunoassays have been used frequently in the literature to quantify concentrations of analytes in the blood. Specifically, multi-plex immunoassays, including LEGENDplex™ immunoassays, allow for simultaneous quantification of numerous analytes at once. Cytokine concentrations within plasma samples collected at baseline were analyzed using the LEGENDplex Human Th Cytokine (12-plex) multi-plex assay (BioLegend, San Diego, CA, USA; lot number B327881) to generate immune profiles for each participant. Each Th filter plate

contained 16 wells for the standard (STD) curve points of known concentration, in duplicate, which were generated from the Th panel standard cocktail in 1:4 serial dilutions. The bottom standard (C0) contained only assay buffer. Each Th filter plate contained 39 samples, in duplicate, and one reference sample, in duplicate, to test for inter-plate consistency. Following sample and assay preparation, fluorescence data was analyzed through flow cytometry (BD FACSCelesta™), allowing for quantification of 12 cytokines, including: interleukin (IL) IL-5, IL-13, IL-2, IL-6, IL-9, IL-10, IL-17A, IL-17F, IL-22, and interferon-gamma (IFN-ɣ) and tumor necrosis factor-alpha (TNF-α). LEGENDplex Data Analysis Software Suite was used to analyze flow cytometry data files (raw fcs. files) and to generate summary reports to be used for downstream analysis.

2.2.1 Quality Control of LEGENDplex Data

A quality control standard operating procedure (SOP) was created to ensure reliability, consistency, and validity of the LEGENDplex data prior to data analysis. First, a visual check of the standard 5-PL curve generated by the LEGENDplex software algorithm was performed to assess if any replicates of the STD curve points were significantly off from the curve. Next, coefficients of variance (CVs) were assessed to ensure that standard curve points did not have a CV > 20. If high CVs were found in STD curve points toward the higher end of the curve, then those curve points were removed. If high CVs were found in STD curve points toward the lower end of the curve (particularly near C0), then all samples with a concentration below the level of detection (LOD) were rerun; this was due to the fact the LOD is generated based on the predicted concentration of the blank (C0) + 3*SD. Subsequently, CVs of the samples were also assessed where it was decided that any samples with more than two detectible analytes with a CV > 20, or one detectible analyte with a CV > 30, were rerun. Original samples were run on plates 1-8 and

rerun samples were on plates 9 and 10. Once all Th plates were performed, overall undetection (UND) rates across all plates for all 12 analytes was examined and it was decided that IL-13 would be removed from all future analyses due to the UND rate exceeding 50% (Figure 1). This is in conjunction with other studies that have found plasma IL-13 to be detectible in less than 50% of human participants (Doucet et al., 2013).

Batch effect was also considered during the QC process. Batch effects are characterized as any non-biological or technical factors that may contribute to variance between different groups of samples (Luo et al., 2010). To visually confirm the presence of a batch effect, or in this case a plate effect, Principal Component Analysis (PCA) was performed. PCA were performed using base R 1.4.1717 (R Core Team, 2022) functions. To reduce batch effects, batch effect correction was performed. Batch effect analyses were done using the sva (v3.40.0; Leek et al., 2021) package in R. The ComBat function allowed for reduction of batch effects in the concentration data collected from each Th cytokine plate using Bayes frameworks (Johnson et al., 2007). Sample concentration data was cleaned and log2 normalized prior to batch effect removal.

2.3 Statistical Analyses

2.3.1 Principal Component Analysis (PCA)

PCA was used to visually analyze if the baseline concentrations of Th cytokines clustered by relapse status. Briefly, PCA is a technique used for visualizing large multi-dimensional datasets, such as cytokine data. PCA reduces the dimensionality of the data to the first few principal components which allows the data to be more easily interpreted in a two-dimensional space. Most commonly, principal components 1 and 2 (PC1 and PC2) are used as they capture the majority of the variability in the dataset. The data can then be coloured by a variable of

interest, which will allow us to visually identify clusters. The amount of overlap between clusters indicates the uniqueness of the clusters, with high overlap indicating less unique clusters and low overlap indicating more unique clusters. For these analyses, baseline cytokine concentration data were coloured by relapse status. Minimal or significant overlap between relapse, unstable, and ultrastable clusters would indicate that the data does or does not cluster by relapse status.

2.3.2 Kruskal-Wallis Tests

Residuals of the baseline cytokine data were visually confirmed to not be normally distributed through quantile-quantile plots (Figures 3-4). Kruskal-Wallis tests, a non-parametric equivalent to a one-way analysis of variance, was used to determine whether the mean baseline cytokine concentration was significantly different between at least two of the relapse groups. Kruskall-Wallis tests were performed using the *rstatix* (v0.7.0; Kassambara et al., 2021) package in R. Kruskall-Wallis tests were later used when examining whether mean clinical variable scores (GAD7, QIDS-SR, and SHAPS) at baseline were significantly different between immune biotypes (Figure 7).

2.3.3 Hierarchical Clustering

Hierarchical clustering (HC) was performed to identify immune-driven subtypes using the baseline cytokine data. Agglomerative HC is a form of clustering analysis that combines individual data points to form a hierarchy of clusters based on similarity. The Euclidean distance was used as the dissimilarity metric. Ward's minimum variance method (Ward D2) was a criterion applied that grouped together observations with the objective of reducing the amount of variance within each cluster. HC was performed using the *rstatix* (v0.7.0; Kassambara, 2021) package in R. A heatmap was generated using the *pheatmap* (v1.0.12; Kolde, 2021) package to allow for visualization immune-related clusters. To examine if the clusters generated by HC were

related to relapse status, relapse status was added as a column annotation for visual confirmation.

2.3.4 Chi-squared Test of Independence

The chi-squared test of independence was used to examine whether there is a relationship between relapse status and the immune clusters (biotypes) generated by hierarchical clustering analysis. The *chisq.test* function in base R was used to determine independence and to provide analytical confirmation this relationship as observed in the heatmap.

3.0 RESULTS

3.1 Baseline Demographic and Clinical Characteristics

Table 1. Baseline demographic and clinical characteristics of participants (n=95) who provided plasma samples at baseline.

		N	%
Sex	Male	58	61.1
	Female	37	38.9
Relapse status	Relapse	27	28.4
	Unstable	29	30.5
	Ultrastable	39	41.1
Medication during study	Monotherapy	44	46.3
	Combination therapy	43	45.3
	No antidepressants	8	8.43
		Mean ± SD	Range
Baseline Measures	Age (in years)	36.4 ± 12.6	20-64
	MADRS	5.1 ± 3.7	0-13
	GAD7	3.0 ± 3.7	0-18
	QIDS-SR	4.8 ± 3.0	0-16
	SHAPS	22.7± 5.7	14-38

Abbreviations: MADRS, Montgomery Asberg Depression Rating Scale; GAD7, Generalized Anxiety Disorder 7-Item Self-Report; QIDS-SR, Quick Inventory of Depressive Symptomology Self-Report; SHAPS; Snaith-Hamilton Pleasure Scale, SD, standard deviation.

3.2 Detection Rate of Analytes in Samples

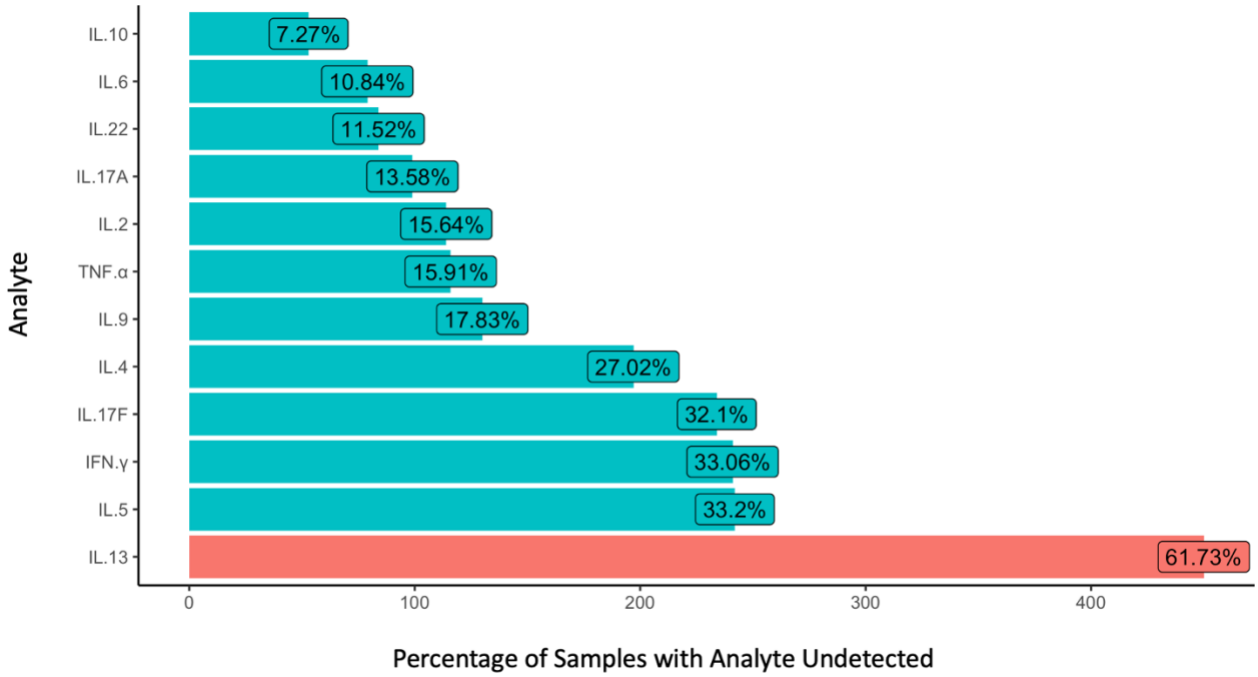

Figure 1. Bar plot showing number of undetected samples per analyte. X-axis represents the number of undetected analytes across all 8 original sample plates. Y-axis represents the analyte.

3.3 Batch Effect Analyses

To visually identify the presence of a batch effect between plates, PCA was performed. The first two principal components were plotted on a PCA biplot and were coloured by plate. Separation of colours indicates a batch effect (Reese et al., 2013), which is observed in our data. Batch effect correction was performed in R using the *sva* package (v3.40.0; Leek et al., 2021). Figure 1 provides visualization of the data pre and post batch correction.

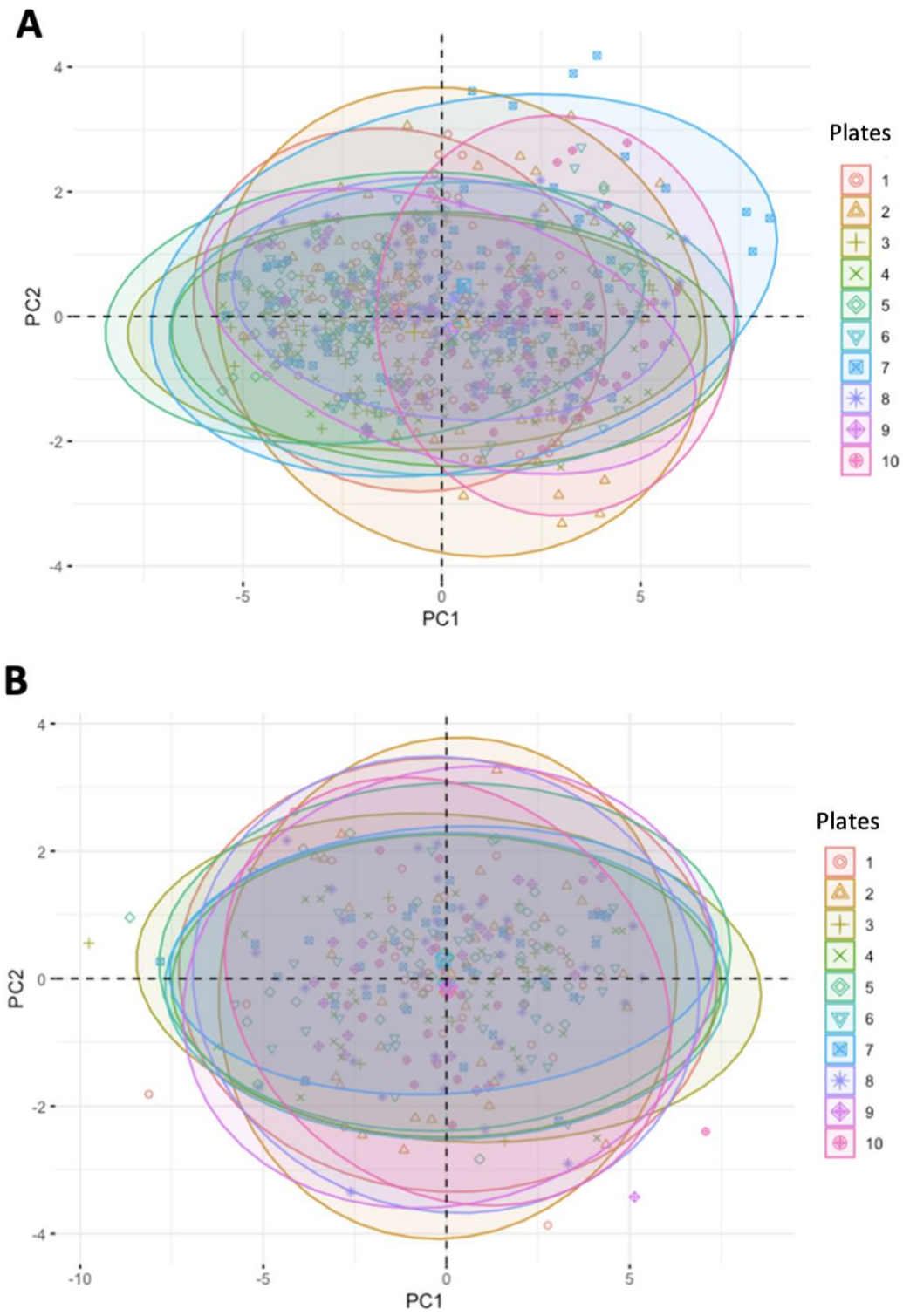

Figure 2. Th cytokine concentration pre- and post-ComBat correction for batch effects. A) PCA prior to batch effect reduction. B) PCA after batch effect reduction. Individual points represent each sample concentration, coloured by plate.

3.4 Baseline cytokine data clustering by relapse status

PCA was performed using cleaned, batch corrected, log2 transformed baseline cytokine data. A scree plot was generated to best determine how to analyze the highly multi-dimensional baseline cytokine data in two-dimensions. The scree plot revealed that PC1 and PC2 best represented the variability in the dataset and therefore was used in future analyses (Figure 2). PCA biplots show clusters of data based on their similarity to show how the data may cluster according to a particular variable. When the data was coloured by relapse status in the biplot, there is significant overlap between the relapse, unstable and ultrastable clusters (Figure 2). Observations such as this suggest that baseline cytokine composition cannot alone serve as a reliable predictor of future relapse status. Interestingly, in the biplot, there are two unique cytokine groups that can be observed. Each arrow, or loading, represents the contribution of each cytokine to either PC. When loadings on the biplot appear close together, forming a small angle between them, indicates that these loadings are positively related. The first cytokine group observed is composed of IL-5, IL-6, IL-10 and IFN-ɣ, and the second cytokine group is composed of IL-2, IL-9, IL-17A, IL-17F, IL-22, IL-4, and TNF-α (Figure 2). Although the cytokine data does not appear to be related to relapse status specifically, it does seem to cluster into two unique groups. Residuals of the baseline cytokine data were visually identified to not be normally distributed through quantile-quantile plots (Figures 3-4), therefore a non-parametric equivalent to an ANOVA was used, such as the Kruskal-Wallis test. To confirm the visual observation that the cytokine data does not cluster by relapse status, the Kruskal-Wallis test was performed to provide analytical confirmation. The Kruskal-Wallis test was performed to compare the effect of relapse status (relapse, unstable, and ultrastable) on baseline immune phenotype (concentrations of IL-5, IL-2, IL-6, IL-9, IL-10, IL-17A, IL-17F, IL-22, IL-4, IFN-ɣ,

and TNF-α, individually). The Kruskal-Wallis test revealed that there was not a statistically significant difference between any of the baseline cytokine concentrations between at least two relapse status groups ($p < 0.05$, Table 2). These results indicate that none of the immune cytokines measured were reliably related to relapse status. This suggests that baseline measurements of Th cytokines do not appear to predict future changes in relapse status in this population. Further power analyses are required to confirm this observation.

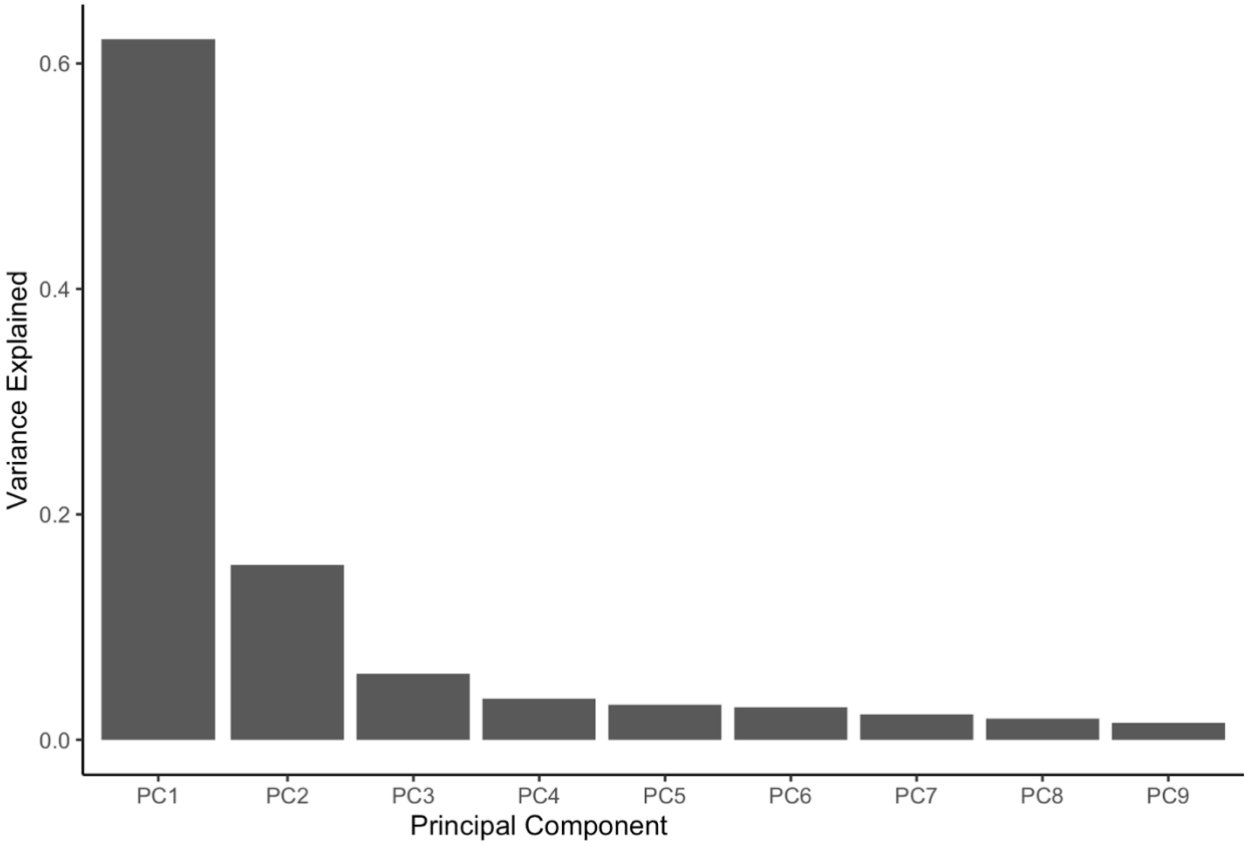

Figure 3. Scree plot of principal components. Y-axis displays the proportion of variance explained per principal component, using the Euclidean distance metric. X-axis contains the principal components.

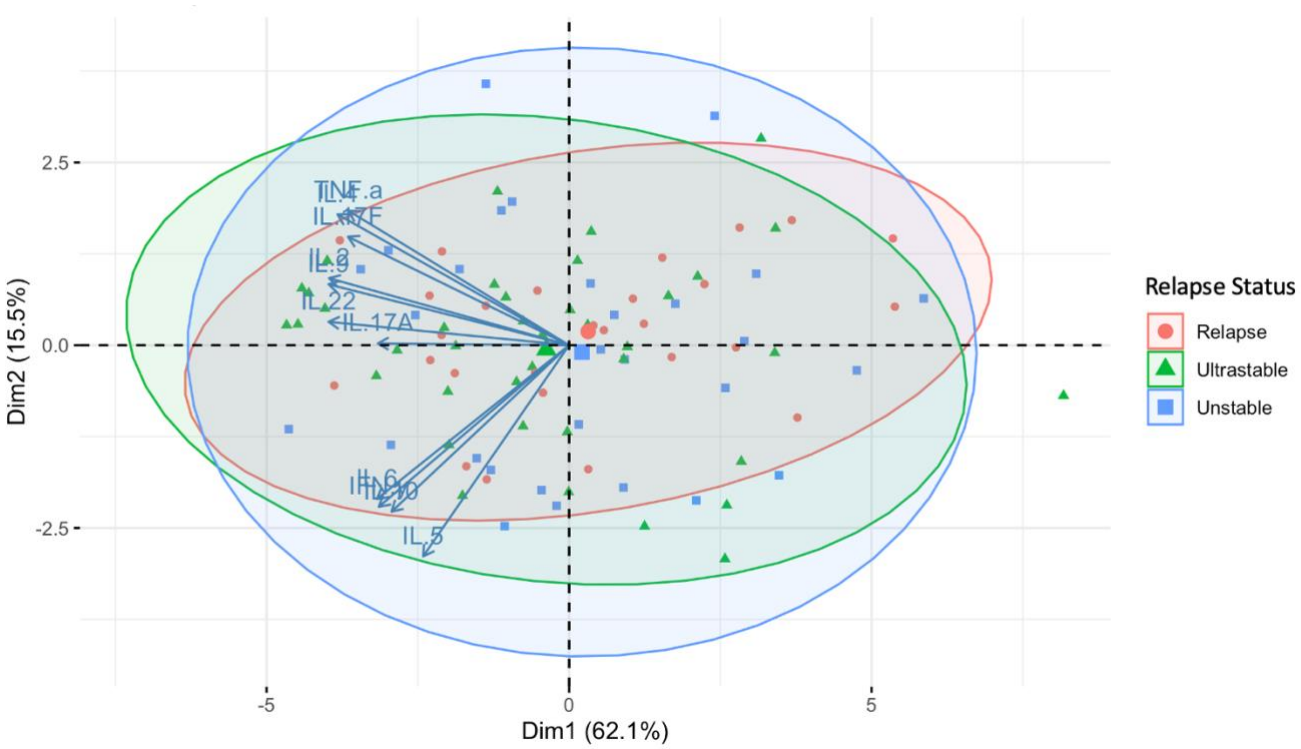

Figure 4. Principal Component Analysis biplot of baseline cytokine data. Includes score plot and loadings plot. Score plot shows significant overlapping of relapse status clusters. Loading plots reveal two unique cytokine clusters. Each point represents one participant.

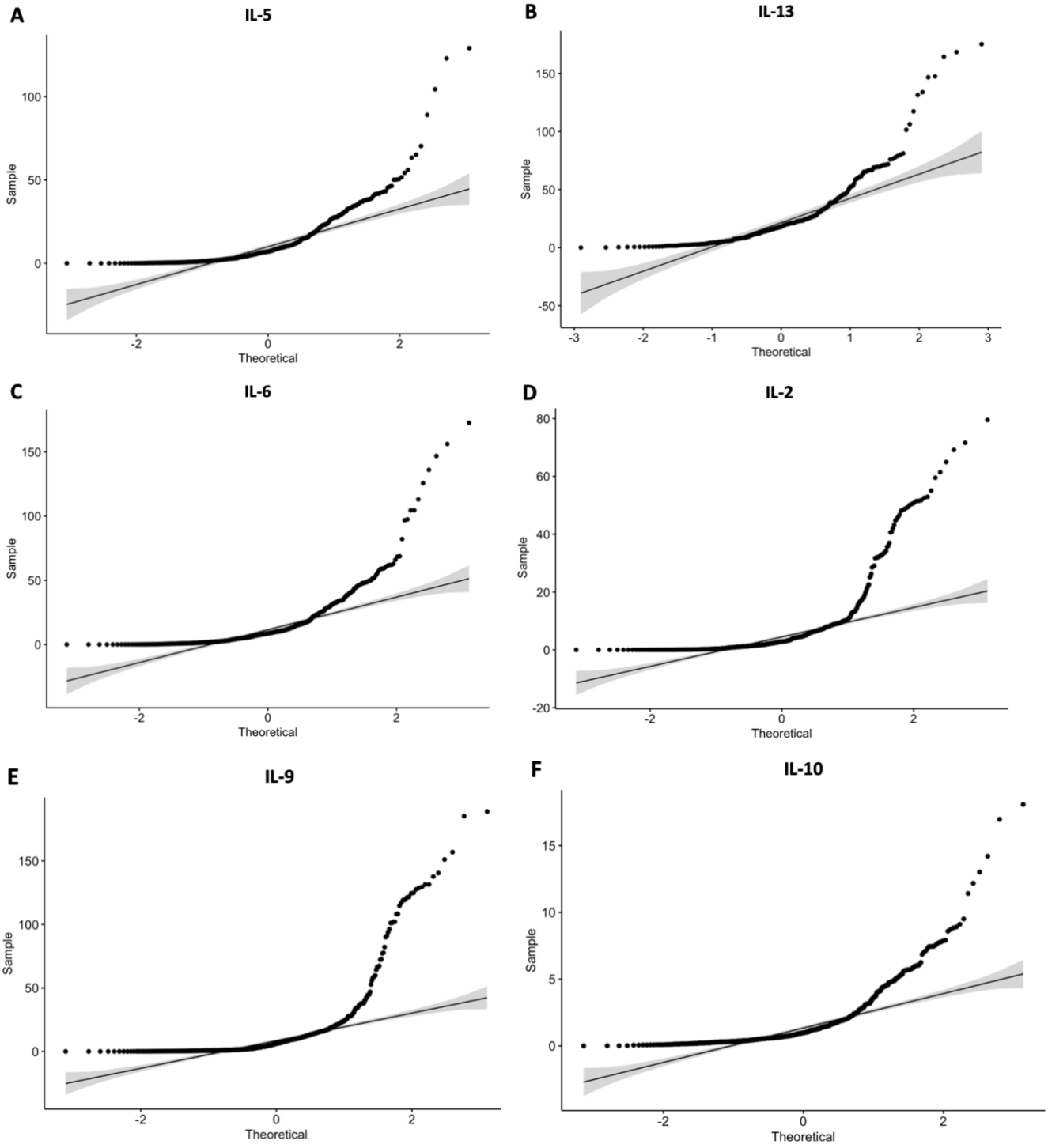

Figure 5. Quantile-quantile plots for IL-5, IL-13, IL-6, IL-2, IL-9, and IL-10 (A-F).

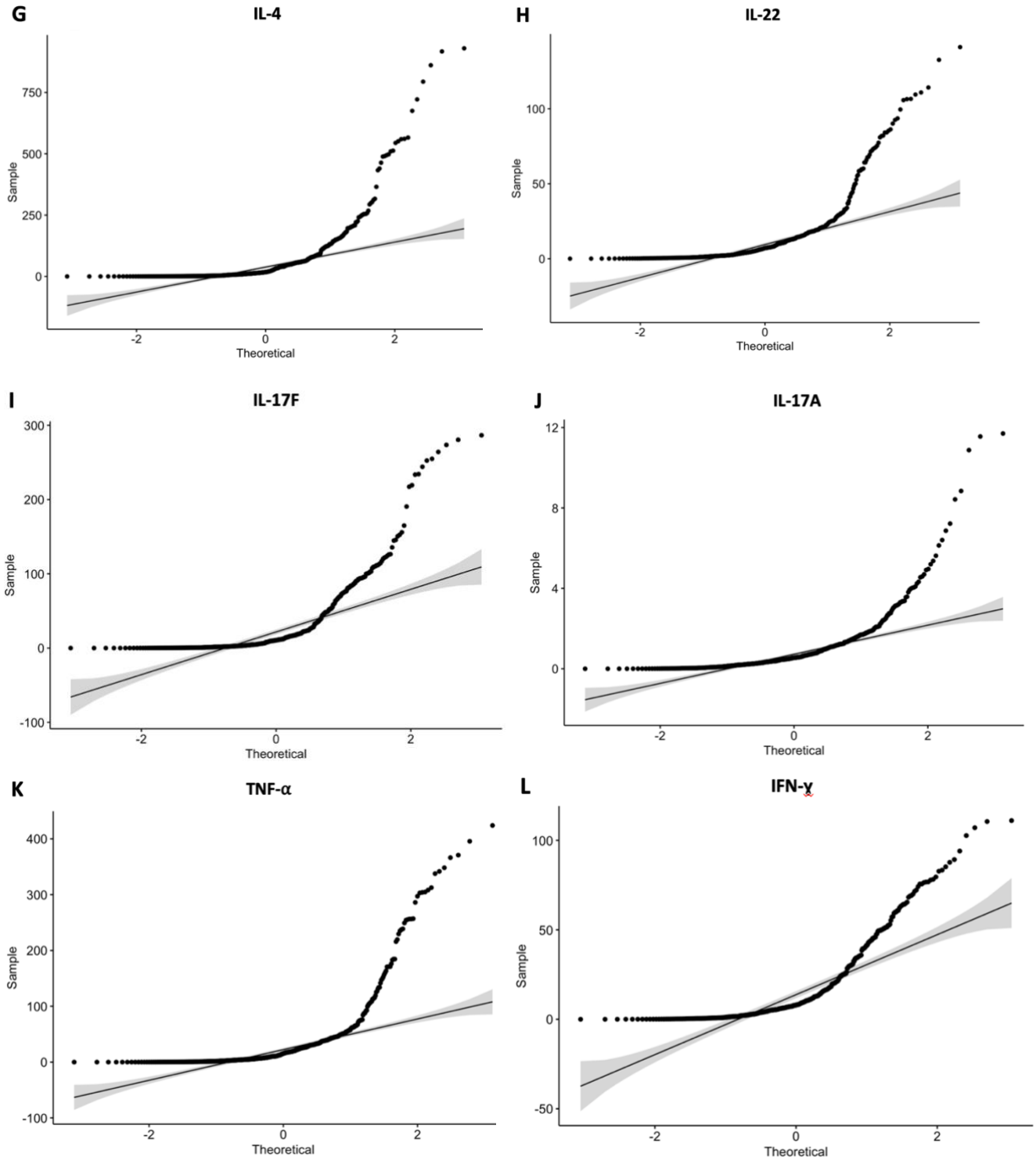

Figure 6. Quantile-quantile plots for IL-4, IL-22, IL-17F, IL-17A, TNF-α, and IFN-ɣ (G-L).

Table 2. Results of Kruskal-Wallis test between baseline cytokine concentrations and relapse status.

Cytokine	Chi-square value ($x^2(2) =$)	Significance (p-value =)
IL-5	2.089	0.352
IL-2	0.056	0.972
IL-6	5.006	0.082
IL-9	3.592	0.166
IL-10	0.841	0.657
IL-17A	0.359	0.836
IL-17F	4.386	0.112
IL-22	1.492	0.474
IL-4	3.720	0.155
IFN-ɣ	2.218	0.320
TNF-α	1.582	0.453

3.5 Identification of immune biotypes and cytokine clusters using hierarchical clustering

A heatmap was generated using the baseline cytokine data to identify immune-driven subtypes that resulted from hierarchical clustering analysis (Figure 3). A colour scheme was added to identify observations ranging from low to high concentrations, which revealed three unique immune clusters that represent immune biotypes (Figure 3), representing low, medium, and high inflammation (Figure 4). The high immune biotype is characterized by increased plasma levels of cytokines, with majority of individuals nearing the top of the colour scheme (red/orange). The low immune biotype is characterized by the lowest levels of cytokines observed, with the majority of individuals nearing the bottom of the colour scheme (blue). Annotation column was added for relapse status to help visually the relationship with immune biotype (Figure 3). As seen in the heatmap, the observations in the relapse status annotation column does not appear to align with the 3 biotypes identified. To provide analytical confirmation for this observation, the Chi-Square Test of Independence was performed. The variables were confirmed to be independent, therefore there is no relationship between immune biotype and relapse status [χ^2 = 1.2, p = .875].

Hierarchical clustering also produced two dendrograms, one representing the clustering of the cytokine data into the three immune biotypes, and the other representing the clustering of the cytokines themselves, forming two cytokine clusters. The cytokine dendrogram (Figure 5) confirms the presence of the two cytokine clusters observed in the PCA biplot (Figure 2); cytokine cluster 1 (IL-5, IL-6, IL-10 and IFN-ɣ) and cytokine cluster 2 (IL-2, IL-9, IL-17A, IL-17F, IL-22, IL-4, and TNF-α). A correlation matrix was created to show the correlation coefficients between all Th cytokines. The relationship between any two cytokines belonging to the same cluster is stronger than between any two cytokines belonging to different clusters.

Overall, three unique immune biotypes and two cytokine groups were identified. These immune biotypes are defined by differing levels of inflammation but were not related to relapse status.

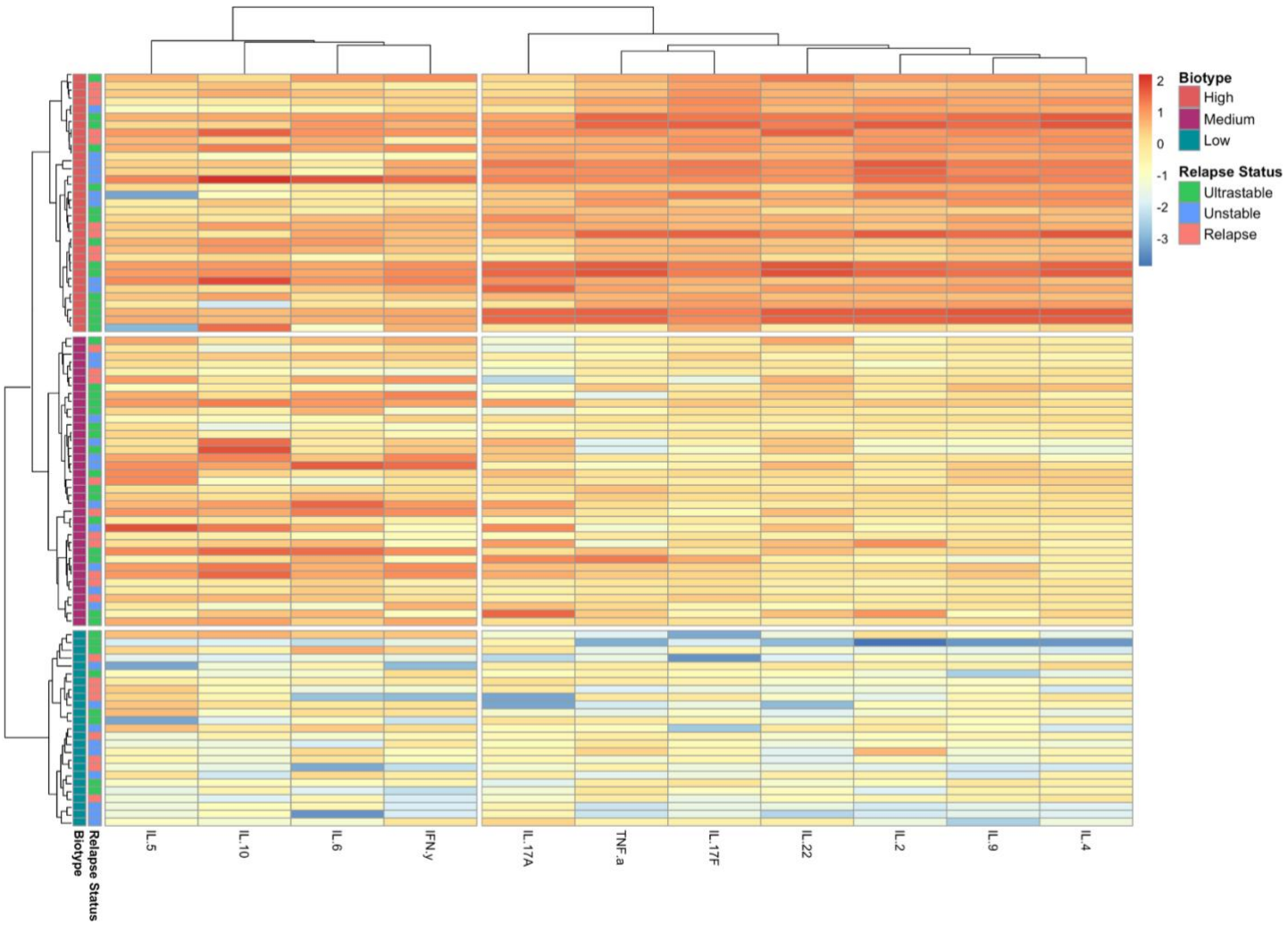

Figure 7. Heatmap of baseline cytokine data showing results of the hierarchical clustering analysis. Y-axis contains column annotations for relapse status, age, sex, and medication use, in addition to the cluster dendrogram for immune biotype. X-axis contains cluster dendrogram for cytokine cluster. Colour scheme of the represents low (blue) to high (red) cytokine concentration. Participants are shown per each row.

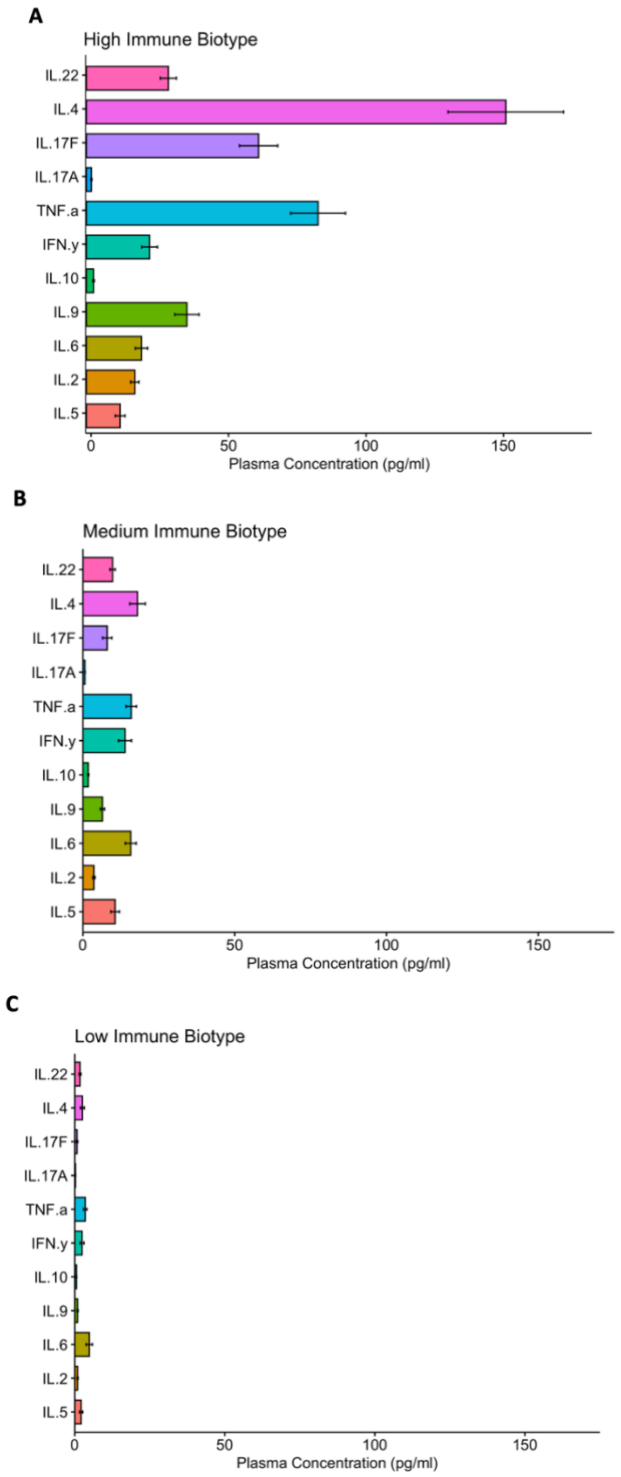

Figure 8. Bar plots of average plasma cytokine concentration (pg/ml). Plots are represented by each immune biotype. X-axis displays average plasma cytokine concentration in pg/ml at baseline for all participants. Y-axis displays the analytes. A) High Immune Biotype (high inflammation). B) Medium Immune Biotype (medium inflammation). C) Low Immune Biotype (low inflammation).

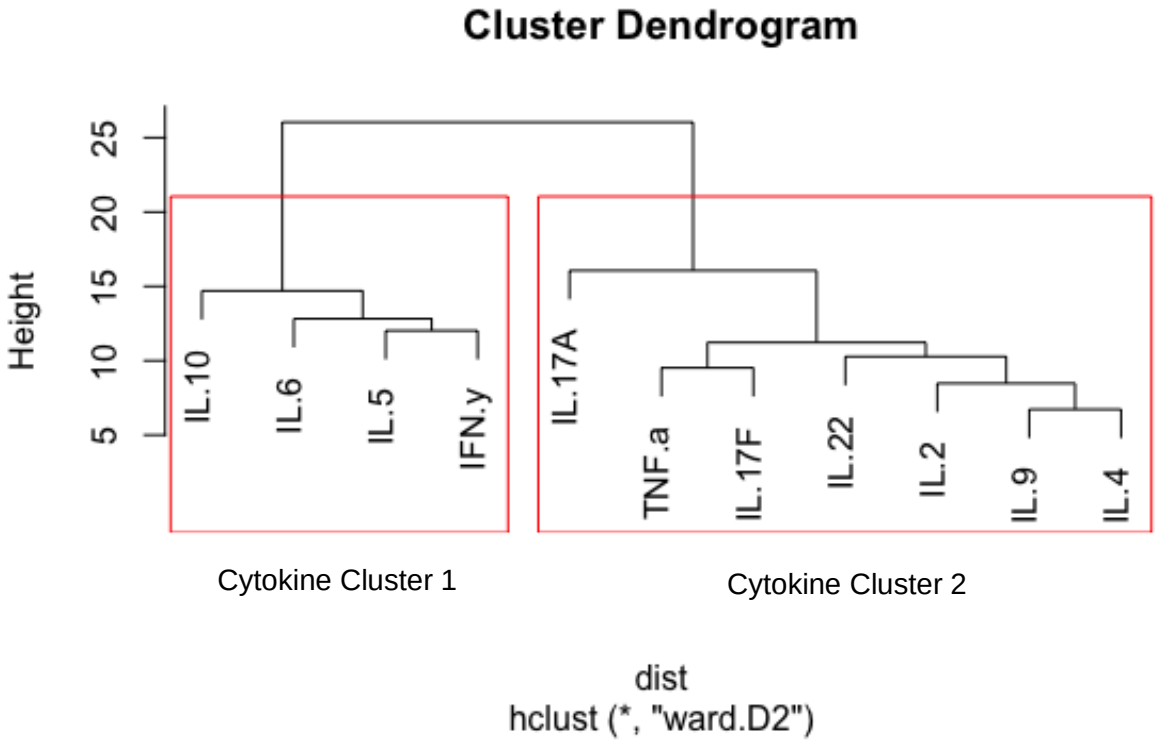

Figure 9. Cluster dendrogram of Th cytokines. Cytokine cluster 1 (IL-5, IL-6, IL-10 and IFN-ɣ) and cytokine cluster 2 (IL-2, IL-9, IL-17A, IL-17F, IL-22, IL-4, and TNF-α). Ward's minimum variance (ward.D2) method for hierarchical clustering.

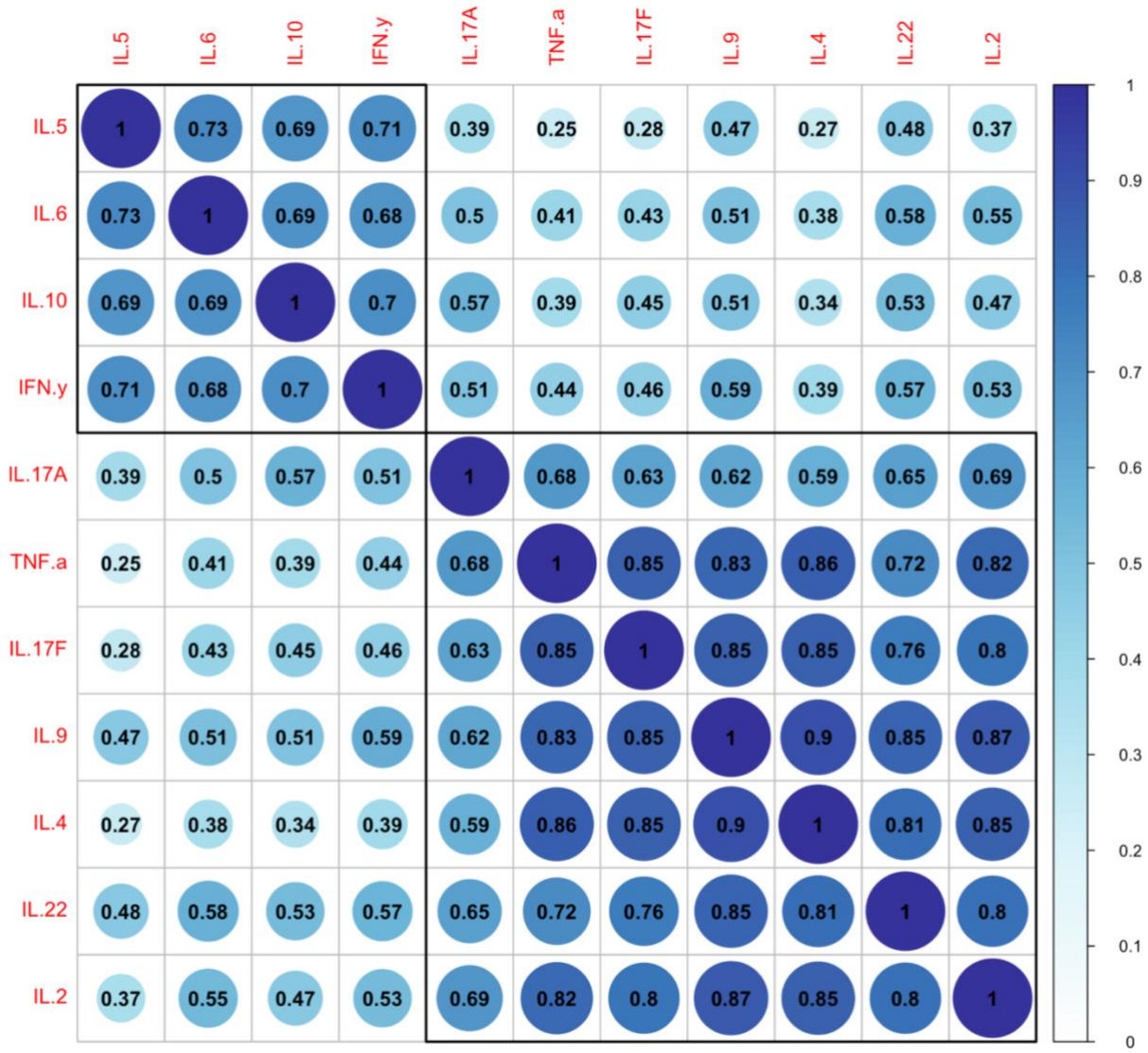

Figure 10. Correlation matrix of Th cytokines. Cytokine cluster 1 (IL-5, IL-6, IL-10 and IFN-ɣ) and cytokine cluster 2 (IL-2, IL-9, IL-17A, IL-17F, IL-22, IL-4, and TNF-α) outlined in black boxes. The scale represents the strength of the correlation between two cytokines. 0 representing no relationship, 1 representing a perfect positive correlation.

3.6 Immune biotypes in relation to clinical phenotypes

Baseline immune biotypes were explored further in relation to clinical phenotypes, specifically anxiety (GAD7), depressive severity (QIDS-SR) and Snaith-Hamilton Pleasure Scale (SHAPS). It has already been confirmed that immune biotype was not related to relapse status. Therefore, Kruskal-Wallis tests were performed to compare whether sample means of clinical variables were statistically significant between the three immune biotypes previously identified by hierarchical clustering analyses (Figure 4). There was no statistically significant difference found between any of the three biotypes (low, medium, and high) for all three clinical variables (GAD7 ($p = 0.87$), QIDS-SR ($p = 0.59$) or SHAPS ($p = 0.92$)) (Figure 7). These results confirm that immune biotype is not related to baseline clinical variables.

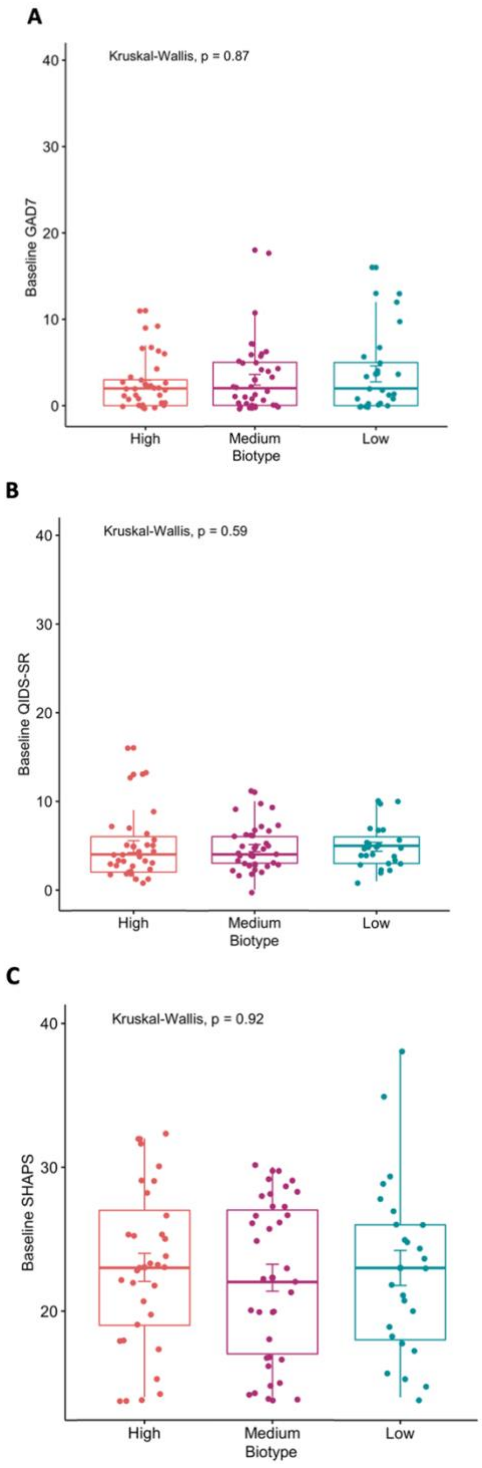

Figure 11. Relationship between immune biotypes and clinical variables. Y-axis represents baseline clinical score. X-axis represents immune biotype. A) Kruskal-Wallis test results between immune biotype and GAD7 ($p = 0.87$). B) Kruskal-Wallis test results between immune biotype and QIDS-SR ($p = 0.59$). C) Kruskal-Wallis test results between immune biotype SHAPS ($p = 0.92$). Each point represents one participant.

DISCUSSION

4.1 Overview

The main objective of this project was to identify baseline predictors of relapse in a population of individuals with MDD by investigating baseline clinical and immune data. A secondary objective was to explore the immune composition of MDD individuals to stratify them into more homogenous groups and characterize them with clinical correlates of inflammation. Previous studies have provided evidence of baseline inflammation predicting future depressive relapse in BD (Bond et al., 2016) and a psychiatric cohort with histories of MDD or BD (Freeman et al., 2018). If baseline immune composition could predict future relapse in MDD, then we would have seen a significant relationship between baseline concentrations of 11 Th cytokines and relapse status. Using PCA and KW, it was observed that there was no relationship between baseline immune composition and relapse status. However, two distinct cytokine clusters were discovered in the PCA biplot. These results demonstrate that although baseline plasma cytokines do not group according to relapse status, newly discovered cytokine clusters may be biologically relevant in this population. The cytokines belonging to each of these clusters are highly correlated with others in the same group. In addition, we have successfully shown the presence of three distinct immune biotypes, representing low, medium, and high inflammation of these Th cytokines through hierarchical clustering analysis. These analyses also validated the presence of the two cytokine clusters observed previously. These findings implicate the importance of the immune system when exploring this highly heterogenous disorder as it helps to stratify this population into distinct subtypes. The three immune biotypes were explored further with regard to anxiety and anhedonia, two clinical correlates of inflammation in MDD, in an attempt to characterize these biotypes. However, no relationship was found between any of the

three biotypes with baseline measures of anxiety (GAD7) or anhedonia (SHAPS). No relationship was found between baseline depression severity (QIDS-SR) with the biotypes, which would make sense in this population as all individuals included in the analyses were considered "clinically healthy" as they were not in relapse at baseline (MADRS < 14). As such, depression severity would be expected to not differ between the three biotypes. By using data collected from a stable, homogenous population that later was stratified into three different relapse groups, we were able to identify three distinct immune biotypes characterized by differing levels of two groups of Th cytokines.

4.2 Highly Correlated Subnetworks of Th Cytokines Characterize MDD Biotypes

To the best of our knowledge, this is the first report utilizing immunological subtyping to characterize the immune profiles of a population of MDD individuals and successfully stratify them into three distinct immune biotypes. Previous evidence has supported the relationship between MDD and inflammation, with depressed individuals having altered levels of peripheral cytokines in the blood (Dahl et al., 2014; Dowlati et al., 2010; Haapakoski et al., 2015; Hernández et al., 2008; Hiles et al., 2012; Howren et al., 2009; Köhler et al., 2017; Liu et al., 2012; Maes et al., 1997; Piletz et al., 2009; Simon et al., 2008). Our findings expand upon these previous studies by providing preliminary evidence for differing levels of immune dysfunction in MDD individuals, characterized by low, medium, and high inflammation. Notably, we utilized a panel of Th cytokines that have been linked to the development and maintenance of major depression (Anjum et al., 2020; Gazal et al., 2015; A.-M. Myint et al., 2005; Schwarz et al., 2001). Given the highly correlated nature of these cytokines in their respective clusters, high levels of one cytokine are therefore associated with high levels of the others. This finding is supported by previous literature that highlights the important balance between Th1 and Th2

cytokines, as altered levels of both types have been found in individuals with MDD (A.-M. Myint et al., 2005; Schwarz et al., 2001). In cytokine cluster 1, IL-10 was found to be highly correlated with IL-5, IL-6, and IFN-ɣ. Anti-inflammatory cytokines, namely IL-10, are often increased in depression and may work through a negative feedback loop to help regulate the pro-inflammatory response (Roque et al., 2009). Recent work by Anjum and colleagues suggests that IL-10 is released in response to an immune challenge such as an alterations to the Th1/Th2 balance (Anjum et al., 2020). In addition, animal models of depression have demonstrated a depressive-like phenotype in IL-10 knockout mice and reversal of these behaviours with administration of IL-10 (Mesquita et al., 2008). Additionally, when faced with immune challenge induced by LPS-injection, mice exposed to a chronic mild stress model of depression demonstrated increased IL-10 production (Kubera et al., 2001). The above studies highlight the highly interconnected nature of pro-/anti-inflammatory Th cytokines in depression and support our finding of two highly correlated cytokine clusters.

However, it is important to note that no concrete biological conclusions can be drawn from our study alone. What can be concluded is that these cytokine clusters represent highly correlated sub-networks in depression that characterize unique immune biotypes in MDD. Given that the cytokines in each cytokine cluster are highly correlated, future studies should investigate whether all cytokines necessarily must be measured or if one cytokine in the cluster is representative. One potential method to do so is through feature selection. Given the high-dimensionality of the cytokine data used in our study, feature selection would allow for selection of relevant cytokines while reducing redundancy in the data by removing cytokines that do not add any additional information outside of the currently selected cytokine(s) (Kumar & Minz, 2014).

4.3 Immunological Subtyping Identified Three Immune Biotypes

Recent advances in the field of depression research have focused on identifying data-driven subtypes utilizing a host of biological data, with the overarching goal being to identify more homogenous subtypes within MDD. Of note, different biotypes have been identified using altered functional connectivity within clinically-significant brain regions in MDD (Chen et al., 2023; Fatt et al., 2023; Liang et al., 2020), white matter abnormalities (Liang et al., 2019), and SNP-genotyping data (Nguyen et al., 2022; Yu et al., 2017). A number of these studies have also found associations with clinically-relevant features, including treatment outcomes (Fatt et al., 2023), neurocognitive impairments (Liang et al., 2019), and anxiety (Yu et al., 2017). These findings provide evidence for the existence of distinct and clinically significant groups within the MDD population.

As for our study, we are the first study to the best of our knowledge that utilized immunological subtyping and identified three distinct immune biotypes using data-driven analyses of baseline Th cytokine data. Previous studies have successfully linked peripheral inflammation to clinical measures of anhedonia (Bekhbat et al., 2022; Dunjic-Kostic et al., 2013; Freed et al., 2019; Rush et al., 2016) and anxiety (Wang et al., 2022). However, it is important to highlight that our study did not find associations of our biotypes with clinical measurements, including anxiety and anhedonia, as measured by GAD7 and SHAPS. These clinical measures may have been able to characterize our biotypes, such as a high anxiety or high anhedonia group. A potential explanation for this observation was that all participants were considered “clinically healthy” at the time of data collection and analysis. At baseline, all participants had a MADRS score less than 14, meaning that they were not in relapse, and were ultrastable in their clinical presentation, and as such, clinical scores of anxiety and anhedonia may have reflected this.

However, this observation does support the ongoing discourse regarding stratification of MDD, as efforts have shifted away from clinical features and symptom presentation alone (Arnow et al., 2015; Rush et al., 2008), and toward emphasizing the use of biological data. Another potential explanation for these findings may be due to the relatively small sample size of our participant population. Future studies should further explore the relationship between biotype and clinical measures of anhedonia and/or anxiety in a larger sample.

Biological subtypes in MDD may have important implications with respect to treatment response and disease course. In particular, biotypes have been identified using baseline functional connectivity in MDD and one of these subgroups has successfully been linked to remission and response rates to antidepressant treatment (Fatt et al., 2023). Findings such as these suggest that biological subgroups may not only be helpful in addressing the heterogeneity that may complicate treatment of MDD, but also has applications in improving treatment and prevention in MDD. Therefore, future analyses should focus explore these immune biotypes in relation to treatment response and other relevant clinical measures.

4.5 LIMITATIONS AND FUTURE DIRECTIONS

It is important to highlight some limitations of our study that may have influenced our findings. One feature of the Wellness Monitoring for MDD study is that it is an observational study, meaning no study-related treatments or interventions were performed. Participants continued their regular antidepressant regimens according to their physician; therefore, medication use was not controlled for. This may have influenced our results due to an interaction between the immune system and medication use. Given that levels of inflammatory mediators are elevated in the blood of individuals with MDD, a main mechanisms of action for antidepressant treatments is by blocking the effect that these cytokines may have on the brain

(Hannestad et al., 2011). A recent systematic review and meta-analysis explored the effect of selective serotonin reuptake inhibitors (SSRIs), a class of antidepressants, on levels of peripheral inflammatory markers in participants with MDD, including IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-ɣ (Wang et al., 2019). Researchers found that SSRIs reduced peripheral levels of IL-1β, IL-6, TNF-α and IL-10 (Wang et al., 2019). Other meta-analyses further support this observation with other classes of antidepressants (Goldsmith et al., 2016; Köhler et al., 2018; Strawbridge et al., 2015; Więdłocha et al., 2018). With this in mind, it is plausible that the antidepressants that the participants were taking throughout the duration of the study may have affected levels of peripheral cytokines. Additionally, previous analyses conducted by the CAN-BIND team showed that participants in the monotherapy and combination therapy groups relapsed more compared to participants that were not on any anti-depressant regimen (Foster, personal communication). Notably, the majority of the participant population were on some form of medication, with 46.3% of the participants on monotherapy and 45.3% on combination therapy. As a result, 91.57% of the participants demonstrated an increased risk for relapse, which may be a contributing factor as to why we did not find a relationship between baseline immune composition and relapse status. Given this, it would be valuable to further explore this relationship in a population in which medication use is controlled for. Another important limitation to note from our study is that we only included plasma cytokine data that was collected at baseline in our analyses. By focusing on one timepoint, we do limit our ability to examine the stability and validity of these immune biotypes and cytokine clusters over time. Future analyses should focus on exploring data from more timepoints to validate our findings on a longitudinal basis.

4.6 CONCLUSION

Overall, the results from our study provide insight into the immune-brain interactions that play a role in MDD. Our findings have shown that immune composition may serve as an important factor in parsing heterogeneity that is observed in this disorder through identification of distinct immune biotypes and highly interconnected cytokine subnetworks in major depression. The potential for our immune biotypes to be of significance is supported by recent efforts that have identified biotypes in MDD and have linked them to clinical variables, such as treatment response. Future investigations should focus on validating our immune biotypes and exploring their clinical utility. Considering various limitations or confounding factors that may have influenced our results, the above findings are preliminary. By further elucidating and stratifying the immune-brain interactions that occur in psychiatric illness, we are equipped to better understand the potential for immune composition to serve as a tool in the treatment of MDD.

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5.2 R Packages

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