# Cytokines and psychiatric symptoms in patients receiving inpatient treatment: The relationship between changes in immune activation and symptoms of mental distress. A 12-week follow-up study of patients with mental health disorders.

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**Abbreviations**

ACTH: Adrenocorticotropic hormone

AN: Anorexia nervosa

ANN: Atypical anorexia nervosa

BBB: Blood brain barrier

BD: Bipolar depression

BMI: Body mass index

BN: Bulimia nervosa

CCL2: C-C motif chemokine ligand 2

CD14: Cluster of differentiation 14

CI: Confidence interval

CNS: Central nervous system

COX: Cyclooxygenase

CPTSD: Complex post-traumatic stress disorder

CRH: Corticotropin-releasing hormone

CRF: Corticotropin-releasing factor

CRP: C-reactive protein

CXCL4: Chemokine (C-X-C motif) ligand 4

DAMP: Damage-associated molecular pattern

ECT: Electroconvulsive therapy

GR: Glucocorticoid receptor

GWAS: Genome-wide association study

HPA: Hypothalamic-pituitary-adrenal

HSCL-90R: Hopkins Symptoms Checklist-90 Revised

ICD-10: The 10th revision of the International Statistical Classification of Diseases and Related Health Problems

IDO: Indoleamine 2,3-dioxygenase

IFN: Interferon

IgA: Immunoglobulin-A

IgM: Immunoglobulin-M

IKK: IκB kinase

IL: Interleukin

IL-1RA: Interleukin-1 receptor antagonist

iNOS: Inducible nitric oxide synthase

LOD: Limit of detection

LPS: Lipopolysaccharide

MAOIs: Monoamine oxidase inhibitors

MCP-1: Monocyte chemoattractant protein-1

MDD: Major depression disorder

MINI: Mini International Neuropsychiatric Interview

ML: Maximum likelihood

MMOL/L: Millimol per liter

NF-κB: Nuclear factor-kappa β

NO: Nitrox oxide

NSAIDs: Non-steroidal anti-inflammatory drugs

PAMP: Pathogen-associated molecular pattern

PTSD: Post-traumatic stress disorder

REML: Restricted maximum likelihood

SAM: Sympathomedullary

SD: Standard deviation

SE: Standard error

SSRI: Selective serotonin reuptake inhibitors

TDO: Tryptophan 2,3-dioxygenase

TLR: Toll-like receptor

TLR4: Toll-like receptor 4

TNF: Tumor necrosis factor

TRYCATs: Tryptophan catabolites

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**Summary**

**Background:**

Many psychiatric diseases are related to changes in the immune system. Most studies on this topic have used a cross-sectional design, with a focus on depressed patients, and have compared levels of cytokines in psychiatric patients with those in healthy volunteers. Less is known about how the relationship between cytokines and psychiatric symptom changes over time in patients receiving inpatient treatment.

**Aims:**

We aimed to investigate the levels and trajectories of cytokines and mental distress in a mixed sample of psychiatric patients across 12 weeks of inpatient treatment. Also, we wanted to explore the cytokine and psychiatric symptom patterns in different patient categories, also taking the use of anti-inflammatory drugs into account.

**Methods:**

This study had a cross sectional and longitudinal design. The data consisted of blood samples and psychometric questionnaires collected three times during a period of 12 weeks of psychiatric inpatient treatment at Modum Bad Psychiatric Center. Data were collected a few days after admission ($T_0$), about halfway through the stay ($T_1$), and approximately 1-2 days before discharge ($T_2$). Serum blood samples were collected at all three time points and analyzed for seven cytokines and one chemokine chosen because they are known to be related to psychiatric diseases. The cytokines were IL-1β, IL-1RA, IL-6, IL-10, IL-17A, IFN-γ, TNF-α and chemokine MCP-1. Cytokine measurements were performed using Bio-Plex xMAP technology (Bio-Rad, Austin, Texas, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA). Three self-reporting inventories were utilized in this study. The Hopkins Symptom Checklist-90 Revised (HSCL-90R) questionnaire quantifies the level of symptom severity by mean score, referred to as the Global Severity Index (GSI). This questionnaire was administered at $T_0$, $T_1$ and $T_2$. The Beck Depression Inventory-II (BDI-II) quantifies the level of depression, and was collected at $T_0$ and $T_2$. The Alcohol Use Disorders Identification Test (AUDIT) identifies alcohol consumption, drinking behavior, and alcohol-related problems during the past year, and was collected at $T_0$.

**Results:**
At baseline, stratified on trauma experience, the traumatized patients in the minimal/mild depression group and the traumatized in the severe depression group had higher levels of IL-1RA than the non-traumatized ($p = 0.046$ and $p = 0.047$, respectively). Also in the severely depression group, those with trauma experience had higher levels of TNF-α than those without trauma experience ($p = 0.029$).

In the longitudinal analysis with cytokines as dependent variables we found that the slopes (interaction with time) of IL-1β, MCP-1 and TNF-α developed differently between the patients with post-traumatic stress disorder (PTSD) and patients without PTSD ($p = 0.025$, $p = 0.011$, $p = 0.008$, respectively). There was a significant main effect of IL-1RA in patients with PTSD when compared to patients without PTSD.

In the same longitudinal analysis, using GSI as dependent variable, we found that in the general patient group there was a significant main effect of cytokine IL-1RA ($p = 0.005$), chemokine MCP-1 ($p = 0.020$) and having PTSD disorder ($p = 0.002$) on GSI. In the stratum who did not use anti-inflammatory drugs, we found significant main effects of IL-1RA ($p = 0.023$), chemokine MCP-1 ($p = 0.018$) and having PTSD disorder ($p = 0.014$) on GSI score. There were no associations between any of the independent variables on GSI in the stratum who used anti-inflammatory drugs. Time interaction did not show any significant results in either of the two strata.

**Conclusions:**
Psychiatric symptom reduction was achieved during psychiatric treatment. The levels of IL-1β, MCP-1 and TNF-α in PTSD patients developed differently than patients without PTSD despite symptom reduction, with the PTSD patients showing increasing levels. In contrast to the PTSD patients, patients with depression, anxiety or eating disorder exhibited reduced levels of MCP-1 and IL-1RA across time. These findings suggest that PTSD patients have a different neuroimmunological pattern of recovery than non-PTSD patients. Levels of IL-1RA and MCP-1 in patients who did not use anti-inflammatory drugs were associated to the level of GSI. This suggests a possible mediating effect of anti-inflammatory drugs on psychiatric symptoms for patients in treatment.

**Sammendrag (Norwegian)**

**Bakgrunn:**
Mange psykiske sykdommer er relatert til endringer i immunsystemet. De fleste studier som er gjort i dette feltet har hatt tverrsnitt-design med fokus på deprimerte pasienter, og med en sammenligning mellom nivåer av cytokiner hos pasienter og hos friske frivillige. Det eksisterer lite kunnskap om hvordan forholdet mellom cytokiner og psykiske symptomer endrer seg over tid hos pasienter som mottar behandling.

**Mål:**
Vi hadde som mål å undersøke nivåer og utvikling av cytokiner og psykiatriske symptomer hos pasienter over en periode på 12 uker med behandling av psykiske lidelser. I tillegg ønsket vi å undersøke cytokiner og psykiatriske symptomer i ulike pasientkategorier. Vi tok også hensyn til bruken av antiinflammatoriske medisiner.

**Metoder:**
Denne studien hadde et tverrsnitt-design og et longitudinelt design. Datamaterialet besto av blodprøver og psykometriske spørreskjemaer samlet inn tre ganger i løpet av en periode på 12 uker med psykiatrisk behandling på Modum Bad. Dataene ble samlet inn noen dager etter innkomst ($T_0$), omtrent halvveis i oppholdet ($T_1$), og ca. 1-2 dager før utskrivelse ($T_2$). Serumblodprøver ble samlet på alle tre målepunkter og analysert for 7 cytokiner og 1 chemokin. Disse biomarkørene ble valgt fordi de er kjent for å være assosiert med psykisk sykdom. Disse cytokinene var IL-1β, IL-1RA, IL-6, IL-10, IL-17A, IFN-y, TNF-a og chemokin MCP-1. Cytokinmålinger ble utført ved hjelp av Bio-Plex xMAP-teknologi (Bio-Rad, Austin, Texas, USA) med et Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA). Tre selvrapporteringsskjemaer ble benyttet i denne studien. Hopkins Symptom Checklist-90 Revised (HSCL-90R) spørreskjema kvantifiserer nivået av symptomer med en gjennomsnittlig poengsum referert til som Global Severity Index (GSI). Denne ble samlet inn ved alle tre måletidspunkter. Beck Depression Inventory II (BDI-II) kvantifiserer nivået av depresjon, og ble samlet inn ved $T_0$ og $T_2$. Spørreskjemaet Alcohol Use Disorders Identification Test (AUDIT) identifiserer alkoholforbruk, drikkeadferd og alkoholrelaterte problemer i løpet av det siste året, og ble samlet inn på $T_0$.

**Resultater:**

Tverrsnittanalyser fra $T_0$: Vi stratifiserte pasientene på traumelidelse. Pasienter med traumelidelse og minimal/mild depresjon eller alvorlig depresjon hadde forskjellige nivåer av IL-1RA sammenlignet med de uten traumelidelse (henholdsvis $p = 0.046$ og $p = 0.047$). Også nivået av TNF-α var høyere hos de traumatiserte pasientene med alvorlig depresjon ($p = 0.029$). Det var ingen signifikante assosiasjoner mellom AUDIT-score og depresjonsnivå. I en sammenligning mellom pasienter og friske frivillige hadde pasientene høyere nivåer av MCP-1 og TNF-a.

I den longitudinelle analysen med cytokiner som avhengig variabel fant vi at nivåene av IL-1β, MCP-1 og TNF-α utviklet seg forskjellig for pasienter med PTSD sammenlignet med pasienter uten PTSD ($p = 0.025$, $p = 0.011$, $p = 0.008$, respektivt), hvor PTSD-pasientene hadde stigende kurver. Det var en signifikant hovedeffekt av IL-1RA i PTSD sammenlignet med de uten PTSD ($p = 0.021$).

I den samme longitudinelle analysen, med GSI som avhengig variabel, fant vi at for pasientgruppen i sin helhet så var det en signifikant sammenheng mellom nivået av IL-1RA ($p = 0.005$), chemokin MCP-1 ($p = 0.020$) og med PTSD ($p = 0.002$) på GSI som avhengig variabel. I stratumet som ikke brukte antiinflammatoriske legemidler fant vi at nivået av IL-1RA ($p = 0.023$), chemokin MCP-1 ($p = 0.018$) og det å ha PTSD ($p = 0.014$) var relatert til GSI-score. Det var ingen interaksjoner mellom noen av de uavhengige variablene på GSI i stratumet som brukte anti-inflammatoriske medisiner. Interaksjonen med tid viste ingen signifikante resultater i noen av strataene.

**Konklusjoner:**

Psykiatriske symptomer ble redusert i løpet av behandlingsperioden på Modum Bad. Nivåene av IL-1β, TNF-α og MCP-1 utviklet seg forskjellig mellom pasienter med og uten PTSD, hvor PTSD-pasientenes nivåer steg over tid til tross for opplevd symptomreduksjon. Hos pasienter med depresjon, angst, og spiseforstyrrelser så vi en reduksjon av MCP-1 og IL-1RA over tid. Disse funnene antyder at PTSD-pasienter i behandling utviser et neuroimmunologisk mønster som er annerledes enn hos pasienter som ikke har PTSD. Nivåene av IL-1RA og MCP-1 i pasienter som ikke brukte anti-inflammatoriske medisiner var assosiert med nivået av GSI. Dette antyder at bruken av anti-

inflammatoriske medisiner kan ha en mulig medierende effekt på psykiatriske symptomer hos pasienter i behandling.

# 1 Introduction

According to the World Health Organization (WHO), depression and anxiety disorders are among the 20 top reasons to years lived with disability (YLD) (1). Depressive disorders are ranked as the 4$^{th}$ leading cause for years lived with disability globally which equals 5.8 % of the total global YLD (1). Anxiety disorders are ranked as the 8$^{th}$ leading cause giving a total of 3.4 % of the global YLD. WHO has predicted that major depression will be the number one cause of global disease by the year 2030 (2).

Depression is still a clinical puzzle with an etiology that has proven hard to comprehend, as it is associated with both a hereditary (3) and environmental components (4). Psychiatric drugs target the serotonin, dopamine or norepinephrine signaling, but they are not very effective as more than 50 % fail to respond from treatment (5). Many patients do not respond to the available treatment at all, despite the variety of psychological and pharmacological treatments that are available (2, 6, 7). The key to treating depression might be found elsewhere, perhaps at the biological level within the hypothalamic-pituitary-adrenal (HPA) axis (5).

Psychiatric diseases are heterogenous, the symptoms which characterize the disorders are manifold. Diagnostic screening tools have been developed to identify psychiatric disorders like major depression and anxiety disorders. However, they have limitations, as various clinical symptoms can qualify in the diagnostic process of a disorder, but the clinical symptoms may vary in both significance and presentation (2). Diagnostic categories are explanatory constructs with no clear boundaries, challenging the validity, which may be improved by taking into account neurobiological data (8).

Treatment and assessment of psychiatric diseases may be improved by a better understanding of the neurobiology of psychiatric symptoms and by targeting underlying mechanisms (9). Many patients have increased inflammation, and the phenotype these patients have in common is the exposure to traumatic events, often in childhood. This leaves a biological scar of early exposure to high levels of stress (10). Using biological markers to distinguish subgroups of patients and be able to identify groups of patients who might benefit from the same kind of treatment is warranted. Such knowledge might provide health personnel better remedies in the difficult tasks of diagnosing and treating psychiatric diseases (11).

# 2 Background

In 2015, the point prevalence of depression and anxiety was estimated by the World Health Organization. Depression was diagnosed in 4.4% of the world's population, while 3.6% of the world's population suffered from anxiety disorders (12). Depression was more common in females (5.1%) than in males (3.6%), and the same pattern was seen in anxiety disorders, i.e. more females (4.6%) suffered from anxiety than males (2.6%) (12). Epidemiologic surveys in the United States show that the lifetime prevalence in the USA of specific phobias is 15.6%, social phobia 10.7%, generalized anxiety disorder (GAD) 4.3%, and separation anxiety disorder 6.7% (13). The prevalence of PTSD is approximately similar across countries, but countries affected by war or conflicts show higher rates of PTSD than peaceful countries (14). For instance, Northern-Ireland had a rather high lifetime prevalence of PTSD at 8.8 % in a World Mental Health measure (15), while countries like Japan, Italy, Spain and South Africa range from 1.3 % to 2.4 % life time prevalence (14). The patients in the current study were diagnosed according to the 10$^{th}$ revision of the International Classification of Diseases (ICD-10), and the following diagnoses constitute the four main disorders in this project:

## 2.1 Post-traumatic stress disorder

In ICD-10, PTSD is classified as a subtype of the anxiety disorders, and it is a chronic and severe psychological disorder that only occurs in the aftermath of exposure to a traumatic event (16). PTSD is characterized by an impaired stress response and chronic, low-grade inflammation (17). The disorder is also characterized by heightened vigilance, sleep disturbances, re-experiencing the trauma as intrusive memories (flashbacks) or dreams, unresponsiveness to surroundings, anhedonia, and avoidance of situations reminiscent of the trauma. These symptoms occur as a delayed response to exceptionally threatening or stressful situations, often of a catastrophic nature, which are likely to cause extreme distress in almost anyone. Such events could be for instance natural disasters, war, a serious accident, witnessing the death of others, or being a victim of torture, terrorism or rape (18, 19). PTSD has been criticized for not accounting for those who have survived multiple traumas (20). As a consequence, complex post-traumatic stress disorder (CPTSD) is included as a diagnosis in the ICD-11 (21). CPTSD describes a syndrome observed in survivors of repeated traumatic

experiences, especially childhood abuse, or genocide campaigns, under which escape is no option. In addition to these criteria, individuals with CPTSD will also suffer from disturbances in affect regulation and interpersonal problems (20, 21).

## 2.2. Major depression

Being clinically depressed is reflected as a diagnosis in both DSM-5 (22) and ICD-10, and is a more devastating and severe condition than the common feeling of sadness. In ICD-10, the diagnosis of depression is entitled "F33 Major depressive disorder, recurrent", and excludes single episodes of depression, as well as bipolar disorder (22). The ICD-10 groups the severity of depression into three categories: mild, moderate and severe (F33.0 – F33.2). These groups are based on number of symptoms, severity of symptoms, functional impairment, level of distress and type of symptoms. This differs somewhat from DSM-5, where severity levels are categorized by number of symptoms, the level of distress caused by the intensity of the symptoms, and the degree of impairment in social and occupational functioning (23). In ICD-10, the key symptoms of major depression are persistent sadness or low mood, and/or a loss of interest or pleasure, and/or fatigue or having low energy. At least one of these symptoms must be present most days, and for at least two weeks. The other symptoms are disturbed sleep, poor concentration or indecisiveness, low self-confidence, poor or increased appetite, suicidal thoughts or acts, agitation or slowing of movements, guilt or self-blame. Together, these 10 symptoms define the level of depression, and therapy is based on the severity. The classification is as follows: Not depressed (fewer than four symptoms), mild depression (four symptoms), moderate depression (five to six symptoms), and severe depression (seven or more symptoms, with or without psychotic symptoms). The symptoms should be present for a month or more, and should be present for most of each day (22).

## 2.3 Anxiety disorders

In ICD-10, anxiety disorders are categorized into "F40 Phobic anxiety" disorders, including e.g. F40.0 agoraphobia and F40.1 social phobia, and "F41 Other anxiety disorders", including F41.0 Panic disorders and F41.1 Generalized anxiety disorder and (22). They constitute a

range of disorders with symptoms of irrational worrying about possible future events or specific phobias. A specific phobia (formerly called simple phobia) is an excessive and unreasonable fear caused by the presence or thought of an object or a situation which normally does not represent any real threat, usually leading to avoidance whenever possible (19, 24). The term “anxiety” is closely related to the feeling of fear, but being afraid is more of a rational feeling to current events and not pathological. Fear may, however, develop into anxiety if the feeling is persistent (25).

GAD can be described as excessive worry, persistent anxiety and feelings of apprehension about everyday events and problems, often with symptoms of muscle and psychic tension, causing significant distress and functional impairment. According to ICD-10, a GAD diagnosis requires at least four symptoms to be present most days for at least six months. Some symptoms of GAD are sweating, shaking, dry mouth, breathing difficulties, chest pain, nausea, feeling dizzy, fear of losing control (“going crazy”), passing out or dying, hot flushes/cold chills, numbness and concentration difficulties (24).

Social anxiety disorder, also known as social phobia, includes the core symptom of excessive fear of being judged by others or scrutinized in social situations such as public speaking (26). Such excessive concern about being negatively evaluated may lead to anxiety in social situations (19). The symptoms of social disorders include blushing, dry mouth, and excessive fear of humiliation or of embarrassment. Avoidance of situations is common, and one may have difficulties in relationships or problems at school or work (24).

## 2.4 Eating disorders

Eating disorders constitute a group of severe disorders characterized by pathological eating and weight control behaviors, as well as disturbances of body image perception (27). Eating disorders are classified in ICD-10 as F50.0 Anorexia nervosa (AN), F50.1 Atypical anorexia nervosa (ANN), F50.2 Bulimia nervosa (BN) (28). AN is characterized by extremely low bodyweight and a fear of its increase. Other typical features are deliberate weight loss with a psychopathology concerned about dread of fatness, where the patients impose a low weight threshold on themselves. People with AN often have symptoms of restricted dietary choice, are engaged in excessive exercise, induced vomiting, and the use of appetite suppressants as well as diuretics (18). BN is characterized by repeated binge eating and behaviors aiming to

counteract it (29). BN is a disease which shares many features with AN, such as overconcern with body shape and weight. ANN is a diagnosis which includes some features of BN, but where the overall picture does not justify the diagnosis of BN. The ANN diagnosis may be given for example if the recurrent bouts of overeating or use of purgatives do not result in significant weight change. Alternatively, the diagnosis may be used if the strong concern about body shape and weight is not present (18).

In high-income countries, lifetime prevalence of AN in the general population is reported to be around 1% in women and less than 0.5% in men, while point prevalence has been estimated to be about 0.3-0.5% (28). The age of onset is usually 15-25 years, and average illness duration is about six years. The prevalence is higher in young women, who represent the majority of incidences of AN and BN (30). One in every six or seven young women has an eating disorder, and AN is as common as type 1 diabetes (30).

It has been suggested that pathological pathways might be common to eating disorders and other psychiatric disorders. This implies that novel psychopharmacological drugs used in the future for treating other psychiatric disorders could also be effective in the treatment of eating disorders, given the high degree of comorbidity in psychiatric patients and genetic correlations between psychiatric disorders (31). Genetic findings have suggested that AN and BED are not necessarily psychiatric disorders, but could also be metabolic or immune disorders. This could mean that there are different subtypes of eating disorders, for instance a psychological, a metabolic and an immune subtype of AN, where a possible future treatment intervention might be cytokine targeting (31).

## 2.5 Depression and anxiety and the immune system in an evolutionary perspective

Depression and anxiety disorders are two major contributors to the global burden of disease (12). Given the severity of these diseases, it is reasonable to question why the human genome still contains the genetic alleles for developing such devastating disorders. During the evolution of humans through thousands of years, the innate immune defense has played a crucial part in promoting host survival. The risk of being hunted and wounded by conspecifics and wild animals in a prehistoric environment was presumably rather high. The inflammatory response would have to react fast and mount a strong defense against invaders

crossing the barrier of the skin, releasing inflammatory mediators, and promoting depressive behavior in parallel. At first glance, the subsequent state of depression does not seem to promote survival. However, it has been hypothesized that depressive behavior promotes survival in terms of allocating energy for the immune system to attack pathogens, and withdrawal from others to prevent spreading contagious diseases and to avoid additional pathogen exposure (32).

Across human evolution, the great risk of being attacked by conspecifics in order to achieve hierarchical and reproductive status would naturally induce psychological stress, similar to the principle of a smoke detector, a state of constant alarm. The feeling of stress would thus serve as an early warning signal to the innate immune system, keeping the immune system in an alerted state, ready to fight dangerous pathogens. Stress is linked with depression through the pathways of the immune system (32). More specifically, a genome-wide association study (GWAS) has confirmed that candidate genes for major depression disorder (MDD) play an important role in processes crucial to host defense, for instance pro-inflammatory cytokine signaling (tumor necrosis factor), providing both immunological and behavioral responses to infection (33).

Chronic stress is best known to reduce inflammation through release of glucocorticoids (34), but is frequently also associated with exaggerated levels of circulating inflammatory biomarkers (35). The hypervigilant characteristics of anxiety disorders, which are often co-morbid with depression, also serve to protect the individual from dangerous situations which could lead to traumatic wounds and possibly death. It is therefore reasonable to think that evolution favored those individuals whose genes coded for depressive and anxious behavior, as they would have higher chances of survival in the highly pathogenic and threatening environment of ancestral times (36).

## 2.6 Cytokines

The term “cytokine” refers to a protein made by a cell (“cyto”) that acts (“kine”) on target cells, a term coined by Stanley Cohen in 1974. Cytokines are proteins secreted by cells which transduce signals either on the cytokine-producing cell (autocrine action), e.g. Th2 cells producing IL-4 to stimulate its own growth, or on other target cells nearby (paracrine action) (37) or endocrine action, e.g. TNF-α produced by macrophages in adipose tissue and secreted

into circulation (38). The term cytokine mostly refers to molecules involved in host defense, acting on receptors on leukocytes, usually locally, but sometimes also systemically. Cytokines have a broad range of actions, including cell development, cell differentiation, growth, apoptosis, chemotaxis and neurotransmission (37, 39). They are pleiotropic of nature and they show redundant and synergistic effects, all depending on the nature of defense and the target tissues (40). The actions of cytokines can be divided into four different responses according to the nature of the immunologic threat. This decides which cytokines are produced, and these responses are cytotoxic, cell-mediated, humoral or allergic. A cascade of responses is seen in response to cytokines, and cytokines often synergize to express optimal function (41).

The innate response of the immune system is an inflammatory response where the host acts immediately in cases of invading microbes in attempts to limit infection and kill invaders. The skin, lungs, and intestinal tract each provide a first line of defense against microbial invasion. Keratinocytes of the skin, alveolar epithelial cells and cells in the gastrointestinal tract all contain preformed Interleukin-1α (IL-1α), IL-18, IL-33, as well as members of the IL-36 subfamily. These cytokines are pre-made and are thus readily available upon injury or if invaders like virus or bacteria have passed the natural barriers. Although the term "cytokines" was coined in 1974, the discovery of the first cytokine took place in 1957, when interferon (IFN) was recognized as antiviral activity (37).

The cytokines are classified as type 1 or type 2 cytokines according to their chemical structure and interacting receptors. All type 1 cytokines share similar three-dimensional structures, but only share limited amino acid identity. Type 1 cytokines (i.e. the IL-2, IL-4, IL-6, IL-12 families) contain four α-helical bundles and are either classified as short-chain or long-chain four α-helical bundles, according to the length of the α helices. The type 2 cytokines (i.e. the IFN-α/β, IFN-γ, IL-10 families) differ in structure from type 1 cytokines by having for instance one or two more helices (37).

However, there exists another nomenclature of cytokine classification; here, they are classified according to function. This type of classification has proven more useful in clinical and experimental practice. The first group in the classification is the pro-inflammatory cytokines (e.g. IL-1, IL-6, tumor necrosis factor-alpha), which are released from the monocyte/macrophage lineage. These play an important role in non-specific innate immune defense. Second, the T helper 1 (Th1) cells of the immune system produce specialized sets of

cytokines (e.g. IL-2 and IFN-γ) that induce cellular cytotoxic immunity. Third, the T helper 2 (Th2) cells produce anti-inflammatory cytokines (e.g. IL-4, IL-10). Fourth, some cytokines are referred to as Th3, and this group includes members of the transforming growth factors (e.g. the TGF-β family), which are strongly immunosuppressive cytokines. These two nomenclatures may lead to confusion, as IL-4 is a type 1 cytokine (i.e. having four α-helical bundles), but is functionally a type 2 cytokine since it is produced by Th2 cells, while IFN-γ is a type 2 cytokine which is produced by Th1 cells. Most cytokines are pleiotropic (i.e. mediating more than one function) (37).

The term "chemokines" is a contraction of "chemotactic cytokines", a term which conveys that chemotaxis (i.e. the movement of leukocytes from the lumen into a damaged area) is the predominant characteristic of these small heparin-binding proteins. The first chemokine was discovered in 1977 by Wu and colleagues, and more than 50 human chemokines and 20 chemokine receptors have been identified since (42). Chemokines are grouped into two main categories, inflammatory and homeostatic chemokines, and are secreted in response to signals such as pro-inflammatory cytokines. The inflammatory chemokines are involved in recruiting monocytes, neutrophils, and lymphocytes in response to inflammation and tissue injury. The homeostatic chemokines are involved in non-immunologic tasks of regulating organ development, more like housekeeping functions (43), such as directing leukocytes within lymphoid organs, in the bone marrow and the thymus during hematopoiesis (44).

Details of the cytokines we assessed in the current study are presented below. The reason for assessing these particular cytokines is because many studies have previously found these cytokines to be altered in patients with psychiatric disorders, however, these studies were mostly cross-sectional. Thus, it was reasonable to believe that we would find elevated levels or abnormal patterns over time when assessing cytokines known to be associated with psychiatric disorders. In addition, cytokine IL-17A has only recently been investigated in depression, hence studies on this cytokine in the context of psychoneuroimmunology is scarce (45).

*IL-1β*

The IL-1 family comprises 11 members, 7 of which are pro-inflammatory (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) while 4 are anti-inflammatory (IL-1RA, IL-36RA, IL-37, IL-38) (46). More than any other cytokines, the IL-1 family plays a fundamental role in the non-specific innate response to infection, such as the release of antibodies and cytotoxic T-lymphocytes. Pro-inflammatory cytokines like IL-1β decrease the production of serotonin (5-HT) by stimulating the activity of indoleamine 2,3-dioxygenase (IDO). IDO exists in macrophages, and converts serotonin to tryptophan, which is further metabolized down the kynurenine pathway to kynurenic acid and quinolinic acid (47). Neutralizing IL-1β or blocking the IL-1 receptor is mostly an anti-inflammatory action (37). Numerous studies have found IL-1β to accompany MD (48).

*IL-1RA*

The anti-inflammatory cytokine IL-1RA is structurally similar to IL-1β, but lacks the agonistic characteristics. It binds to the same IL-1 receptors as IL-1β, but does not induce an IL-1-like response (49). IL-1RA is produced by mononuclear cells (50) and is found circulating in humans where it serves as a brake on inflammation driven by IL-1α or IL-1β (37). Studies have reported IL-1RA to be elevated in MD (48, 51) and it has also been suggested that IL-1RA possibly might be used as treatment of inflammatory and neurodegenerative processes (52).

*TNF-α*

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine classified within the large and important TNF family, as a Th1 cytokine, a family which comprises approximately 20 ligands and 28 receptors. TNF represents 2 proteins primarily derived from mononuclear phagocytes (TNF-α) and lymphocytes (TNF-β). TNF-α is also produced by neutrophils, lymphocytes, NK cells, endothelial and mast cells (53). Similarly to IL-1β, TNF-α stimulates the IDO enzyme, which shunts tryptophan away from serotonin production, a mechanism which has been thought to induce depression by several researchers and in numerous research

papers (48, 54-56). The primary purpose of TNF-α is antitumor immunity through cytotoxic effects on cancer cells and by stimulating antitumor immune responses (41). TNF-α also interacts with endothelial cells to induce intracellular adhesion molecules and vascular cell adhesion molecules. This allows for granulocytes migrating into inflammatory loci (41). TNF-α is responsible for cachexia, which is a condition that occurs in chronic infections and in cancer. TNF-α is also involved in vascular leakage, negative inotropic effects, and is the primary endogenous mediator of toxic shock and sepsis (57). Serum and plasma levels of TNF-α are often reported to be higher in patients with depression (38), as well as in patients with AN and PTSD (58, 59), but heterogeneity between studies is large (58).

*MCP-1*

A potent chemotactic factor for monocytes is monocyte chemoattractant protein-1 (MCP-1), also referred to as CCL2 in literature, depending on the nomenclature used, and first identified in 1977 (42). It is one of the key chemokines that regulate migration and infiltration of monocytes and macrophages, memory T lymphocytes and NK cells (60). Composed of 76 amino acids, being 13 kDa in size, MCP-1 belongs to a family comprised of at least four members, MCP-1, -2, -3 and -4 (43). MCP-1 is produced by a variety of cell types, including endothelial, fibroblasts, epithelial and smooth muscle cells, as well as the central nervous system (CNS) cells astrocytes and microglia, although mainly by monocytes and macrophages (60). MCP-1 secreted in response to pro-inflammatory cytokines, oxidative stress or growth factors, attracts leukocytes which express the appropriate chemokine receptors towards areas with high concentrations of chemokines (37). MCP-1 is an important candidate in linking the peripheral and central inflammation, as it orchestrates neuroinflammatory crosstalk and drives the immune cell migration. A recent meta-analysis has linked MCP-1 with depression (11).

*IL-6*

IL-6 is a pro-inflammatory cytokine produced by a variety of cells, such as T and B lymphocytes (61), monocytes and macrophages in response to infections or tissue injury by stimulation of pathogen-associated molecular patterns or damage-associated molecular

patterns (62, 63). IL-6 is responsible for activation of acute phase, immune and hematological responses by hepatocytes, leukocytes, and hematological cells. These processes are crucial for mounting a solid immune response, thus IL-6 is one of the key cytokines, playing a pivotal role of maintaining homeostasis (62, 63). IL-6 is well-documented as an important cytokine in the context of various psychiatric disorders (64, 65) and traumatic stress (66), it is elevated in depressed subjects compared to healthy controls (48) , and its use as a possible biomarker for PTSD (67) and AN (64) has been proposed. IL-6, together with other biomarkers, has also been found to be useful for correctly classifying patients with bipolar depression (BD) and MDD with 98.1 % accuracy (68).

*IL-10*

Anti-inflammatory cytokine IL-10 was identified in 1989 by Mosmann and colleagues (69). It belongs to the Class II cytokines. It is produced by T cells and by virtually all T cell subsets (e.g. Th1, Th2, regulatory T cells). It is a potent anti-inflammatory cytokine, exhibiting its anti-inflammatory capabilities by activating regulatory T cells, which suppress other immune cells, thereby limiting or suppressing immune function. IL-10 also suppresses excessive inflammatory response, it suppresses the effector function of macrophages, T cells and NK cells, and it blocks cytokine synthesis in macrophages (70). IL-10 also inhibits production of IFN-γ from Th1 cells (69, 71). This anti-inflammatory cytokine has recently been proposed to be a primary immune-regulatory mechanism of MDD (68). IL-10 has been found to be both elevated and reduced in previous studies on depression patients, a variation probably being related to differences between plasma and serum concentrations, as well as between methods for cytokine analyses. For example, IL-10 has been found to be reduced in studies not using enzyme-linked immunosorbent assay (ELISA) for cytokine analysis, and to be elevated in studies using ELISA (38). This suggests that the various materials and methods currently in use in this field of research adds to the complexity of finding potential biomarkers for psychiatric disease, as studies are generally not directly comparable when different methods and materials are being used.

*IL-17A*

IL-17A is a profound pro-inflammatory cytokine, a signature cytokine of the T helper 17 (Th17) cells. IL-17A is produced by innate immune cells residing in the skin, gut and lung, like NK cells and neutrophils, amongst others (72). Literature suggests IL-17A plays a role in clearing pathogens which were unsuccessfully handled by Th1 and Th2 cells. A key role of IL-17A is recruiting, activation and migration of neutrophils (72). There are still rather few studies on IL-17A in the context of psychiatric disorders, although some research on IL-17A and depression exists (45). Little is known about the Th17/IL-17A contribution to depression, suggesting IL-17A deserves more attention in this regard. However, a recent study proposed IL-17A as a marker of treatment resistance in MDD (73). It is believed that Th17 cells target neurons, microglia and astrocytes in the brain and thereby contribute to neuroinflammation. More knowledge on this might provide a future therapeutic intervention in treatment of depression. Moreover, the source of Th17 cells in depression remains unclear, but the synthesis is related to the composition of gut microbiota in the small intestine (45). All in all, IL-17A seems like an exciting and under-investigated cytokine which needs more attention.

IFN-γ

Pro-inflammatory interferon IFN-γ belongs to the Class II cytokines. IFN-γ increase the activity of natural killer lymphocytes and amplifies macrophage activity in order to kill and consume pathogens (74). IFN-γ is produced by T cells, NK cells, macrophages and dendritic cells, and it is a major activator of antimicrobial macrophage functions, including release of reactive nitrogen and oxygen intermediates (75). Interestingly, IFN-γ is reported to be lowered in patients with depression compared to healthy controls (38). This contrasts the other cytokines/chemokine assessed in this study, which often have been found to be higher in such patients compared to healthy controls (52, 76). The finding on lowered levels might be specific to depression disorders, as IFN-γ has been found to be elevated in women with severe anxiety (77).

## 2.7 The associations between mental distress, psychiatric disorders and biological markers

### 2.7.1 Early development

An increasing amount of research during the 1980s suggested that biomarkers of the immune system were altered following reactions to stress, bereavement and depression (78, 79). The biomarkers assessed at the time would be for instance lymphocytes, where studies showed abnormal mitogen-induced lymphocyte blastogenesis (80, 81). Following the increasing awareness of cytokines and their biological properties, cytokines have in recent decades been intensively studied in the field of psychoneuroimmunology research, as they serve as good indicators of immune system activity (82).

The term “macrophage theory of depression” was coined in an article by Ronald Smith in 1991 (83), where it was postulated that IL-1, produced by excessive secretion of macrophages in various diseases, correlated with increased symptoms of depression. Further, it was hypothesized in the mid-1990s by Michael Maes and colleagues that an increased monocytic production of cytokines IL-1β and IL-6 accompanies major depression (84). This increasing production would contribute to HPA axis hyperactivity, as well as impairing the serotonin metabolism (54). Ultimately, it would lead to behavioral symptoms associated with depression (84), collectively referred to as “sickness behavior” (76). These findings were further consolidated in a systematic review by Dantzer and Kelley, who summed up the past 20 years of research on cytokine-induced sickness behavior (85). They pointed out that Lucile Capuron was the first researcher to systematically investigate at the psychological level the symptoms of patients who had received cytokines in treatment of cancer or hepatitis C. Capuron deconstructed the symptoms experienced by such patients into two main categories. All patients developed neurovegetative symptoms (i.e. fatigue, loss of appetite, sleep disorders), which were resistant to treatment by the current, widely-used anti-depressives. The second group consisted of those who in addition developed depressed mood, anxiety and cognitive dysfunction (86). Capuron also found that patients who had had one month of cytokine treatment, and who had a markedly high score on the Montgomery-Åsberg depression scale (MADRS) at end of treatment, also had high scores at the baseline measurement. Having emotional symptoms or sleep disturbance at baseline, in addition to

low social support, was found to be predictive of the severity of depressive symptoms after end of cytokine treatment (87).

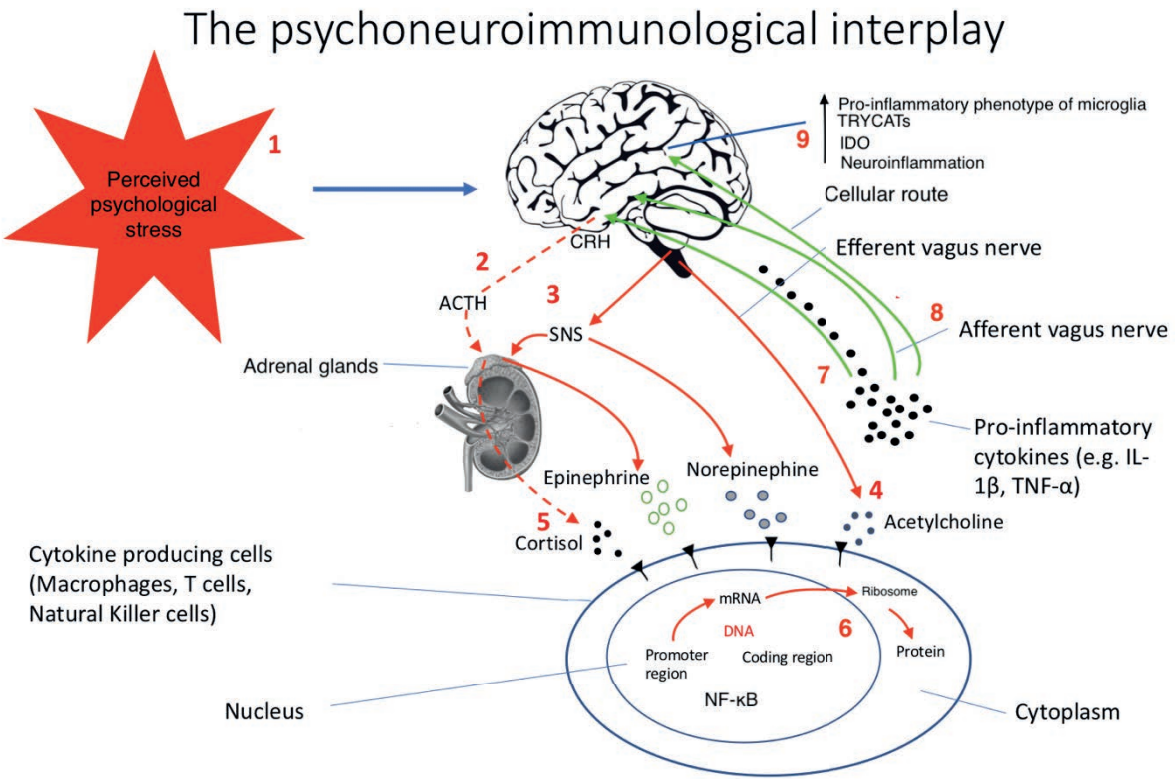
### 2.7.2 Cytokines in the etiology of psychiatric disorders

Increasing evidence has suggested that neurobiological mechanisms underlie a range of psychiatric disorders (88). MDD, anxiety disorders, PTSD and alcohol use disorders (AUD) are complex disorders where etiology is only partly understood, but it is now a well-established fact that the immune system plays a part in these diseases (89, 90). Studies which have assessed TNF-α, IL-1β, IL-6 and anti-inflammatory cytokine IL-1RA have found these markers to be elevated in MDD (48, 91), schizophrenia (92), bipolar disorder (59), post-traumatic stress disorder (PTSD) (93), eating disorders (27) and anxiety disorders (94). An increase in circulating and brain cytokine levels has been consistently reported in both animal models of MDD as well as among human subjects when compared to healthy volunteers (54, 95).

Debate still exists around whether the observed inflammatory augmentation simply accompanies or rather causes the depressive state, and which cytokines may be primary in the pathophysiology of MDD (48, 54, 76, 96). Several studies have found a low-grade chronic inflammation, characterized by several increased inflammatory markers, among them IL-6, TNF-α, IL-1RA, and MCP-1, when comparing groups of patients with MDD to healthy controls (48, 97). This is also supported by a recent systematic review and meta-analysis, which stated that depressed people have higher levels of IL-6 and TNF-α compared to controls (98). Studies report that cytokines may promote depressive-like symptoms (97). These results, together with a systematic review suggesting pro- and anti-inflammatory cytokines to seemingly be related to a trait of mood disorders, but possibly also a state (99), are significant findings about cytokines. These findings point to the importance of doing longitudinal studies, as intra-individual changes in the cytokine levels are related to state depression (100, 101). The variation in cytokine levels cannot be captured in cross-sectional studies. Cytokines may cross the blood-brain barrier, and cytokine IL-1β may affect the brain through the vagus nerve (102). Not only is trauma exposure an important contributor to depression, but having a pattern of repeated cycles of alcohol intake interferes with microglia and neuro-signaling in the CNS in terms of altering their response by enhancing production

of pro-inflammatory cytokines. This subsequently affects the organism in various ways through the chronically elevated inflammation (103, 104).

Figure 1. The psychoneurommunological interplay




Abbreviations: CRH: Corticotropin-releasing Hormone. ACTH: Adrenocorticotropic hormone. SNS: Sympathetic Nervous System. mRNA: Messenger Ribonucleic Acid. NF-κB: Nuclear Factor Kappa Beta. TRYCATs: Tryptophan Catabolites. IDO: Indoleamine 2,3-dioxygenase. This figure was made by the PhD candidate inspired by the work of Slavich & Irwin (105) and others (106, 107).

The following elaboration follows the numbers in the figure: When social stressors are perceived (1), the HPA axis is enabled, with the production of ACTH. ACTH is induced by the anterior pituitary gland in response to the stressor, and in response to production of CRH in the hypothalamus (36). Pathways of the sympathetic nervous system and the efferent vagus nerve induce secretion of norepinephrine and acetylcholine (3 & 4), promoting cytokine production in immune cells. ACTH enables production of cortisol and epinephrine (adrenaline) in the adrenal glands (5). The receptors in cytokine producing cells are affected

by cortisol, epinephrine and acetylcholine. Cortisol and acetylcholine have anti-inflammatory effects, but over time stress may become chronic, subsequently leading to glucocorticoid receptor (GR) resistance and an enhanced activation of pro-inflammatory cytokines (108). Next, epinephrine in turn activates the intracellular transcription factor nuclear factor-kappa β (NF-κB) pathway, resulting in change in cell function, i.e. the production of pro-inflammatory cytokines (6) (105, 109). The cellular route (7) indicates the passing of cytokines in the areas in the brain (circumventricular organs, structures characterized by permeable microvasculature) which are not covered by the BBB (110). Here, cytokines slowly diffuse through the BBB to the brain side of the barrier (111). The upregulation of peripherally produced pro-inflammatory cytokines by the cytokine producing cells in turn activate afferent vagus nerve fibers (8), which constitute a fast transmission of neurocognitive and behavioral messages to the brain. Such messages are information that regulate mood, motor activity, motivation and sensitivity to social threat (105). The production of cytokines by microglia in the brain is in turn stimulated, which relays and amplifies the action of cytokines, also contributing to neurodegeneration, reduced neurogenesis, and less available tryptophan (9).

TLRs in macrophage-like cells, which reside in the circumventricular organs and in the choroid plexus (i.e. cerebrospinal fluid producing cells in brain ventricels), respond to circulating pathogen-associated molecular patterns by producing pro-inflammatory cytokines (112). In conclusion, the activation of all these immune-to-brain pathways ultimately results in pro-inflammatory cytokine/chemokine production by microglial cells (113), one example being chemokine MCP-1 which attracts myeloid cells (for instance activated monocytes) to the brain (36).

### 2.7.3 Trauma, stress and cytokines

Even those exposed to maternal depression in utero have been found to exhibit elevated levels of CRP in young adult life (114). There is also some evidence of individuals being exposed to medically-related inflammation in childhood, for instance through common infections or autoimmune disorders, showing increased risk of depression in adulthood (115). Together, all these lines of evidence indicate there are subgroups of depressed patients who

have elevated inflammatory biomarkers, owing to exposure to stress and/or inflammation in childhood or in utero.

When trauma is experienced, the HPA axis is activated, with subsequent release of cortisol. Acute-phase reaction cytokines (IL-1, IL-6) cross the blood-brain barrier (BBB), which triggers the HPA axis response in an attempt to reduce the risk of excessive inflammation and thereby protect the individual from such a state (116). As for the ongoing debate regarding whether increased circulating inflammatory markers are caused by or cause mental illness, for PTSD it has been suggested that the inflammatory response following trauma exposure contributes to the symptoms (117). This is in line with the growing evidence on psychological stress stimulating cytokine production, which plays a role in the etiology of anxiety and depression (118). Numerous research papers have reported that PTSD is associated with excessive amounts of inflammatory actions of the immune system (117, 119). However, in a recent prospective study on anxiety disorders, people with PTSD were found to exhibit decreased levels of IL-6 after a 5-year follow-up period. IL-6 is a complex cytokine with both pro- and anti-inflammatory properties with the anti-inflammatory effect particularly prominent in the acute phase of the stress response. Finding lower levels of IL-6 than in non-PTSD patients has been hypothesized to be due to long-term suppression of the anti-inflammatory response of IL-6 due to a hypersensitive HPA axis, although the exact mechanism is still unknown (120).

### 2.7.4 Low grade inflammation and anxiety

Inflammation has been found to be associated with anxiety disorders in some research (121), and there is evidence of different levels of inflammation across the various anxiety disorders (122). A recent, large population cohort study found these disorders, except social anxiety, to be significantly associated with elevated CRP levels. In particular, people with panic disorder with agoraphobia exhibited higher levels of CRP than people with the other anxiety disorders (123). This was also found in another recent large prospective study of the general population with a 5-year follow-up, where levels of high-sensitivity CRP suggested that chronic low-grade inflammation could be a consequence of agoraphobia. The study suggested a direct association between anxiety disorder at baseline and chronic low-grade inflammation at follow-up, thus suggesting that inflammation is a consequence rather than a risk factor for

anxiety disorders. The study did not find any evidence of chronic low-grade inflammation as a predictor of future anxiety disorders (120). A recent case-control study in patients with GAD showed that these patients had higher levels of the pro-inflammatory cytokines TNF-α and IFN-γ, and lower levels of the anti-inflammatory cytokine IL-10 (124) when compared to healthy controls. Maes and colleagues showed in 1998 that subjects with anxiety had higher levels of IFN-γ and lower IL-10 levels than subjects without anxiety (125). Another study found the production capacity of several cytokines in patients with anxiety disorders to be positively associated with severity of anxiety symptoms, even after taking lifestyle and health factors into account (90). In conclusion, research suggests the immune response to be involved in anxiety disorders.

### 2.7.5 Pro-inflammatory cytokines and eating disorders

The evidence for elevated pro-inflammatory cytokines seems clear in many psychiatric disorders, such as depressive disorders, but the evidence is not so clear in eating disorders. Previous results are conflicting. Some studies suggest a pro-inflammatory state, while other studies indicate reduced capacity to produce certain cytokines, e.g. IL-2 (126). Further, IL-6 has been found to be elevated in AN, and to be higher in obese subjects compared to healthy controls, and higher levels of IFN-γ have been found in AN than in healthy controls (127). One study on cytokines and chemokines in patients undergoing treatment for eating disorders found that patients reported a moderate level of depression (mean scores on the BDI-II of 22.6), but exhibited few of the immunologic abnormalities often found in depression patients (e.g. elevated IL-6) (128). Rather than elevated cytokine levels, the immunologic properties in people with eating disorders seem to demonstrate a different nature. It has been suggested that the common characteristics of patients with AN, such as loss of adipose tissue and excessive exercise, may attenuate the production of cytokines. However, one characteristic of patients with eating disorders distinguishes this group from all other psychiatric disorders, namely that the serum of patients with AN contains a stimulatory factor or factors for cytokine production that compensates for lower production of cytokines by peripheral blood mononuclear cells (126). One might hypothesize that reduced expression of cytokines would render the subjects susceptible to infections, but it seems to be the opposite for AN patients; individuals with AN tend not to get infected by common infections such as colds or flu (127,

129). Further, AN and BN patients have been reported to be significantly more depressed and anxious than healthy, normal-weight controls, as indicated by scores on the BDI (130). However, despite the relative level of depression symptoms found in these patients, it has for decades been unclear if depression in eating disorder patients is related to cytokine levels, as previous studies have found no such relationship (127). A recent meta-study summed up numerous studies on AN and BN patients, and concluded that levels of TNF-α and IL-6 were specifically higher in AN patients than in healthy controls (27). The levels did not differ between patients with BN and healthy controls. Also, the levels of IL-1β and transforming growth factor beta (TGF-β) did not differ between healthy controls and any eating disorder group. As cytokines can access the brain via humoral, neural and cellular pathways (131), they have an effect on learning, memory, and behavior through several pathophysiological mechanisms (132). Cytokines have been linked with body weight and the regulation of body weight (133), as shown by the correlation between IL-6 and body mass index (BMI) (134). Another recent meta-analysis found TNF-α, IL-1β and IL-6 to be elevated in patients with AN when compared to healthy controls, and also found that concentrations of these cytokines did not change after weight gain (135). Given the fact that these cytokines are also elevated in other psychiatric disorders, the clinical impact of these findings is not so clear. This implies that this inflammatory response is rather unspecific and not a trait found exclusively in eating disorders, but rather a marker of overall illness severity (27).

### 2.7.6 Direction of causality

There is still a lack of evidence regarding the direction of causality in psychoneuroimmunology, i.e. does poor mental health lead to heightened inflammation, or does a chronic state of inflammation lead to poor mental health? Research has shown that the use of interferon-alpha leads to depression when treating patients with hepatitis C and cancer (136), implying a causal effect in at least one direction. Interferon-α has therapeutic benefits in these somatic diseases, but may also cause neuropsychiatric complications. For instance, using interferon-α as a medical agent induces the production of pro-inflammatory cytokines. Consequences are depletion of tryptophan and activation of the HPA axis, which are direct effects of increased circulating levels of pro-inflammatory cytokines (86).

Some have found cytokines to decline in parallel with depressive symptoms in patients receiving psychiatric treatment (101). Others have found that cytokine TNF-α was not only heightened, but also increased during the course of treatment in PTSD patients receiving psychotherapy (137). As TNF-α is a pro-inflammatory cytokine which contributes to the inflammatory response, the increasing levels of TNF-α suggests that the PTSD patients exhibit a condition of increased inflammation while undergoing inpatient treatment. It is too early to draw any conclusions as to why this phenomenon has been seen in PTSD, as too few studies have yet reported such a finding.

Several studies have reported no associations between depressive symptoms and inflammation (48). A recent study in China found no associations between CRP and depressive symptoms among elderly and middle-aged people (138). Also, it has been reported that various subtypes of depression do not differ in cytokine levels from healthy controls (139).

## 2.8 Alcohol and cytokines

Alcohol (ethanol) modulates the immune system in several ways. The brain is one of the major targets of ethanol, and chronic and acute intoxication alters brain structure and function, and may lead to neurodegeneration. Even low concentrations of ethanol have been found to up-regulate the inflammatory mediators iNOS (inducible nitric oxide synthase) and NO (nitrox oxide) from amino acid l-arginine, as well as cytokines (140, 141). Ethanol consumption activates the enzyme complex IκB kinase (IKK) which propagates the cellular response to inflammation through phosphorylation of the inhibitory IκBα protein, resulting in separation of IκBα from NF-κB. This process frees the NF-κB transcription factors, allowing it to migrate into the nucleus and activate genes encoding for cytokines, thus promoting inflammation (142). Alcohol alters the gut microbiota, leading to an enteric dysbiosis and intestinal bacterial over-growth (143), and also increases gut permeability. This results in translocation of LPS into systemic circulation and interferes with Kupffer cells (hepatic macrophages) in the liver (144). The Toll-like receptor-4 (TLR4) and Cluster of differentiation-14 (CD14) receptor, found on the Kupffer cells, are activated by the presence of LPS, activating transcription of pro-inflammatory cytokines like TNF-α, and hepato-

protective cytokines like IL-6 and anti-inflammatory IL-10, and increasing oxidative stress, which has several effects, one of them being increased fibrosis and eventually liver cirrhosis (145). People with alcohol dependency are known to have increased levels of circulating antibodies immunoglobulin-A (IgA) both with and without active liver disease (144). Also, immunoglobulin-M (IgM) may be increased during active phases of liver disease, for instance alcoholic hepatitis, as well as in inactive phases such as quiescent cirrhosis, but to a lesser extent. The elevated levels of antibodies, however, do not imply an increased ability to battle infections. This is reflected by e.g. the fact that the level of B cells does not correlate with increased levels of immunoglobulins (146). In fact, people with AUD are immunodeficient, and have an increased risk of contracting infectious diseases (147). The increased level of antibodies may be related to dysregulation of antibody production, possibly through modulating cytokine expression. The effect of alcohol on the immune apparatus is dose-dependent, and one way of distinguishing the effect has been described in the literature as acute and chronic exposure to alcohol consumption (144).

Acute consumption reduces the responses in both the innate and adaptive immune system, decreasing the monocyte and dendritic antigen presentation capacity and the pro-inflammatory cell activation pivotal to innate immune activation. In contrast, the immune response to chronic alcohol exposure is increased pro-inflammatory activation. Alcoholic hepatitis in those with chronic alcohol consumption responds to the alcohol levels by increasing the production of monocytes and macrophages, ultimately resulting in increased levels of TNF-α, IL-1, IL-6 and chemokine interleukin-8 (IL-8) (144).

The consumption of alcohol renders the gut wall leaky, allowing passage of LPS from the intestinal lumen into circulation (103), challenging immune cells to release pro-inflammatory cytokines. These peripherally produced cytokines enter the brain and stimulate glial cells to produce CNS cytokines, resulting in alcohol-induced neuroinflammation as a consequence. It is at this junction the consumption of alcohol translates into alterations of mental health. The persistent neuroinflammation precipitates cognitive and behavioral responses (148), a consequence of factors like activation of signaling pathway NF-κB and microglia releasing MCP-1 that attracts monocytes into the CNS. Pro-inflammatory cytokines are produced in the CNS with the increased expression of IDO, where the downstream metabolites (TRYCATs) are likely to be the cause of inflammatory mood alterations, such as depression (149). After years of research on both animals and humans, there is still no real consensus as to if alcohol

consumptions predisposes to development of MDD or if it the other way around (149). It has been suggested that the link between alcohol and MDD probably goes both ways, as bidirectional causality has been demonstrated (149). However, a more robust association has been found in the direction from AUD to MDD, suggesting what one might call “alcoholic depression” (148).

## 2.9 Anti-depressive, anti-inflammatory drugs and cytokines

Antidepressants, both selective serotonin reuptake inhibitors (SSRIs) as well as monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) act by altering the serotonin and norepinephrine action (150). However, they have also been found to exhibit anti-inflammatory effects, as shown by reduced levels of IFN-γ, IL-1β, IL-6 and/or an increased level of IL-10 (52, 58). Anti-inflammatory drugs (NSAIDs) have been found to have better anti-depressive effects than placebos, either as monotherapy or as add-on treatment to anti-depressants (151), but the effects of NSAIDs on depression are inconsistent due to methodological heterogeneity (152). NSAIDs as a group are structurally diverse and differ in pharmacokinetic and pharmacodynamic properties. Nevertheless, they have a common mode of action; they inhibit the production of prostaglandins by blocking the COX enzyme. This results in analgesic, antipyretic and anti-inflammatory benefits, and also gives rise to a risk of gastrointestinal bleeding (153). It has been suggested that anti-inflammatory drugs are not likely to benefit all depressed patients, and that the challenge rather lies in identifying which subgroups of patients actually respond to anti-inflammatory interventions (152). The increasing awareness of the immunomodulatory properties of these drugs suggests that they should be accounted for in studies of psychiatric patients by controlling for them or by stratification. Exclusion of patients using such drugs could be characterized as unethical, given the fact that these drugs are increasingly used in patients worldwide and their effects in various populations therefore should be scrutinized (154).

The responsiveness to drugs with predominantly serotonergic action (e.g. escitalopram) has been found to be poor. A fast step-up to dopaminergic (e.g. bupropion) or gluamatergic (e.g. ketamine) drugs, or  a combination of an anti-depressant with an anti-inflammatory drug (e.g. infliximab, minocycline or fish oil), has been suggested (155). It has been postulated that the

reason why serotonergic drugs lack effectiveness on depression outcome, and thus may be successfully combined with glutamatergic drugs, is the breakdown of tryptophan down the kynurenine pathway with various neuroactive compounds which aggravates glutamatergic neurotransmitter imbalances (156, 157). More research is needed due to rather heterogenous study designs, publication biases and underpowered studies (155), but the available literature collectively point in the direction that low-grade inflammation plays a role in the outcome of anti-depressant therapy in patients with MDD (155).

## 2.10 Immune biomarkers in assessment and as predictors of response

A biological marker (biomarker) is defined in medical literature as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (158). Biomarkers are further categorized into two groups, i.e. diagnostic biomarkers and treatment biomarkers. Diagnostic biomarkers are useful in distinguishing the presence or absence of a disorder, while treatment biomarkers may be used to predict treatment response. But biomarkers are also categorized into trait and state markers. Trait biomarkers are persistent and reveal disease which exists before the onset of pathology, during pathologic processes, and after remission. Such biomarkers are useful in predicting which individuals are at risk of developing disease. On the other hand, state biomarkers are temporary, short-lived markers related to the clinical condition. This means that such biomarkers may be present at the onset of a condition, but normalized following remission (159).

The current study encompasses basic research with the possible clinical applications of aiding diagnosis, monitoring treatment and identifying novel treatment targets. The notion of using biomarkers as a means of identification of diagnoses and possibly subsamples within diagnoses has been proposed previously (160), but at present there is no approved biomarker as part of a diagnostic criteria for any psychiatric disorder (159). A recent systematic review and meta-analysis concluded that certain chemokines measured in blood, among them CCL2 (also known as MCP-1), CCL3, CCL4, CCL11, CXCL4, CXCL7 and CXCL8, are altered in depression and can be used to discriminate between people with and without depression (11). However, a meta-analysis on biomarkers in depression, bipolar disorder and schizophrenia

showed that variation in molecules was similar among disorders, suggesting that this reflects transdiagnostic systemic consequences of psychiatric illness (161).

Immune changes have been shown to be present among patients with various psychiatric disorders (162-164). In cross-sectional studies, the studies show a snapshot of the immunologic profile. More longitudinal studies must be conducted to further investigate the state versus trait relationship of inflammatory biomarkers. It still is an enigma whether or not abnormal cytokine levels could be a state or a trait, and whether or not the immune alterations are a product or a cause of the psychiatric disorder (119). The scarcity of longitudinal studies and the diverting results in previous studies made a good argument for conducting the current study, assessing cytokines both cross-sectionally and longitudinally in a mixed psychiatric population, further scrutinizing the field of psychoneuroimmunology. It has been postulated that clinicians might have a high misdiagnosis rate owing to the fact that certain psychiatric diseases have several symptoms in common, for instance MDD and BD (165). Despite previous research suggesting a transdiagnostic inflammatory state (161), it is too early to rule out that inflammatory biomarkers could be useful as a means of assisting clinicians in the complex task of diagnosing patients. It has been proposed that biomarkers could be used as a future method to distinguish between different disorders (119). A discovery of blood sample profiles might form a basis for future immunotherapy-based interventions. Yuan and colleagues also pointed out that trait biomarkers can be useful for monitoring prognosis and possibly to predict treatment outcome (119). With improved methodologies, this could in the future be a possibility, although we are not there at the moment.

# 3 Aims

## 3.1 General aim

To gain knowledge about the relationship between levels of circulating cytokines and mental distress in patients with various psychiatric disorders over the course of 12 weeks of treatment.

## 3.2 Specific aims

1. To explore the association between circulating peripheral cytokine levels and degree of depressive symptoms, taking trauma and alcohol into account (Paper I).

2. To gain insight into whether PTSD diagnosis modulates cytokine levels and development over time. In addition, to study this in the light of the use of anti-inflammatory drugs (Paper II).

3. To explore the relationship between cytokine levels and level and development of mental distress in psychiatric patients in treatment. Further, to examine the potential role of anti-inflammatory drugs in this regard (Paper III).

# 4 Material and methods

## 4.1 Methods

### 4.1.1 The DARCY studies

The current study forms one branch of a larger ongoing study entitled the Depression Alcohol Research on Cytokines (DARCY), led by Innlandet Hospital Trust. The DARCY research group's research on cytokines has been an active project for several years, and the first wave of the DARCY studies started with data collection for a cross-sectional study in Nepal (166-170). With the cross-sectional results as a basis, a longitudinal approach was the logical step forward, and the Modum Bad DARCY project was designed and planned. The data collection started in March 2015 and ended in April 2016 when 147 patients had submitted three blood samples and reported their corresponding psychiatric symptoms. In the following sections the material and methods of the study are detailed and the PhD candidate's contribution is stated.

### 4.1.2 Modum Bad Psychiatric Center

Modum Bad Psychiatric Center is a specialized high-threshold facility located in rural Norway in the municipality of Vikersund. The facility treats patients who have been ill for years, who have tried treatment elsewhere with no or little success, and who are referred to Modum Bad Psychiatric Center as tertiary care. Due to limited capacity, the facility predominantly accepts patients living within the boundaries of Health Region South-East. Patients at Modum Bad Psychiatric Center are offered a 12-week inpatient treatment program consisting of psychotherapy treatment and psychoeducation both in groups and individually. As the patients have tried treatment previously without achieving recovery, they are to be regarded as a treatment-resistant patient group. They are referred here either by their general practitioner, by their psychologist, or by their local District Psychiatric Center. The facility does not specifically treat patients with substance use disorders (SUD), but some patients may have SUD as a comorbid condition. Patients with active SUD or who are psychotic are

referred to institutions elsewhere. The staff consists psychiatrists, psychologists, psychiatric nurses, social workers, art therapists, and pastoral staff.

### 4.1.3 Patient recruitment

The patients were recruited from the depression, anxiety, eating and trauma units at Modum Bad Psychiatric Center. The patients were approached in groups. They were given a 15-minute oral presentation, and a written consent form with information about the study was handed out. These sessions took place during one of each patient's very first group sessions, usually 2-4 days after enrolment. Altogether, 249 patients were approached during the recruitment period. Participation in the current study was not directly related to the treatment and did not yield any kind of benefit except the contribution to science, so it was anticipated that about 50% of the patients would agree to participate. Of the 249 patients approached, the number who consented to participate was 148 (59%). The reasons patients gave for not wanting to participate were mainly fear of pain from venipuncture, and that participating felt burdensome when it came in addition to the treatment program. Others disliked the fact that there was no possibility of giving individual feedback on results from blood sample analyses. One individual chose to withdraw her consent, and the number of participants was thus reduced to 147. Further, the nature of the immune system is primarily to protect the individual from invaders like viruses, bacteria and fungi. An activated immune apparatus likely suggests an attack has been launched against detrimental invaders, and we did not want to include participants with suspected ongoing infection. As a way of ruling out such patients, we identified and excluded the patients with cytokine levels above the $95^{th}$ percentile in each cytokine at $T_0$.

In this way, 19 patients were removed from all analyses, and the number of patients dropped to 128. One female patient was unable to give blood due to failed venipuncture, and was excluded from all blood sample analyses, giving a total number of 127 patients in the blood sample analyses. Papers I and III thus comprised data provided by 92 women (72%, mean age 39.04 years, SD 11.26) and 36 men (28%, mean age 49.06 years, SD 9.36), giving a total of 128 patients (the abovementioned female patient participated in analyses not involving blood samples). In Paper II, residual diagnostics was performed by plotting the random effects, which resulted in exclusion of four outliers (see the statistics section for details). This

gave a total of 88 women (71%, mean age 38.98 years, SD 11.31) and 36 men (29%, mean age 49.06 years, SD 9.36), thus 124 patients in total (123 patients in the blood sample analyses).

### 4.1.4 Healthy volunteers

A group of 19 healthy volunteers was used in paper I for comparison with the cytokines in the patients at $T_0$. This group was not the optimal group for comparison, as they were men only and younger than the patients. The female gender and higher age are known to be associated with different cytokine levels (171, 172). We selected the 19 youngest men of the patient sample for the comparisons, a procedure which introduces selection bias. These factors taken together are weaknesses which should be taken into consideration when interpreting results.

## 4.2 Material

### 4.2.1 Assessment of symptoms and diagnoses

All patients were interviewed by trained psychologists or psychiatrists using the Mini-International Neuropsychiatric Interview (MINI) (173). The MINI interview is a short diagnostic structured interview developed in France and the USA. It is well-designed to be used by non-specialized interviewers, it focuses only on current disorders for the sake of brevity, and it has been found to provide reliable diagnoses within a short time frame (174). The diagnostic interviews were performed during the first week of admission. This part of the data collection was an established routine at the facility and did not represent any difference from a regular stay without participation in the DARCY study. The MINI interview results in a diagnosis in the 10$^{th}$ revision of the International Classification of Diseases and Related Health Problems (ICD-10) (173). There was also a combination of psychometric questionnaires and clinical judgment taken into consideration when disorders were assessed. There were 54 patients with only one disorder, and 59 who had two or more disorders. There were 15 patients with missing diagnosis and 14 with missing trauma status due to missing

data. These 15 patients were excluded from the analysis involving diagnoses. Disorders within F30-39 were treated as one variable of mood disorders. Disorders within the range of F40-49 were merged to one variable of anxiety disorders. In Papers II and III, patients with the F43.1 PTSD diagnosis were excluded from the anxiety disorders group and categorized as one specific group. Disorders within F50.0-F50.9 were merged to one variable of eating disorders. The diagnoses were recorded by the staff in the computer software Checkware® and were thus made available for entering into the statistical software. In addition, the patients completed various self-report questionnaires either on a computer or on a digital tablet. These self-report sessions were all part of the established routine at the facility, except for the second self-report session, which was exclusively for participants in the current study. This session was arranged by adding a self-report session for HSCL-90R (175) in Checkware® at approximately halfway of the stay. The patients were then notified by the staff about filling out the questionnaire. The patients filled out the questionnaires by themselves at the designated time points, however, they always had the clinical staff close for assisting whenever needed. The staff followed up patients who had difficulty remembering to fill out the questionnaires, and the staff also readily answered any questions from the patients. The questionnaires and corresponding sessions are described in the following:

The 21-item Beck Depression Inventory (BDI-II) assesses the level of depressive symptoms during the two-week period prior to the interview with the therapist (176). The Norwegian validated version was used (177, 178). The psychometric properties of the BDI-II are adequate (179). The Cronbach's alpha coefficients for the BDI-II at treatment initiation and before discharge were 0.91 and 0.93, respectively. Each of the 21 items is scored as 0 to 3. Based on the average score, we categorized the answers into three levels of depression severity: Minimal and mild depression (score 0-18), moderate (score 19-29), and severe (score 30-63). This questionnaire was filled out at $T_0$ (on one of the first days of the stay) and at $T_2$ (just before discharge). There were 11 patients who did not fill out BDI-II at $T_0$ and 12 at $T_2$. Only the $T_0$ measurement was assessed in this study.

General symptom severity was assessed using the HSCL-90R questionnaire. The patients filled out the questionnaire three times during their stay: At $T_0$, at the halfway stage ($T_1$), and at $T_2$. The 90 questions measure the level of general distress for the last 7 days. Each question ranges from 0 to 4, corresponding to "not at all", "a little bit", "moderately", "quite a bit", and "extremely". Mean score is calculated for the HSCL-90R, referred to as the Global

Severity Index Index (GSI) (180). The HSCL-90R has been found to provide valid evaluation of the severity of symptoms in a broad range of psychiatric patients (181). Based on previous literature, we set the cutoff score for caseness at 0.85 (182). The Cronbach's alpha coefficients for the GSI scores at admission, halfway and at discharge were 0.973, 0.979, and 0.983, respectively.

The Alcohol Use Disorder Identification Test (AUDIT) is a 10-item screening test designed to identify harmful, hazardous or possible alcohol dependence the last 12 months. The AUDIT has proven able to detect DSM-IV alcohol dependence and DSM-IV alcohol use disorder (AUD) when compared with semi-structured clinical interviews (183). Some examples of questions are: "How often do you have a drink containing alcohol?" and "How many drinks containing alcohol do you have on a typical day when you are drinking?" The scores range from 0 to 4. In these examples, a score of 0 refers to "never" and "1-2", respectively. A higher score refers to a more severe drinking pattern, and in these two examples, a score of 4 means "4 times a week" and "10 or more". The cutoff scores for harmful or hazardous drinking were set at 8 for men and 6 for women (184). The Cronbach's alpha coefficient for AUDIT at admission was 0.86. This questionnaire was filled out at $T_0$.

Trauma history was recorded by therapists at the facility as part of the anamnestic interview. There were five questions regarding trauma exposure: 1) Has the patient been exposed to sexual assaults in childhood? 2) Has the patient been exposed to physical abuse in childhood? 3) Has the patient during childhood experienced other traumatic events which have led to severe problems later in life? 4) Has the patient been exposed to sexual assaults or abuse in adulthood (after 18 years of age)? 5) Has the patient in adulthood experienced other traumatic events which have later led to severe problems?

### 4.2.2 Pharmacological data

Receiving pharmacological therapy was unrelated to being included in the current study. The choice or the use of the various drugs was in no way affected by the participation. The pharmacological data was recorded by the staff in the medical cardexes and was available to the PhD candidate for manually entering into the statistical software.

### 4.2.3 Blood sample collection and preparation

The patients had their blood samples drawn in the laboratory at Modum Bad Psychiatric Center. The blood sampling was conducted between 8:00 and 9:00 am for all patients at $T_0$, $T_1$ and $T_2$, except for one of the groups in the depression unit, who gave their blood samples between 12:00 am and 3:00 pm (16 patients). Vacuette 8 ml serum containers were used for blood collection. These were turned upside-down approximately 8-10 times immediately after the blood was drawn and set to rest in a blood tube stand for a minimum of 30 minutes and a maximum of 1 hour. They were then centrifuged in a Kubota 2420 swing-out centrifuge set at 10 minutes. The centrifugation power reached 1917 g, and the centrifugal process was conducted at room temperature. Finally, the blood was drawn with 1 mL single-use pipettes into Nunc tubes before they were set to rest at -80° C until assay.

### 4.2.4 Cytokine and chemokine measurements

The frozen blood samples were brought by the PhD candidate to Oslo University Hospital Ullevål, Oslo, Norway. The samples were analyzed there by the laboratory staff. All samples were analyzed in one batch. Due to queue on the laboratory facilities, it took approximately 2 months from the samples were handed in until they were analyzed. Prior to this, the storage period from collection until assessment for the entire blood sample material varied from one year until approximately 2 months. Previous research on the effects of long time storage of serum samples in -80° C suggests that most cytokines remain stable within a two year period (185). However, IL-17 in serum samples has been reported to start degrading within one year of storage in -80° C, and IL-1β, IL-6, and IL-10 have been found to degrade up to 50 % or less of baseline value within 2-3 years of storage (186). This suggests that the storage period in the current study of approximately one year did not affect the quality of the samples, except possibly for IL-17A. Literature has also suggested that batch testing of samples is feasible, as most cytokines are stable across various storage conditions (187).

We had 7 cytokines and 1 chemokine analyzed. The cytokines were IL-1β, IL-1RA, IL-6, IL-10, IL-17A, IFN-γ, TNF-α and the chemokine was MCP-1. They were chosen based on the available literature on the neuroimmune correlates of psychiatric disorders: Cytokine IL-17A

was chosen due to some reports relating it to be elevated in women with severe anxiety and depression (77), as well as reporting it not to be correlated with MDD (188). The other 6 cytokines, and chemokine MCP-1, are well-known for their presence in many psychiatric disorders (48). However, the majority of research is conducted cross-sectionally. Thus, we took it a step further and explored these biomarkers with a longitudinal design. Cytokine analysis is rather expensive, so investigating biomarkers previously reported to be associated with psychiatric disease was considered a safe approach giving high possibility of positive results. The choice of cytokines is further elaborated on in section 1.4.

Four cytokines had rather high values under the limit of detection (LOD) (> 55%) and were excluded from the study. We had adequate results in cytokine IL-1β, IL-1RA, TNF-α and MCP-1. All blood samples were thawed on ice, vortexed, and then spun down a tube with 250 µl serum at 14,000 × g for 10 min at 4° C, before dilution (1 + 4) and further processing. Cytokine measurements were performed using Bio-Plex xMAP technology (Bio-Rad, Austin, Texas, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA), powered using Bio-Plex Manager (version 6.0.1) software. The assay was performed according to the manufacturer's instructions, but an additional standard point was included. To achieve more reliable results, individual sets of samples from patients were run in the same assay, all samples were assayed in duplicate and a magnetic plate washer was used during assay set up. The StatLIA software package (version 3.2, Brendan Scientific, Carlsbad, California, USA), incorporating a weighted, five-parameter logistic curve-fitting method, was used to calculate sample cytokine concentrations. Longitudinal controls were used in order to validate inter-assay variation: IL-1β (18.1), IL-1RA (10.2), MCP-1 (6.7) and TNF-α (7.4). The inter-assay percent coefficient of variability (CV) in parentheses is a measure of variation between plates, where a lower figure is better. Any figure below 21% is considered acceptable. The mean inter-assay percent CV for all blood sample plates was 10.4%. The unit of measurement was picograms per milliliter (pg/mL). The LODs were 0.01 pg/mL for IL-1β, 3 pg/mL for IL-1RA, 0.76 pg/mL for MCP-1, and 0.02 pg/mL for TNF-α. In paper II, cytokine levels below LOD were imputed with 1% of the mean value. At $T_0$ we performed 64 (50.4%) imputations for IL-1β, one (0.8%) for IL-1RA, nine (7.1%) for MCP-1 and 53 (41.7%) for TNF-α. One patient did not have the first blood sample collected. At $T_1$ there were 62 imputations (51.7%) for IL-1β, no imputations for IL-1RA, five (4.2%) for MCP-1 and seven (5.8%) for TNF-α. Seven patients did not attend blood sampling at $T_1$. At $T_2$ we had 64 imputations (53.8%) for IL-1β, no imputations for IL-1RA, eight (6.9%) for

MCP-1 and 45 (38.8%) for TNF-α. Eleven patients did not attend blood sampling at $T_2$. The imputed values were: IL-1β = 0.003 pg/mL, IL-1RA = 0.4 pg/mL, MCP-1 = 0.03 pg/mL, and TNF-α = 0.05 pg/mL.

The imputation rates for cytokine IL-1β ranged between 50.4 % to 53.8 % in the three blood sampling session. As these imputation rates exceeded 50 %, they might seem arbitrary. Other studies sometimes dichotomize the cytokine as being detectable or not detectable if the number of zero values exceed 50 % (101, 189), however it has occurred that researchers set a cutoff level at 75 % (190, 191). We decided to keep IL-1β, despite that the imputation rate exceeded 50 %, since we wanted to examine this particular cytokine due to its frequent use in the literature on cytokines and psychiatric disorders.

However, the following radical changes were conducted for paper III: We excluded IL-1β as well as TNF-α after reviewers in the peer review process strongly encouraged us to do so. This concern was due to the high level of imputations in these two biomarkers. Further, we chose to replace the values below the LOD with the LOD, rather than replacing these values with 1 % of the mean. In conclusion, we kept IL-1RA and MCP-1. These biomarkers had fewer than 6 % of the values below the LOD. Taking all three blood sampling occasions into consideration, 423 samples were collected. For IL-1RA, one value was imputed (0.2 %). For MCP-1, 25 values were imputed (5.9 %). We excluded the remaining 6 cytokines due to a rather high number of values below LOD. There were 194 (45.9 %) values below LOD for IL-1β, 148 (35.0 %) for TNF-α, 200 (47.3 %) for IL-6, 226 (53.4 %) for IL-10, 384 (90.8 %) for IL-17, and 332 (78.5 %) for interferon-gamma (IFN-γ).

### 4.2.5 Ethical considerations

All patients enrolled at Modum Bad Psychiatric Center are above 18 years of age. The patients who decided to participate gave their written consent after having received 15-minute oral information and a written brochure explaining the procedures involved. The first author provided this information in one of the first group sessions of the patient stay, and usually received the signed consent forms 1-2 days after the group sessions. Participation was not in any way related to treatment. This meant that declining or accepting to participate

would not affect the stay or the treatment in any way. The patients were free to withdraw from the study at any time. Further, there was no reward for participating except from contributing to science. The risks of participating could have been an increased susceptibility to skin infection due to perforation of the skin, or painful experiences from the venipuncture. No patients reported infectious wounds. A few patients reported the venipuncture as a little painful, but still chose to contribute. All patients were treated anonymously at group levels when results were presented in papers and in other contexts such as oral presentations at conferences. In this way, the privacy of each participant was maintained. The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics prior to data collection (reference number 2014/2189).

### 4.2.6 The PhD candidate's independent contribution to the thesis

The following tasks were conducted by the PhD candidate:

- Patient recruitment
- Collection of blood samples
- Implementation of the HSCL-90R questionnaire at halfway of treatment
- Statistical analyses
- Drafting and revision of all three manuscripts

## 4.3 Statistics

### 4.3.1 Bivariate analyses

The Mann-Whitney *U* Test was used for comparing non-normally distributed dependent variables between patient groups. For the continuous variable age, Spearman's Rho correlation was used. The Kruskal-Wallis one-way analysis of variance was used for comparing continuous variables across the multiple groups variable BDI-II. Pearson's chi-square test was used for analyses of categorical dependent and independent variables. The Wilcoxon signed rank test was used to assess differences in GSI score between groups and between different measurements within groups. Log transformation was initially tested, but did not affect the results. In addition, the Kolmogorov-Smirnov test remained significant after log transforming. Thus, we chose to use the raw data instead of the log-transformed data.

### 4.3.2 Multilevel models

In Papers II and III, we used data from all three measurement points. Multilevel modeling was chosen as the statistical method for longitudinal analysis, as this is the appropriate method for analyzing repeated measures of patients over time (also known as "mixed models") (192). In paper II, the repeated measurements of cytokines from all three time points were treated as one continuous, dependent variable. In Paper III, the repeated measurements of HSCL-90R GSI from all three time points were treated as the continuous dependent variable, while the cytokines were treated as independent, continuous variables. The models were run with one cytokine as covariate, together with the time variable as predictor variable. These models were in other words adjusted for time. The multilevel models were however tested with adjustment for sex and age, as sex and age are known to be related to cytokines and psychometry. This adjustment did not affect the results. These analyses are nevertheless presented in supplementary table 5 in paper III. Little research is done on the impact on GSI across time. The field of immunology and psychometry with a longitudinal design is a rather unexplored field. Thus, we considered this study as exploratory by character, and did not present the results adjusted for multiple hypothesis testing. It has been emphasized by statisticians that Bonferroni adjustment at best has limited application in biomedical research (193), and other researchers have previously followed the advice not to

use Bonferroni correction in their published work (194). However, section 4.4 in this thesis presents revised analyses adjusted with Bonferroni correction.

Importantly, when dealing with multilevel models with time-varying predictors, the raw scores at the time points represent a mixture of effects between- and within subjects-effects, and this could lead to biased estimates if not disentangled (195). The within-subjects effects in the context of the current study refer to the expected change in a patient's GSI levels when assessing the subject-specific change in cytokine values relative to the population average during the 12 weeks of treatment. However, as there were only three measurement points, this effect is not very informative and does not represent the focus of interest in this study. Rather, the interest lies in the between-effects, i.e. how the effect of higher levels of cytokines affects the dependent variable GSI. Initially, an empty multilevel model with GSI as dependent variable was run to assess the intraclass correlation coefficient (ICC). The ICC in the empty multilevel model was 0.69. The interpretation is that 69 % of the variation in the GSI was explained by between-subjects effects (i.e. at level 2).

Nevertheless, we disentangled the within-subjects effects as well as the between-subjects effect (195). As it turned out, there were no significant within-subjects effects. The within- and between-subjects effects are shown in paper III, supplementary table 5. The mean values of each individual's cytokine levels were computed in order to obtain the between-subjects effects of cytokines on GSI. In Paper II, the cytokines from all three measurement points constituted four dependent variables. The independent variables were dichotomous, i.e. the estimates of the main effects represent how much higher the estimates are for the group coded as 1 (patients with PTSD) than the group coded as 0 (patients without PTSD).

The covariance matrix was set to unstructured in Paper II because there were two random effects (intercept and slope). The variance for each, as well as the covariance between them, had to be estimated, and the unstructured covariance matrix implies that all variances and covariances were allowed to be freely estimated. There was only one random effect (the intercept) in Paper III, so the covariance was set to identity, since the value of the random intercept in each patient was independent from random intercepts in other patients. The multilevel analyses in Paper III were adjusted with restricted maximum likelihood (REML) estimation, which is an adjustment for small samples. A full information maximum likelihood test (abbreviated as FIML or simply maximum likelihood, ML) may result in

biased estimates in small samples, while REML incorporates uncertainty in the estimation of the fixed effects. However, ML was used in likelihood ratio tests for testing nested models, as REML is not allowed in such tests. In Paper II, robust standard errors were applied since the cytokines were severely skewed with non-normally distributed errors (196). Further, in paper III, there were conducted a couple of linear multilevel regression analyses which often are referred to as simple slopes analysis. These are conducted by separating the data by group, and the individual regression equations are estimated with the cytokine regressed on GSI, giving a p-value which indicates if the slope is significantly different from zero (197).

### 4.3.3 Fixed and random effects

The analyses were conducted with fixed and random effects. The combination of fixed and random effects in statistical literature is referred to as "mixed models" or "mixed effects models") (192). The random effects adjust the fixed effects for random variation, allowing levels and slopes of the patients' cytokines to vary. The nature of the data made us decide to incorporate random effects into the model specifications. This was due to the significant variation between the patients' initial values of the dependent variables, seen by visual inspection, and also due to the fact that repeated measurements in patients are dependent, the residuals from the same patients are more likely to be related than residuals from different patients (195). There was also significant random intercept variation in all multilevel analyses (95% confidence intervals did not include zero, and the estimates of random intercepts divided on the standard errors were above 2 SD, thus $p < 0.05$). Next, it was desirable to be able to generalize the results from this study to a broad psychiatric population suffering from the same conditions (e.g. to determine whether the patients characteristics may be common to PTSD patients in general). This is also one advantage of assessing fixed and random effects. A model with only fixed effects (i.e. intercept and slope) treats all patients as having one mean level and one mean slope, thus not allowing for variation between patients. This would probably not be a good approach, as a situation where different patients have the same levels and development rarely occurs in real life.

A random effects approach could include simply a random intercept, or it could include a random slope as well. A random intercept allows the patients' cytokine levels to vary across the first measurement. The random slope allows the patients' cytokine levels to differ across

time, taking every single patient's slope (i.e. development over time) into account, rather than treating all patients as having one mean cytokine level over time (i.e. a fixed slope). All these considerations were handled by analyzing the data with a stepwise comparison of models with random intercept and a model with random intercept and random slope. As these models were nested (i.e. the more complex model included all the effects of the simpler model), the fit of the models was assessed using likelihood ratio tests, and the -2 log likelihood test and the Bayes information criterion for the best model fit were presented in addition. The best model fit in Paper III was the model with random intercept. In Paper II, the most complex model had the best fit, i.e. random intercept and random slope.

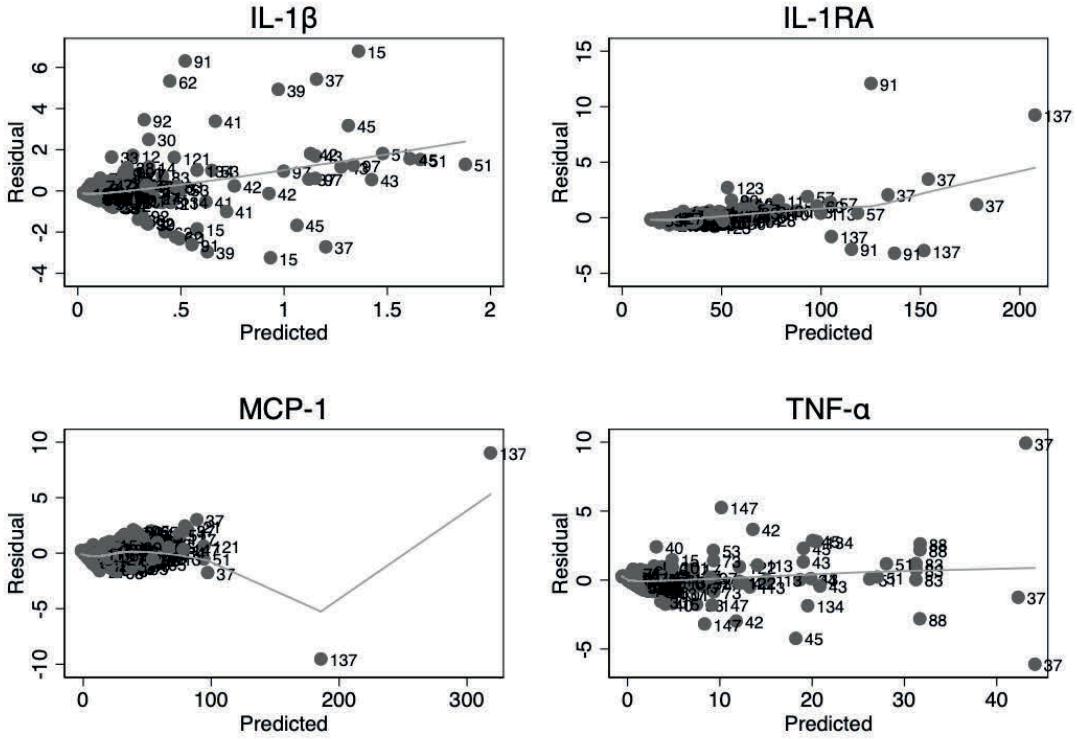
### 4.3.4 Outliers

Another assumption is that the errors (i.e. deviations of the observed value from the unobservable value, for example a population mean) must be normally distributed. To meet this assumption for Paper II, robust standard errors were applied, and in addition, all models were visually inspected for outliers using scatter plots. This resulted in the exclusion of four patients, as these patients had extreme levels in one cytokine each, biasing the predictions. We did not apply robust standard errors for Paper III, since the mean levels (obtaining the between-subjects effect) of the cytokines were computed, ultimately not resulting in outliers affecting the p-values, as was the case in Paper II. Further, the dependent variable of GSI scores in Paper III did not have any outlying residuals. Thus, it was more appropriate not to exclude the above-mentioned four patients in Paper III.

In the figures on the next page, the predicted estimates of each cytokine and of the GSI together with residuals are shown, all patients flagged with numbers.

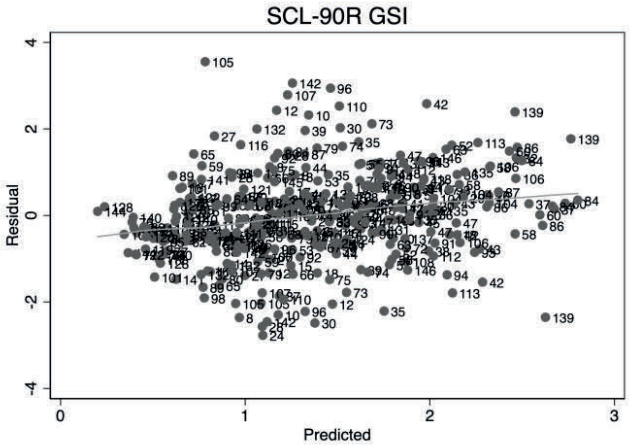
There were several interesting characteristics in these plots. The patients numbered 15, 37, 91 and 137 were removed from all analyses which treated cytokines as dependent variables, due to extreme divergence from the assumption of normally distributed residuals. There was also evidence of heteroscedastically distributed residuals in the cytokine scatter plots, as the residuals were not equally distributed around zero.

Figure 2. Predicted estimates and residuals of cytokines/chemokine




This was not apparent in the HSCL-90R GSI plot, where residuals showed no heteroscedastic distributions (below).

Figure 3. Predicted estimates and residuals of GSI

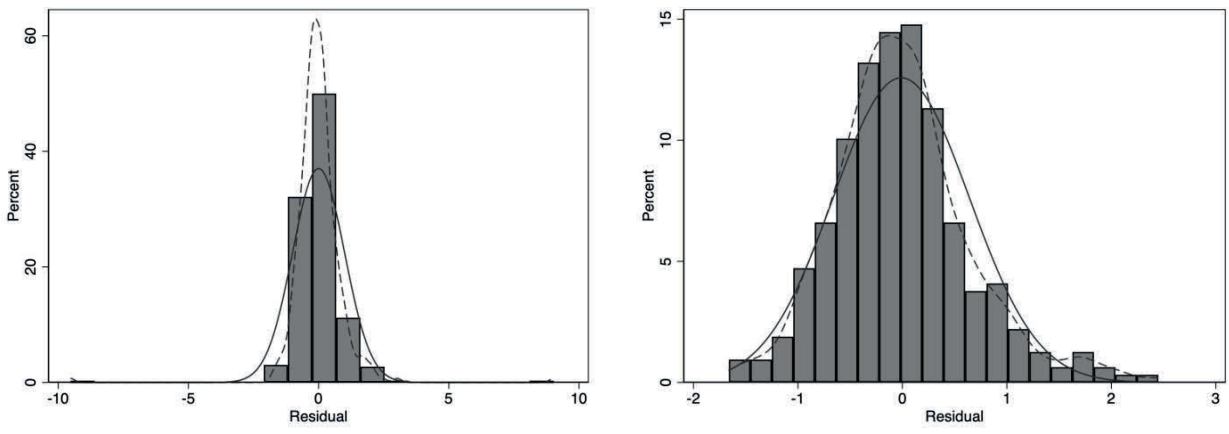



A multilevel regression analysis requires the residuals (i.e. differences between observed and estimated values) from the measurement points to be homoscedastically distributed, i.e. that the distance of the residuals to the regression line is approximately equal across time. The

presence of heteroscedastically distributed residuals was formally confirmed by likelihood ratio tests for IL-1β ($\chi^2(2) = 14.01$, p = < 0.001), IL-1RA ($\chi^2(2) = 55.02$, p = < 0.001), and TNF-α ($\chi^2(2) = 7.20$, p = 0.027). For MCP-1 ($\chi^2(2) = 3.27$, p = 0.195) and SCL-90R GSI ($\chi2(2) = 1.01$, p = 0.603), the non-significant p-values indicated that the assumption of homoscedastic residuals was met. Consequently, the analysis allowed for heteroscedastic distribution in IL-1β, IL-1RA and TNF-α.

Further, when plotting subject-specific predicted values, as done here, the expectation is that there should be no systematic trend in the plot. All residuals should hover around a horizontal line at zero. This could give rise to concern as to whether there is a quadratic effect in the predicted values. However, the predicted values in these plots are based in part on random effects, and the estimates of random effects and the estimates of the residuals are correlated. This phenomenon is known as residual confounding. Virtually any plot of multilevel models with random effects will produce a plot of residuals by predicted values with an apparent trend. This is simply a product of the way residuals and predicted values are computed. Therefore, the slightly upward trend is not necessarily problematic.
The exclusion of the four divergent cases resulted in a more normal distribution of residuals. To give one example, the following two histograms show the distribution of residuals in MCP-1 before (left) and after (right) exclusion of extreme observations.

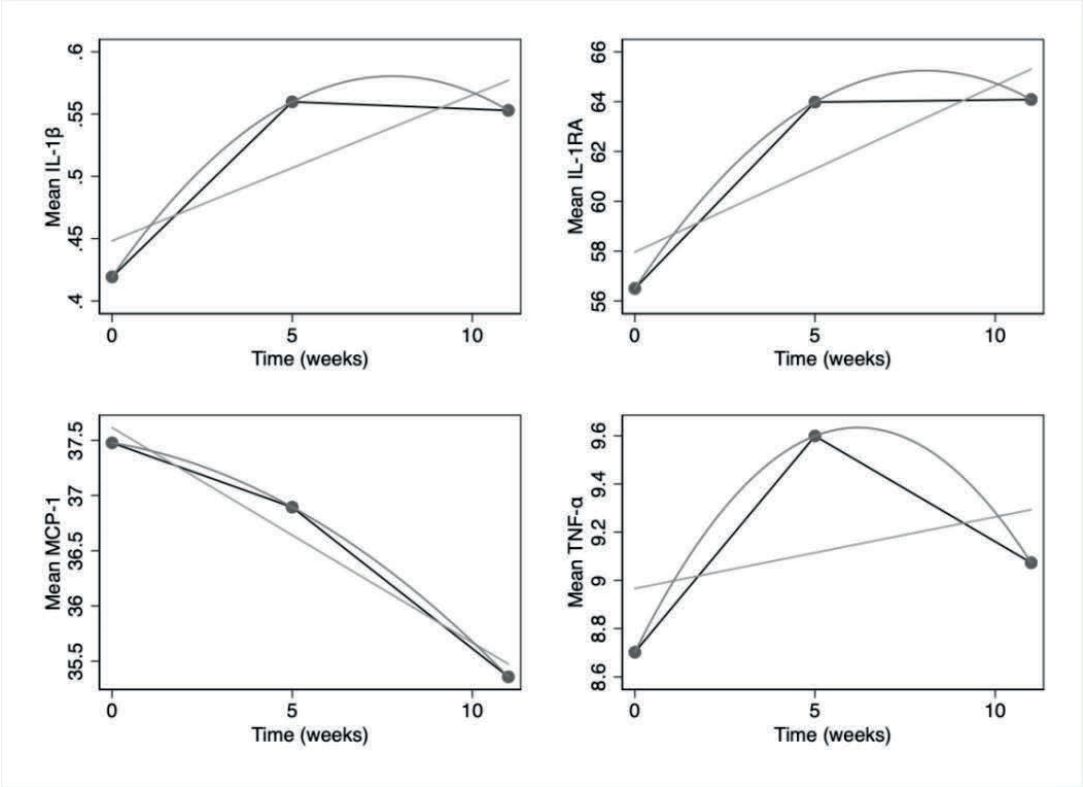
Figure 4. Normality plots of predicted values of MCP-1 before and after exclusion of outliers. Gauss curves and kernel density curves (dashed lines) showing the distribution shapes.

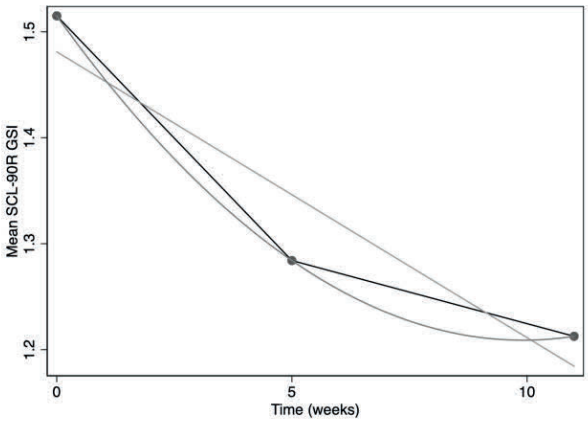


4.3.5 Linearity

Variables in mixed models also should be linear, i.e. “linear mixed effects models”. This implies that the value of X, when increasing by one, exerts an effect on Y which is linear in shape, thus either increasing or decreasing at a constant rate. Linearity was assessed by visually inspecting the predicted values using linear plots. Also, assumptions were tested formally by running linear models and quadratic models and comparing these using likelihood ratio tests. The first step included plotting the mean values of each dependent variable at each measurement point, together with a linear regression (“best fit”) line as well as a quadratic regression line. The benefit of the quadratic model is that it is appropriate for non-constant amounts of change in Y per unit change in X.

Figure 5. Mean values, linear regression and curvilinear regression of cytokines/chemokine and GSI across the treatment period

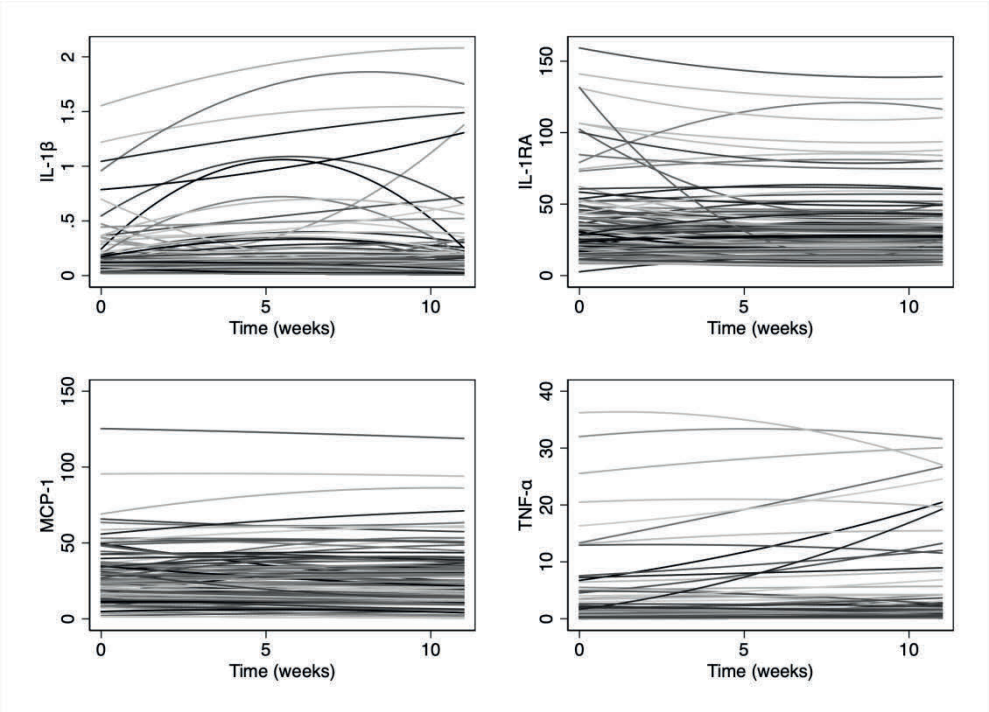


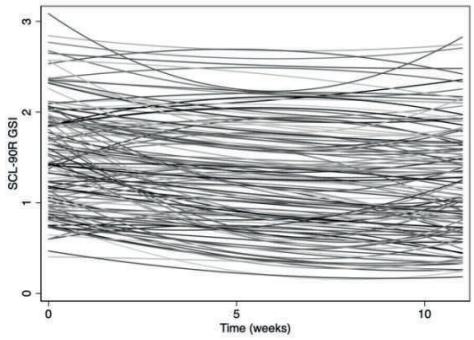



The plots above might indicate that the variables may not have been linear in relation to time, as seen from the curved lines. This issue was explored further by running multilevel models using a squared term for time (i.e. quadratic models). These multilevel models were non-significant for all cytokines, the chemokine MCP-1, and the GSI ($p = < 0.05$). The variance of the quadratic random effects was very small (data not shown), which indicated that it was probably not necessary to use a quadratic term.

The quadratic models were, however, visually inspected. The following plots are regression plots with quadratic predicted values in each patient across time.

Figure 6. Curvilinear (quadratic) regression lines of all patients across the treatment period






Likelihood ratio tests were conducted to formally assess whether the linear or quadratic model would fit the data better. The likelihood ratio test for IL-1β: $\chi^2(3) = 5.18$, $p = 0.159$. For IL-1RA: $\chi^2(4) = 45.49$, $p = < 0.001$. For MCP-1: $\chi^2(4) = 3.07$, $p = 0.546$. For TNF-α: $\chi^2(4) = 8.43$, $p = 0.038$. For GSI: $\chi^2(3) = 7.02$, $p = 0.071$. To conclude, the likelihood ratio tests favored the linear model for IL-1β, MCP-1 and GSI, and the quadratic model for IL-1RA and TNF-α. However, given the weak and non-significant main effects of the quadratic model (data not shown), and the somewhat modest degree of curvature in the quadratic regression plots (plots not shown), in addition to the fact that running the analysis with either linear or quadratic models did not significantly alter p-values (data not shown), it was decided to reject the more complex quadratic model and retain the parsimony of the linear model. The statistical package SPSS (SPSS version 23 for Windows (SPSS Inc., Chicago IL, USA) was used for all analyses in Paper I, while the statistical package STATA (StataCorp. 2015. Stata Statistical Software: Release 15. College Station, TX: StataCorp LP) was used for Papers II and III.

# 5 Results

## 5.1 Paper I. The effect of trauma and alcohol on the relationship between level of cytokines and depression among patients entering psychiatric treatment

The patients were stratified by trauma experience and by their level of depression. Patients with minimal/mild depression, and those with severe depression, had different levels of IL-1RA ($p = 0.046$ and $p = 0.047$, respectively). Also, the level of TNF-α was higher in the traumatized patients with severe depression ($p = 0.029$). There were no significant associations with depression score when stratifying by under and over cutoff on the AUDIT questionnaire. The patients' drinking severity, as measured by the AUDIT, differed across the three categories of the BDI-II. The highest AUDIT score was seen in the group with moderate depression ($p = 0.009$). When classifying the patients according to mood disorder, anxiety disorder, eating disorder and trauma history, the depression scores did not differ. There were however a higher number of patients using anti-inflammatory drugs, anti-depressives, and having trauma history in the severely depressed category. The IL-1β level was higher in the group without mood disorders than in those who had mood disorders ($p = 0.037$). The IL-1β level was higher in the patients with anxiety disorder when compared to those without anxiety disorder ($p = 0.008$). Both IL-1RA and TNF-α were higher in patients with a history of trauma than in those who had no trauma experience ($p = 0.048$ and $p = 0.033$, respectively). Finally, we compared the cytokines and the chemokine MCP-1 from 19 healthy, young, male volunteers with the 19 youngest male patients in our clinical sample. The chemokine MCP-1 and the cytokine TNF-α were significantly higher in patients ($p = 0.012$ and $p < 0.001$, respectively).

## 5.2 Paper II. PTSD patients show increasing cytokine levels during treatment despite reduced psychological stress

Levels of IL-1RA were higher in PTSD patients than in patients without PTSD ($p = 0.021$). The trajectory of IL-1β developed differently over time in PTSD patients compared to patients without PTSD ($p = 0.025$), as did the trajectories of MCP-1 ($p = 0.011$) and TNF-α ($p = 0.008$). In a bivariate analysis of the first measurement, performed shortly after admission to treatment, users of anti-inflammatory medications had higher levels of IL-1β ($p = 0.038$). The same patients had a tendency towards elevated levels of TNF-α ($p = 0.062$), and those with a PTSD diagnosis tended to have higher levels of IL-1RA ($p = 0.074$). Also, symptom severity, as measured by the GSI score, was subject to bivariate analysis to examine differences in levels between PTSD and non-PTSD patients at each time point. In addition, we ran bivariate analyses for each stratum separately, from $T_0$ to $T_1$, $T_1$ to $T_2$, and from $T_0$ to $T_2$, to assess simple, descriptive differences in levels. Levels between PTSD and no PTSD were different at $T_0$ ($p = 0.007$) and $T_2$ ($p = 0.001$). Patients without PTSD decreased from $T_0$ to $T_1$ ($p = 0.018$), from $T_1$ to $T_2$ ($p = 0.003$), and from $T_0$ to $T_2$ ($p < 0.001$). Patients with PTSD decreased from $T_0$ to $T_1$ ($p = 0.024$) and from $T_0$ to $T_2$ ($p = 0.051$).

In the general patient sample, users of anti-inflammatory drugs had higher levels of IL-1β ($p < 0.05$) than non-users. To further scrutinize the relationship between PTSD, cytokines and anti-inflammatory drugs, we stratified on PTSD and ran the anti-inflammatory drug variable as main effect and in interaction with time. Patients with PTSD who used anti-inflammatory drugs had different development of IL-1β ($p < 0.007$) and TNF-α than PTSD patients who did not use such drugs. Patients without PTSD who used anti-inflammatory drugs had different development of of MCP-1 than those who did not use such drugs ($p = 0.025$).

## 5.3 Paper III. Cytokine concentrations are related to level of mental distress, but not the development, in inpatients not using anti-inflammatory drugs

Patients who used anti-inflammatory drugs had higher levels of cytokine IL-1RA and chemokine MCP-1 than those who did not use anti-inflammatory drugs ($p < 0.001$ for MCP-1 and $p = 0.026$ for IL-1RA). There were also significantly more patients without PTSD and without anti-depressive drugs who were also not using anti-inflammatory drugs ($p < 0.001$ and $p = 0.008$, respectively).

In the multilevel analysis, we found a significant main effect of IL-1RA ($p = 0.005$), MCP-1 ($p = 0.020$) and having PTSD disorder ($p = 0.002$) on GSI. The patient's age was related to the slope of GSI, indicating that older patients improve more in mental distress over time. Stratifying on the use of anti-inflammatory drugs, we found no associations between any of the independent variables in neither level nor development of GSI in those who used such drugs. In the stratum who did not use such drugs, we found significant main effects of IL-1RA ($p = 0.023$), MCP-1 ($p = 0.018$) and having PTSD disorder ($p = 0.014$) on the GSI score. Time interaction did not show any significant results in either of the two strata, indicating that the PTSD and non-PTSD patients did not differ in GSI scores across time, and the cytokine/chemokine was not associated with GSI development across time. Next, we found that the simple slope of the GSI in patients not using anti-inflammatory drugs declined significantly throughout treatment, as it differed significantly from zero ($\beta = -0.03$, $SE = 0.005$, ($p < 0.001$). However, the simple slope of the GSI in the users of anti-inflammatory drugs did not significantly decline over time ($\beta = -0.01$, $SE = 0.01$, ($p = 0.149$).

Lastly, we further assessed the group of patients who did not use anti-inflammatory drugs by categorizing them by the level of IL-1RA and MCP-1, with their corresponding GSI score on the Y axis. The slopes of GSI scores declined throughout treatment for all patients, and the slopes differed from zero ($p < 0.001$).

## 5.4 Additional analyses

This section is dedicated to additional analyses that we did not see fit for the published papers. Some analyses are alternate versions of the published analyses.

In table 1, paper I, we categorized the BDI-II scores into three categories. The scores were classified as minimal/mild depression (score: 0-19), moderate depression (score: 20-28) and severe depression (score: 29-63). Statistical power is generally stronger when using continuous variables rather than dichotomized. That table is hereby presented with continuous BDI-II dependent variable and analyzed using the Mann-Whitney *U* Test for categorical independent variables and Spearman's rho correlation coefficient for the continuous independent variables.

**Table 1. Clinical characteristics of the included patients according to level of depression. Depression severity was measured by the Beck Depression Inventory-II**

| **Variable** | | **BDI-II** | |
|---|---|---|---|
| **Demography** | **n** | **Median** | **P-value** |
| Women | 82 | 29 | 0.306 |
| Men | 35 | 26 | |
| Age (years) [a] | 117 | 44 (0.023) | 0.810 |
| **Alcohol use** | | | |
| AUDIT score [a+b] | 99 | 3 (-0.240) | 0.021* |
| **Medication** | | | |
| Anti-inflammatory drugs (yes) | 16 | 31.5 | 0.706 |
| Anti-inflammatory drugs (no) | 101 | 28 | |
| Anti-depressants (yes) | 36 | 31 | 0.453 |
| Anti-depressants (no) | 81 | 27 | |
| **History of trauma** | | | |
| Childhood trauma (yes) | 57 | 27 | 0.311 |
| Childhood trauma (no) | 46 | 31 | |
| Adult trauma (yes) | 55 | 29 | 0.528 |
| Adult trauma (no) | 48 | 28 | |
| Any trauma (yes) | 78 | 28.5 | 0.528 |
| Any trauma (no) | 26 | 28.5 | |
| **Main diagnosis [d]** | | | |
| Mood disorder (yes) | 30 | 24.5 | 0.009** |

| | | | |
|---|---|---|---|
| Mood disorder (no) | 73 | 31 | |
| Anxiety disorder (yes) | 51 | 29 | 0.456 |
| Anxiety disorder (no) | 52 | 28 | |
| Eating disorder (yes) | 17 | 38 | 0.028* |
| Eating disorder (no) | 86 | 26.5 | |
| MDD (yes) [e] | 11 | 23 | 0.098 |
| MDD (no) | 106 | 29 | |

Notes: [a] Spearman´s rho correlation coefficient for continuous independent variables. [b] AUDIT: Alcohol Use Disorders Identification Test. [c] Mood disorders: ICD-10 F30-34. Anxiety disorders: ICD-10 F40-F44. Eating disorders ICD-10: F50-F50.3. [d] MDD: Major Depression Disorder, classified by the International Statistical Classification of Diseases and Related Health Problems-10 (ICD-10). * = P-value < 0.05. ** = P-value < 0.01. No p-values were significant after applying Bonferroni correction with corrected p-value of 0.004.

AUDIT is negatively correlated with BDI-II score (p = 0.021), meaning that a more heavy drinking pattern is associated with being less depressed. This is in line with the categorical BDI-II analysis in table 1, paper I, where AUDIT was different across the three categories of BDI-II. According to the BDI-II questionnaire, those with mood disorders were less depressed than those who did not have mood disorders (p = 0.009). Those with eating disorders were more depressed than those who did not have eating disorder (p = 0.028). Applying Bonferroni correction by dividing the 12 hypothesis tests on alpha level of 0.05 gives an adjusted p-value at 0.004, leaving no significant findings.

In the three papers we excluded four cytokines. These were IL-6 (median = 0, mean = 2.45, SD = 9.60), IL-10 (median = 0, mean = 3.29, SD = 14.12), IL-17A (median = 0, mean = 0.27, SD = 1.29), and IFN-γ (median = 0, mean = 2.19, SD = 9.06). However, instead of excluding them, these cytokines could have been dichotomized into non-detectable and detectable and analyzed using Pearson's chi-square test. This approach, similar to table 2 in paper I, only with dichotomization of the four excluded cytokines, is presented in table 2 (next page):

**Table 2. Frequencies and porportions of patients with detectable and non-detectable levels of cytokines according to clinical characteristics of the included patients.**

| Variable | IL-6 | | | IL-10 | | | IL-17A | | | IFN-γ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Not detectable | Detectable | P-value | Not detectable | Detectable | P-value | Not detectable | Detectable | P-value | Not detectable | Detectable | P-value |
| | n (%) | | | n (%) | | | n (%) | | | n (%) | | |
| **Demography** | | | | | | | | | | | | |
| Men [a] | 22 (31.4 %) | 14 (25.5 %) | 0.464 | 19 (25.3 %) | 17 (34.0 %) | 0.295 | 34 (28.8 %) | 2 (22.2 %) | 1.000 | 33 (29.5 %) | 3 (20.0 %) | 0.553 |
| Women | 48 (68.6 %) | 41 (74.6 %) | | 56 (74.7 %) | 33 (66.0 %) | | 84 (71.2 %) | 7 (77.8 %) | | 79 (70.1 %) | 12 (80.0 %) | |
| Age (years, SD) [b] | 40.2 (12.1) | 44.3 (10.8) | 0.066 | 41 (11.4) | 43.5 (12.1) | 0.224 | 41.9 (11.6) | 40.6 (13.0) | 0.612 | 42.2 (11.6) | 39.1 (12.1) | 0.374 |
| **Psychometrics** | | | | | | | | | | | | |
| **AUDIT** [c] | | | | | | | | | | | | |
| Under cutoff score | 38 (69.1 %) | 33 (70.2 %) | 0.902 | 39 (65 %) | 32 (76.2 %) | 0.227 | 69 (70.4 %) | 3 (50.0 %) | 0.293 | 68 (71.6 %) | 4 (44.4 %) | 0.092 |
| Over cutoff score | 17 (30.9 %) | 14 (29.8 %) | | 21 (35 %) | 10 (23.8 %) | | 29 (29.6 %) | 3 (50.0 %) | | 27 (28.4 %) | 5 (55.6 %) | |
| **BDI-II** [d] | | | | | | | | | | | | |
| Minimal/mild | 11 (15.7 %) | 15 (27.7 %) | | 14 (18.7 %) | 12 (24 %) | | 24 (20.3 %) | 3 (33.3 %) | | 21 (18.8 %) | 6 (40.0 %) | |
| Moderate | 28 (40.0 %) | 14 (25.5 %) | 0.137 | 26 (34.7 %) | 16 (32 %) | 0.771 | 40 (33.9 %) | 2 (22.2 %) | 0.603 | 40 (35.7 %) | 2 (13.3 %) | 0.111 |
| Severe | 31 (44.3 %) | 26 (47.3 %) | | 35 (46.7 %) | 22 (44 %) | | 54 (45.8 %) | 4 (44.4 %) | | 51 (45.5 %) | 7 (46.7 %) | |
| **Medication** | | | | | | | | | | | | |
| **Anti-inflammatory** | | | | | | | | | | | | |
| No | 60 (85.7 %) | 46 (83.6 %) | 0.748 | 66 (88 %) | 40 (80 %) | 0.222 | 100 (84.8 %) | 8 (88.9 %) | 0.737 | 95 (84.8 %) | 13 (86.7 %) | 1.000 |
| Yes | 10 (14.3 %) | 9 (16.4 %) | | 9 (12 %) | 10 (20 %) | | 18 (15.3 %) | 1 (11.1 %) | | 17 (15.2 %) | 2 (13.3 %) | |
| **Anti-depressants** | | | | | | | | | | | | |
| No | 45 (64.3 %) | 39 (70.9 %) | 0.434 | 50 (66.7 %) | 34 (68 %) | 0.876 | 79 (67.0 %) | 5 (55.6 %) | 0.486 | 74 (66.1 %) | 10 (66.7 %) | 1.000 |
| Yes | 25 (35.7 %) | 16 (29.1 %) | | 25 (33.3 %) | 16 (32 %) | | 39 (33.1 %) | 4 (44.4 %) | | 38 (33.9 %) | 5 (33.3 %) | |
| **History of trauma** | | | | | | | | | | | | |
| **Childhood** | | | | | | | | | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 32 (50.0 %) | 17 (37.0 %) | 0.175 | 39 (57.4 %) | 10 (23.8 %) | 0.001 *** | 47 (45.2 %) | 2 (25.0 %) | 0.267 | 46 (46.9 %) | 3 (21.4 %) | 0.089 |
| Yes | 32 (50.0 %) | 29 (63.0 %) | | 29 (42.7 %) | 32 (76.2 %) | | 57 (54.8 %) | 6 (75.0 %) | | 52 (53.1 %) | 11 (78.6 %) | |
| **Adulthood** | | | | | | | | | | | | |
| No | 32 (49.2 %) | 19 (42.2 %) | 0.469 | 31 (45.6 %) | 20 (47.6 %) | 0.151 | 47 (45.2 %) | 5 (62.5 %) | 0.636 | 49 (49.5 %) | 3 (23.1 %) | 0.084 |
| Yes | 33 (50.8 %) | 26 (57.8 %) | | 37 (54.4 %) | 22 (52.4 %) | | 57 (54.8 %) | 3 (37.5 %) | | 50 (50.5 %) | 10 (76.9 %) | |
| **Any trauma** | | | | | | | | | | | | |
| No | 21 (32.3 %) | 8 (17.4 %) | 0.078 | 21 (30.9 %) | 8 (18.6 %) | 0.151 | 28 (26.7 %) | 1 (12.5 %) | 0.377 | 28 (28.3 %) | 1 (7.1 %) | 0.111 |
| Yes | 44 (67.7 %) | 38 (82.6 %) | | 47 (69.1 %) | 35 (81.4 %) | | 77 (73.3 %) | 7 (87.5 %) | | 71 (71.7 %) | 13 (92.9 %) | |
| **Main diagnosis** | | | | | | | | | | | | |
| **Mood disorder** | | | | | | | | | | | | |
| No | 41 65.1 %) | 38 (80.9 %) | 0.069 | 52 (76.5 %) | 27 (64.3 %) | 0.168 | 74 (71.2 %) | 6 (75.0 %) | 1.000 | 67 (68.4 %) | 13 (92.9 %) | 0.065 |
| Yes | 22 (34.9 %) | 9 (19.2 %) | | 16 (23.5 %) | 15 (35.7 %) | | 30 (28.9 %) | 2 (25.0 %) | | 31 (31.6 %) | 1 (7.14 %) | |
| **Anxiety disorder** | | | | | | | | | | | | |
| No | 39 (61.9 %) | 17 (36.2 %) | 0.008** | 35 (51.5 %) | 56 (50.9 %) | 0.881 | 53 (51.0 %) | 4 (50.0 %) | 1.000 | 54 (55.1 %) | 3 (21.4 %) | 0.023* [d] |
| Yes | 24 (38.1%) | 30 (63.8 %) | | 33 (48.5 %) | 54 (49.1 %) | | 51 (49.0 %) | 4 (50.0 %) | | 44 (44.9 %) | 11 (78.6 %) | |
| **Eating disorder** | | | | | | | | | | | | |
| No | 48 (76.2 %) | 40 (85.1 %) | 0.247 | 51 (75 %) | 37 (88.1 %) | 0.095 | 84 (80.1 %) | 6 (75.0 %) | 0.654 | 79 (80.6 %) | 11 (78.6 %) | 1.000 |
| Yes | 15 (23.8 %) | 7 (14.9 %) | | 17 (25 %) | 5 (11.9 %) | | 20 (19.2 %) | 2 (25.0 %) | | 19 (19.4 %) | 3 (21.4 %) | |

Notes: [a] Categorical variables analyzed with Pearson's chi-square test. Fisher's exact test for values below 5. [b] Mann-Whitney *U* Test. [c] AUDIT: Alcohol Use Disorders Identification Test. [d] BDI-II: Beck Depression Inventory-II. * Significant at the 0.05 level. **Significant at the 0.01 level. *** Significant at the 0.001 level. Bonferroni-corrected p-value = 0.001, childhood traumaand IL-10 are associated after applying Bonferroni correction.

Patients with detectable levels of IL-6 and IFN-γ were associated with having anxiety disorder (p = 0.008 and p = 0.023, respectively). Patients with detectable levels of IL-10 were associated with having experienced childhood trauma (p = 0.001). Further, there are 48 hypothesis tests in table 2 (above). Applying Bonferroni correction by dividing the alpha level of 0.05 on 48 gives corrected p-value of 0.001. With the adjusted p-value of 0.001, IL-10 and the association with childhood trauma is still significant. Further, patients with detectable levels of IL-6 interestingly nearly reached significance in having experienced either adulthood or childhood (or both) trauma (p = 0.078). Not finding significant results here could be due to small group sizes, as there were 29 with no trauma and 82 with trauma experience, possibly a type II error.

In paper I, table 3, we reported mean values. It would have been better to report the median values, as the values were skewed and since we used the Mann-Whitney *U* test to analyze differences between the groups. The median values are presented in the following table:

**Table 3.** Median age ($25^{th}$, $75^{th}$ percentile ) and median levels ($25^{th}$, $75^{th}$ percentile) of peripheral circulating cytokines in healthy volunteers and matched patients

| Variable | | Matched patients n = 19 | Healthy volunteers n = 19 | P-value |
|---|---|---|---|---|
| Age | (years) | 45 (35, 48) | 27 (26, 29) | <0.001 |
| IL-1β | (picograms/mL) | 0.003 (0.003, 0.140) | 0.080 (0.002, 0.130) | 0.977 |
| IL-1RA | (picograms/mL) | 38.800 (18.740, 51.090) | 26.670 (22.530, 52.420) | 0.729 |
| TNF-α | (picograms/mL) | 0.180 (0.046, 0.910) | 0.035 (0.035, 0.035) | <0.001 |
| MCP-1 | (picograms/mL) | 34.530 (23.290, 54.600) | 12.580 (0.213, 27.010) | <0.001 |

Notes: [a] Mann-Whitney *U* test. [b] Patients and healthy volunteers were male.

All three findings were still significant after applying Bonferroni correction with corrected p-value at 0.01. The corrected p-value was found by dividing the alpha level of 0.05 on 5 hypothesis tests. The results were the same as in paper I.

In paper II, table 1, the cytokines/chemokine were presented by mean values with SD and compared between patient groups with the non-parametric analysis Mann-Whitney *U* Test and Kruskal-Wallis one-way analysis of variance for the continuous age variable. Since the cytokines/chemokine were skewed, they should have been presented by medians and $25^{th}$ and $75^{th}$ percentiles. This strategy is presented in table 4 (next page).

**Table 4.** The association between sample characteristics and distribution of cytokine levels at $T_0$.

| Variables | n | IL-1β (pg/mL) Median (25th and 75th percentile) | P-value [a] | IL-1RA (pg/mL) Median (25th and 75th percentile) | P-value | MCP-1 (pg/mL) Median (25th and 75th percentile) | P-value | TNF-α (pg/mL) Median (25th and 75th percentile) | P-value |
|---|---|---|---|---|---|---|---|---|---|
| Gender | | | | | | | | | |
| Female | 87 | 0.003 (0.003, 0.160) | 0.698 | 27.030 (17.430, 39.030) | 0.728 | 20.780 (11.400, 31.920) | 0.046 * | 0.360 (0.046, 2.450) | 0.487 |
| Male | 36 | 0.003 (0.003, 0.130) | | 26.220 (18.090, 45.650) | | 27.675 (15.945, 42.020) | | 0.080 (0.046, 1.370) | |
| Age | | | | | | | | | |
| 18-29 | 25 | 0.003 (0.003, 0.120) | | 20.800 (16.77, 27.84) | | 21.110 (14.950, 38.290) | | 0.046 (0.046, 0.970) | |
| 30-49 | 62 | 0.003 (0.003, 0.140) | 0.584 [b] | 29.750 (18.410, 42.280) | 0.256 | 21.915 (9.790, 33.170) | 0.599 | 0.335 (0.046, 1.410) | 0.425 |
| 30-66 | 36 | 0.055 (0.003, 0.150) | | 24.065 (19.520, 41.175) | | 24.060 (13.815, 37.690) | | 0.615 (0.046, 2.920) | |
| GSI | | | | | | | | | |
| Under 0.85 | 25 | 0.003 (0.003, 0.120) | 0.554 | 28.340 (18.740, 38.800) | 0.945 | 23.010 (13.150, 40.380) | 0.642 | 0.420 (0.046, 1.740) | 0.935 |
| Above 0.85 | 98 | 0.012 (0.003, 0.140) | | 26.700 (17.430, 41.930) | | 22.465 (13.280, 33.440) | | 0.140 (0.046, 2.120) | |
| Anti-inflammatory drugs | | | | | | | | | |
| No | 104 | 0.003 (0.003, 0.130) | 0.038 * | 27.275 (17.435, 41.995) | 0.687 | 22.770 (13.215, 33.305) | 0.732 | 0.080 (0.046, 1.780) | 0.062 |
| Yes | 19 | 0.090 (0.003, 0.300) | | 24.770 (17.460, 34.310) | | 22.210 (10.680, 38.580) | | 1.020 (0.060, 3.410) | |
| Anti-depressive drugs | | | | | | | | | |
| No | 83 | 0.030 (0.003, 0.160) | 0.490 | 27.520 (16.770, 42.060) | 0.602 | 24.340 (14.710, 37.470) | 0.121 | 0.360 (0.046, 2.450) | 0.276 |
| Yes | 40 | 0.003 (0.003, 0.130) | | 26.700 (20.410, 40.665) | | 19.245 (10.505, 31.275) | | 0.060 (0.046, 0.945) | |
| PTSD diagnosis | | | | | | | | | |
| No | 69 | 0.003 (0.003, 0.130) | 0.524 | 23.960 (17.440, 34.920) | 0.074 | 23.290 (14.710, 33.440) | 0.613 | 0.100 (0.046, 1.820) | 0.492 |
| Yes | 39 | 0.003 (0.003, 0.200) | | 31.990 (20.290, 51.090) | | 22.820 (7.750, 40.380) | | 0.420 (0.046, 2.560) | |

Notes: [a] P-values for comparisons of mean ranks computed with the Mann-Whitney *U* Test. [b] P-values for the age variable were computed with the Kruskal-Wallis one-way analysis of variance. * = $p < 0.05$. Abbreviations: GSI = Global Severity Index. PTSD = Posttraumatic stress disorder.

The levels of IL-1β were higher in those who used anti-inflammatory drugs compared to those who did not ($p = 0.038$). The male patients had higher levels of MCP-1 compared to the female patients ($p = 0.046$). Applying Bonferroni correction on table 4 by dividing the alpha level of 0.05 on 24 hypothesis tests gives a corrected p-value of 0.002. This renders both findings not significant.

In paper II, we did not report the simple slopes of the cytokine trajectories in the PTSD and non-PTSD stratas. The multilevel analyses of the dichotomous PTSD variable (PTSD/non-PTSD) in interaction with time, as reported in paper II, showed the differences between the slopes in the two stratas, with the PTSD patients having the higher levels of cytokines (as seen in figure 2, paper II). However, it is also of interest to report simple slope analyses of the PTSD/no PTSD variable with time interaction on cytokine levels in the stratas without the comparison with other groups of patients. In this way, one can examine the strength of the relationship between cytokines and PTSD/no PTSD moderated by time. The significant results of these analyses are:

- The simple slope of TNF-α in patients with PTSD was significantly different from zero ($\beta = 0.20$, $SE = 0.079$, $p = 0.011$), indicating that TNF-α in this stratum significantly increased across time at a rate of 0.20 pg/mL from $T_0$ to $T_2$.
- The simple slope of IL-1β in patients with PTSD was significantly different from zero ($\beta = 0.01$, $SE = 0.006$, $p = 0.026$), indicating that IL-1β in this stratum significantly increased across time at a rate of 0.01 pg/mL from $T_0$ to $T_2$.
- The simple slope of MCP-1 in patients without PTSD was significantly different from zero ($\beta = -0.32$, $SE = 0.135$, $p = 0.016$), indicating that MCP-1 in this stratum significantly declined across time at a rate of 0.32 pg/mL from $T_0$ to $T_2$.
- The simple slope of IL-1RA in patients without PTSD was significantly different from zero ($\beta = -0.28$, $SE = 0.139$, $p = 0.041$), indicating that IL-1RA in this stratum significantly declined across time at a rate of 0.28 pg/mL from $T_0$ to $T_2$.

The simple slopes of MCP-1 and IL-1RA decreased significantly in the patients without PTSD (described above). Together with the declining levels of GSI seen in this group (presented in paper II), these findings are in line with the notion of successful psychiatric treatment corresponding with a reduced pro-inflammatory state (101, 198).

Further, we could also have dichotomized and analyzed the excluded cytokines using mixed models with time-varying HSCL-90R GSI as dependent variable, similar to what was conducted for continuous variables IL-1RA and MCP-1 in paper III. However, no significant results were found neither for main effects nor interaction with time. This means that the patients with detectable cytokine levels did not differ in their reported psychiatric symptom severity on average or in development over time when compared to patients with non-detectable cytokine levels. Main effects of IL-17A: $\beta = 0.161$, SD = 0.128, $p = 0.211$. IL-10: $\beta = 0.046$, $p = 0.554$. IL-6: $\beta = 0.064$, SD = 0.077, $p = 0.408$. IFN-γ: $\beta = 0.054$, SD = 0.089, $p = 0.543$. Interaction with time: IL-17A: $\beta = -0.001$, SD= 0.020, $p = 0.945$. IL-10: $\beta = -0.003$, SD = 0.010, $p = 0.721$. IL-6: $\beta = -0.004$, SD = 0.009, $p = 0.692$. IFN-γ: $\beta = 0.010$, SD = 0.016, $p = 0.548$.

# 6 Discussion

## 6.1 Methodological considerations

### 6.1.1 Study design

Interpretation and discussion of results must always take place on the basis of a careful assessment of the methodologies used. There are several strengths of the current study. The longitudinal design with multilevel models provides information on the development of cytokines and psychiatric symptoms over time. It takes into account that the measurements are not independent, they are correlated, patients with missing values are still included in the model, and the random effects allow for differences in levels and in rate of change that remains unexplained by covariates (192). The longitudinal design with mixed effects models allows for assessing associations between cytokines and psychiatric symptoms across time, but does not allow for concluding on causality. The way this study was conducted gives an impression of how cytokine levels are associated with scores of GSI (Paper III), and vice versa (Paper II) across time. This is a main strength of the study, as it gives insight into how psychiatric symptoms and the immune system correlate during psychological treatment. However, this study is a naturalistic observation study. This means that the environment was not manipulated in any way for the purpose of research, and treatment was not interfered with. This paves the way for confounders, and one cannot draw conclusions on causality.

Although other researchers have assessed the effect of cytokines on mental distress across time, we are unaware of other studies having utilized multilevel models with three measure points across treatment and disentangled the within-subjects and between-subjects effects from the total effects (195). Further, we are unaware of other studies using GSI score as time-varying dependent variable, as well as the associations of the particular cytokine/chemokine IL-1RA and MCP-1. These factors could be regarded as strengths.

It is currently not known whether psychiatric disorders precipitate elevated circulating cytokines or if elevated cytokines precipitate psychiatric disorders. We had no information regarding how long the patients had been sick prior to enrolment at Modum Bad. Neither did

we have previous cytokine samples to compare with. Preferably, this study would have had cytokine measurements and psychometric information from before enrolment. This would possibly have provided more insight into associations between immunology and mental distress.

We excluded 19 patients with cytokine values above the 95$^{th}$ percentile. This approach reduced statistical power and it might have removed interesting findings as well. On the other hand, if those 19 patients with high levels had been included, the findings could have been argued to be confounded by somatic infections. Another weakness was that 16 patients from the Depression ward had their blood samples collected between 12 and 15 pm. Drawing the blood at various time points is probably not a good idea, as cytokines are known to follow circadian rhythms (199). The time of the day affects the kind of cytokines produced, as immune cells possess intrinsic clocks that controls differentiation, development and migration (200). The circadian oscillations are strong in humans, with both the adaptive and the innate immune system peaking at night time during the behavioral resting phase (201). As such, these 16 patients might have had different cytokine levels due to circadian rhytm.

### 6.1.2 Selection bias

Of the 249 patients who were approached, 147 (59%) participated. Of the 102 (41%) patients who chose not to participate in the study, there were 81 women (79%, mean age 36.76 years, SD 11.85) and 21 men (21%, mean age 45.05 years, SD 8.60). The Pearson Chi-Square test showed that the gender distribution in those who did and did not participate was equally distributed ($\chi 2 = 0.699$ (1), $p = 0.403$). The mean age of those who participated was 41.86 years and 38.59 years in those who did not participate. An independent samples T-test showed that the mean age differed between those who did and did not participate ($T = -2.07$, $p = 0.040$). Thus, the age of those who participated was higher, and higher age has been associated with higher basal inflammation level (202), possibly representing a bias between participators and non-participators.

It is reasonable to believe that many of the patients who declined to participate had a higher symptom load than those who did participate, as greater total burden was one reason for not wanting to participate. As we did not succeed in including patients with higher symptom load,

we might have lost patients who could have provided the study with important immunological and psychological information. This is a selection bias too (203, 204).

In the first paper, we utilized a group of 20 young men as healthy volunteers to compare cytokines with the patient sample (163). In our analysis, we excluded one healthy volunteer due to having extreme level of TNF-α. The healthy sample was a convenience sample not particularly designed for comparison with our patients. This group was chosen since we did not have any other control group readily available. However, we tried to minimize the selection bias by comparing the healthy volunteers with the youngest 19 men of the patient sample. Selection bias potentially occurs whenever procedures for selection are applied (205). This kind of bias is common in studies where the convenience sampling method is utilized. The associations between cytokines and psychiatric symptoms will depend upon the kind of people included in the study.

### 6.1.3 Information bias

Systematic errors while obtaining data, as well as when assigning responses into categories, may result in information bias. Two common types of systematic errors are recall bias and misclassification (206), which are both likely sources of bias in most studies, this study being no exception. Patients in the study had been mentally ill for several years, many of them with childhood traumatic experiences. Trauma and depression may lead to cognitive distortions negatively affecting individuals' ability to retrieve specific memory details (207). Indeed, the passing of years is in itself an important factor which contributes to fading memories, ultimately paving the ground for recall bias. The AUDIT questionnaire is based on an individual's drinking pattern for the past year, which suggests the likelihood that the AUDIT results may be prone to recall bias. The trauma questions in the clinical interviews concerned traumatic events from childhood as well as in adulthood. This suggests a strong possibility of recall bias in these questions. However, the HSCL-90R and BDI-II questionnaires only focus on the last week and the past two weeks, respectively. The recall bias is thus likely to be rather low.

Misclassification is a type of bias which might occur when dealing with categorical variables, and the term misclassification has several interpretations. Misclassification occurs when data is collected and categorized (208). In ordinal variables, where the alternative responses are ordered, the respondent might not feel that the alternatives truly reflect his or her current state. This may result in misclassification, i.e. the patient answers in a way which does not reflect the truth. Furthermore, patients might wish to show gratitude and please the staff at the facility, and thus might underreport their symptom severity in for instance the BDI-II or the HSCL-90R GSI questionnaire. The converse is also possible; some might see it as beneficial to report a high degree of symptom severity at discharge, in order to get prolonged sick leave and continued social security benefits. Stigma is also a factor which might lead to underreporting of actual symptom severity. Patients might think people expect a health benefit from having received treatment, and respond accordingly in questionnaires (209). Another form of misclassification (information bias) is the possibility of misunderstanding the self-reporting questionnaires. People who are not trained in using psychometric questionnaires might understand the instructions differently than trained personnel. Literature suggests that self-report assessments produce different results than professionally administered interviews (210). A recent study found that healthy people scored themselves higher in the BDI-II questionnaire when first assessed with self-report, followed by interview-based questionnaires after a short break. On average, a difference of 6 points was found. However, the sample were older (60-87 years, mean age = 67.9 years, SD = 6.6) than patients in the current thesis, and were also regarded as healthy, thus not easily comparable to patients in the current study (210). Little training is required to use the BDI-II, and this is commonly presented as one of its advantages (211).

### 6.1.4 Reliability of laboratory analyses

Reliability refers to the level to which the measurements provide a consistent result whenever repeated. The blood samples were drawn mainly by one person, with additional assistance provided by the bioengineer employed at the laboratory at Modum Bad for a few patients (< 10) who were hard to venipuncture. The bead-based Luminex technology used at Ullevål Hospital, i.e. the Bio-Plex xMAP technology (Bio-Rad, Austin, TX, USA) with a Luminex IS 100 instrument, was utilized for analyzing the serum blood samples. All samples were stored

at -80° C, handled and analyzed by a single employee in the laboratory at Ullevål Hospital. This ensured that all analyses were treated in the same way, assuring a high degree of reliability (212). Several of the cytokines we measured came out with a high amount of undetectable values, i.e. the patient's level was under the LOD. This represents a limitation to the interpretation of results of paper I and II. However, in paper II we chose to keep only IL-1RA and MCP-1, which had almost no zero values.

### 6.1.5 Confounders

There is always a probability of the presence of other, non-measured factors (i.e. a "third variable") confounding the results (213). A confounder is a variable associated with both the independent variable and the outcome variable, which might alter the outcome (214). A history of smoking, as well as current smoking, has in some studies been found to promote pro-inflammatory cytokine production (215, 216). Smoking cigarettes has also been reported to be associated with depression and anxiety (217). These findings taken together suggest that smoking may moderate the relationship between inflammation and mental distress. BMI is also a confounding factor. Adipocytes are known to produce pro-inflammatory markers, adipokines, and the increased secretion of adipokines due to overnutrition is involved in inflammation (218). The patient's weight was not measured, and we do not know if the patients were fasting or had eaten breakfast when blood samples were collected. These issues are limitations to the study. Other confounders in this study is gender, age, and using anti-inflammatory and anti-depressive drugs, all factors which are known to exert an effect on inflammation.

One way of dealing with known confounders is by stratifying on the confounder, as was done in Paper III on those who did and did not use anti-inflammatory drugs. However, some of the strata were rather small in sample size, limiting the application of the results, and interpretation should be done with caution. Stratification deflates the statistical power, increasing the chance of type II errors (explained below). These facts taken together, one might criticize the thesis for possibly not describing what it was intended to describe, i.e. having low internal validity (219), and possibly be influenced by confounding bias.

### 6.1.6 Internal validity

Internal validity refers to how well one can trust that the independent variables actually were the variables which affected the changes in the outcome variables. In this doctoral thesis, the multilevel regression model included the time variable and one covariate variable, and were not adjusted for other variables. However, we tested running the regression models in paper III with adjustment for sex and age, these analyses are included as supplementary table 5 in that paper. Stratified on those not using anti-inflammatory drugs, no significant associations were found (exactly as table 2b in paper III). Stratified on those using anti-inflammatory drugs, only main effect of PTSD was significant ($p = 0.028$). We considered this study to be exploratory by nature, and chose to focus on the predictor variables one at a time to gain a clear picture of the nature of the relationship between the cytokines and GSI.

The questionnaires HSCL-90R and BDI-II are known to have a high degree of validity and reliability (181, 220, 221). The patients in this study had been diagnosed by professionals both in other treatment settings before admission, and tentatively at the one-week pre-clinical assessment stay at the Modum Bad facility, and diagnosis was further assessed during the inpatient stay. The patients filled out well-known psychometric questionnaires as part of the treatment evaluation. This strengthens the validity of the study, as these patients could be regarded as the correct kind of population when assessing symptoms of anxiety, depression, trauma and eating disorders. As described in the methods section, the Cronbach´s alpha for BDI-II and HSCL-90R GSI ranged from 0.91 to 0.98 in the current study.

### 6.1.7 Type I and II errors and Bonferroni correction

All three papers contain a number of comparisons with several hypotheses being tested. This increases the chance of getting false positives (i.e. type I errors, α, alpha). Having too low statistical power might result in false negatives (i.e. type II errors, β, beta). Researchers often correct the issue of type I error by applying Bonferroni correction or by implementing false discovery rate control (222). Correcting for multiple hypothesis testing has been criticized for being unnecessary, as the α and β error rates are considered to be valid in the long run (223). The α error level is the 5% significance level, which indicates that it is acceptable to have a 5% probability of incorrectly rejecting the null hypothesis (type I error). Rothman has stated

that scientists should not penalize themselves by missing out on possibly important findings, and not be so reluctant to explore leads which may turn out to be wrong (223). In fact, Rothman stated that it is preferable not to make corrections for multiple hypothesis testing when data are actual observations on nature, and not random numbers (223). Rothman is not the only one to have this opinion, others share his point of view, it has been remarked that adjusting for multiple testing is a serious issue since it determines whether a result is significant or not (224). Thomas Perneger stated that researchers know that in their career they will reject an amount of true null hypotheses, as well as fail to find true alternative hypotheses, and that this is unrelated to the number of tests (224). Others have stated that the use of Bonferroni correction reduces power, it may increase the type II errors to unacceptable levels, it may contribute to publication bias, and it may ultimately contribute to slowing down the advance of the scientific field, as some reviewers would recommend not to publish if Bonferroni is not applied (225). Other researchers have chosen not to apply Bonferroni correction, considering it to be too restrictive in fields not considered to be well explored (226).

Rothman's advice on not correcting for multiple hypothesis testing was used in this thesis. One could criticize the current thesis for presenting results that are likely a consequence of chance. If Bonferroni correction had been applied, the results would have been different. Bonferroni correction is applied by taking the alpha level of 0.05 and dividing it by the number of hypothesis tests. In the following we present the results with applied Bonferroni correction (203):

In paper I, table 1, there were 12 variables grouped into three categories of the BDI-II questionnaire. The hypothesis tests are to be divided on the alpha level of 0.05. Thus, 0.05 divided by 12 gives a corrected significance threshold level of $p = 0.004$. No p-values are significant after applying Bonferroni correction. Further, table 2 have 48 hypothesis tests. The alpha level of 0.05 divided by 48 gives a corrected threshold level of less than $p = 0.01$ ($p \approx 0.001042$). This removes the four significant results and leaves no results significant in table 2 after correction. In table 3, there are four hypothesis tests. The alpha level of 0.05 divided by four gives a corrected threshold level of $p = 0.0125$. This does not affect the reported results. In figure 1, there are four cytokines and 12 hypothesis tests. The alpha level of 0.05 divided by 12 gives a corrected threshold level of $p \approx 0.0042$. This removes all three significant

findings. Lastly, for figure 2, there were no significant findings, hence no reason to apply Bonferroni correction.

In paper II, table 1, there are 24 hypothesis tests. The alpha level of 0.05 divided by 24 gives a corrected threshold level of 0.002 which removes all significant findings. In table 2, having PTSD is tested as main effect and in interaction with time on each of the four cytokines. This gives a total of 8 hypothesis tests. 0.05 divided by 8 gives a corrected threshold level of $p = 0.006$, and no significant findings. The bivariate analyses of GSI at each time point as well as across time in the PTSD versus no PTSD stratas give a total of 9 hypothesis tests (between each time point and from $T_0$ to $T_1$, and $T_0$ to $T_2$). The alpha level of 0.05 divided by 9 gives a corrected threshold level of $p \approx 0.006$. This leaves the findings on different GSI score of PTSD patients at T2 significant, as well as decreasing GSI score for patients without PTSD from $T_1$ to $T_2$ and $T_0$ to $T_2$. In table 3, the anti-inflammatory variable is tested as main effect and in interaction with time on each of the cytokines. This gives the same results as described for table 2, a corrected threshold level of $p = 0.006$ and no significant findings.

In paper III, table 1, there were five categories of independent variables. The hypotheses in question was whether or not there were different amounts/levels of the categorical and continuous variables when the patients were categorized as users or non-users of anti-inflammatory drugs. There were 7 hypothesis tests. The alpha level of 0.05 divided by 7 gives a corrected threshold level of 0.007. The corrected level removes the significant difference between gender and use of anti-inflammatory drugs. Further, the different levels of IL-1RA and GSI scores between users and non-users are no longer significant. Next, table 2 had 7 variables with a total of 14 hypothesis tests due to analyzing main effects and interaction with time separately. Further, table 2 was divided into table 2a, b and c, which gives a total of 38 hypotheses to be tested as main effects and in interaction with time. The alpha level of 0.05 divided by 38 gives a corrected threshold level of 0.001. This removes all significant p-values. The PTSD diagnosis in table 2a was originally significant with $p = 0.002$, thus barely reaching non-significance in the corrected analysis, suggesting the relation between PTSD diagnosis and level of GSI is a rather robust finding. An increase of type II errors is a possible consequence of introducing Bonferroni correction, but the entire sample has sufficient power to survive multiple hypothesis correction.

Type II errors can be made in significance testing when failing to reject a false null hypothesis or non-significant findings, i.e. not finding an effect which in reality is present. This is related to the power of the test, the probability of correctly rejecting a false null hypothesis, which equals 1-β, where 80% is considered the minimum acceptable level, meaning there is an eight in ten chance of detecting a difference (227). The power of the test is increased by increasing the sample size. In paper III, a statistical power test with the G*Power software, release 3.1.9.4, was done to assess achieved statistical power with the given sample sizes of 99 versus 28 patients in the two stratas (users of anti-inflammatory drugs versus non-users). A power of 80 % is traditionally accepted as being sufficient to detect effects which are present. The power was found to be 71 % with alpha level set at 0.05 and Cohen's *d* of -0.54 (228). This gave a 29 % chance of not finding an effect even if present, suggesting a susceptibility to type II errors.

### 6.1.8 External validity

External validity refers the extent to which the results of a study can be generalized and applied to other people or settings (219). The patients in the current study constitute a rather homogenous sample of individuals who share a common history of having suffered from psychiatric diseases for several years without achieving symptom reduction. Thus, as they were all granted 12 weeks of treatment after completing the one week of assessment at the pre-treatment stay, the patients have passed the same inclusion criteria, i.e. being capable of receiving and benefiting from the kind of treatment the facility offers, as well as having tried treatment before without succeeding. The scarcity of studies which replicates the design of the current study limits the possibility of drawing conclusions on external validity. However, we separated the PTSD patients by the ICD-10 diagnosis F43.1, which gives high external validity to these findings, as they can be generalized to other patients with the same disorder. The generalizability following stratification by diagnosis applies to other findings throughout this thesis too. For example, patients with mood disorder, eating disorder, anxiety disorder and trauma experiences, can and most likely will be generalized for comparison with such patients in other studies. The results can be regarded as being representative of exactly the kind of patients we had in this sample, i.e. treatment-resistant patients who have failed to recover after years of psychiatric disease and treatment without benefit. However, we utilized

a small patient sample which limits the possibility of generalizing the results to a broader range of patients.

## 6.2 Discussion of main results

### 6.2.1 The main results:

The main findings from these studies were that stratified on trauma experience, cytokines were higher in the traumatized patients unregarded to having minimal/mild depression or severe depression. Cytokines developed differently in PTSD patients compared to non-PTSD patients, with PTSD patients showing increasing levels. The patients experienced reduced level of psychiatric symptoms, measured by GSI, across treatment. MCP-1 and IL-1RA were associated with the overall level of GSI in the patient sample as a whole. IL-1RA, MCP-1 and PTSD were associated with the overall GSI level in the sample which did not use any anti-inflammatory drugs, while there were no associations in those who used anti-inflammatory drugs.

### 6.2.2 Associations between trauma, alcohol and cytokines

The cytokines IL-1RA and TNF-α were elevated in those with any kind of trauma history (childhood, adulthood or both). Elevated level of TNF-α correspond rather well to the existing literature on trauma and inflammation, e.g. childhood trauma has been shown to be associated with high levels of TNF-α with trajectories into adulthood (229). As well, higher levels of IL-1RA, together with TNF-α, has been found in a group of mixed psychiatric patients when compared with healthy volunteers in a recent study (230). Also, the expression of these cytokines corresponded with disease severity (230), possibly somewhat comparable to patients in the current study who were traumatized by physical and/or sexual abuse.

We did not find any associations between alcohol use and cytokine levels as measured by the AUDIT questionnaire. The level of alcohol intake was rather low in this sample, which might

explain the lack of associations to cytokine levels. The literature suggests that small amounts of alcohol use might dampen the immune response, while high levels may increase circulating cytokines (231), possible providing a rationale for non-significant associations with cytokines.

### 6.2.3 The contradictory development of cytokines and GSI across time

It was postulated in a review by Dubois and colleagues (99) that inflammatory markers are linked with the severity of the disorder and with treatment resistance, and that cytokine levels seem to parallel various stages of the illness and treatment (99). This is somewhat in accordance with the longitudinal part of this study, as IL-1RA did not change over time in any patients, and IL-1β and TNF-α did not change in patients with mood, anxiety and eating disorder, but all patients achieved symptom relief from treatment. Notably, the PTSD patients exhibited increasing levels of IL-1β and TNF-α, two findings which were not paralleled in the slope of GSI across treatment. This somewhat contradictory finding seems to fill a gap in the literature, as there is a paucity of studies assessing a clinical PTSD sample currently receiving treatment across time. The fact that the patients in the current study were receiving treatment is likely the cause of declining GSI levels across the 12 weeks of treatment. The higher symptom score seen in the PTSD patients, both at baseline and discharge, is consistent with literature showing that PTSD patients exhibit a worse symptom load than many other patient groups (232, 233). Vasiliki Michopoulos stated in a 2017 review that no data as yet prospectively demonstrate that chronic PTSD results in increased inflammation (121). The current study with its longitudinal design demonstrates exactly what Michopoulos called for. In other words, this branch of the DARCY studies demonstrates a rather novel finding and it elucidates a field where more research must be conducted in order to better understand the complex psychoneuroimmunological interface of PTSD and to provide better care for patients with this disorder.

### 6.2.4 Endocrine dysregulation in PTSD

The differences in slopes of cytokines in PTSD versus non-PTSD seen over time suggest that these patients either have an experience during the treatment which fires the inflammatory

response which other patients have not, or the difference could be due to the dysfunctional down-regulating mechanism in PTSD which have been described in some literature (234), however not consistently (235). A triggering experience causing an inflammatory response could be a consequence of receiving talk therapy from the clinical staff, with a possible re-experience of the trauma when giving a detailed elaboration of the traumatic event to the therapist. We did not assess re-experience of trauma in the patients in the current study. However, re-experience of trauma has been proposed as a factor robustly associated with enhanced inflammatory response to stressors in PTSD patients (236), which is supportive to our notion. It is not hard to imagine that such treatment is a tough experience which might be a devastating and painful task for heavily traumatized patients to accomplish. Further, the weak decline of the GSI slope in PTSD patients could imply a trait of treatment resistance. It could also suggest that the immune system might not strongly affect the psychiatric recovery process, i.e. the reduced symptom severity achieved through therapy supersedes the detrimental effects of the pro-inflammatory state somewhat out of control. However, a longer observation time than 12 weeks could have revealed important mechanisms, such as whether the biomarkers would keep increasing or perhaps start decreasing, and whether the pattern of the cytokine/chemokine slopes eventually would coincide with the GSI slope.

PTSD patients have recently been found to have a higher increase in IL-6 across time when exposed to acute mental stress than patients without PTSD, but this pattern was not seen in the slopes of chemokine MCP-1 (236). That is partly in line with the current study. Lima and colleagues found higher inflammatory level in PTSD patients, but with IL-6, a biomarker which had too many values under the LOD in the current study. Our finding on significant differences in the slopes of MCP-1 in PTSD versus non-PTSD was also not seen in the study by Lima and colleagues, who only found significant difference at the baseline measurement of MCP-1. The differences could possibly be due to numerous factors, e.g. serum versus plasma, centrifuging the samples at 4 °C versus room temperature, and different sample sizes (236, 237). Despite different biomarkers being assessed in these two studies, these studies point in the direction of an increased inflammatory state across time in PTSD patients.

There are several alterations in the biology of PTSD patients which could be involved in the process of increasing inflammation during treatment. PTSD patients are known to have alterations in the sympathomedullary (SAM) system and in the HPA axis, affecting production of stress hormones, which in turn interact with the bi-directional relationship

between the CNS and immune system (238). Moreover, PTSD patients are known to have a blunted resting production of cortisol (239) and an increased glucocorticoid receptor (GR) sensitivity, which in turn leads to enhanced negative feedback inhibition of cortisol (234). It has been hypothesized that this mechanism explains why patients with PTSD usually have lower cortisol levels than those with MDD or chronically stressed healthy individuals (234), possibly providing a rationale as to why PTSD patients in the current study do not follow the pattern of other patients at the cytokine level.

Further, the GR expressions in neutrons and lymphocytes are downregulated in PTSD patients, following the chronic stress cascade in which CRH release results in inhibition of negative feedback and down-regulation of glucocorticoid receptors (240). This is specific to PTSD, and it contrasts the biology of depression patients who are known to have less sensitive feedback sensitivity (241). These facts taken together, the PTSD patients suffer from severe and complex biological alterations, contributing to a distinct neuroimmune mechanism of recovery, as demonstrated by the measured cytokines in the current study. Also, the abovementioned biological alterations further serve as one possible explanation to why PTSD and non-PTSD patients react differently to psychiatric treatment.

The fact that we found MDD to differ from PTSD is in line with suggestions in a 2015 meta-analysis, which described significant pathophysiological differences between PTSD and MDD with regard to higher levels of IL-1β, IL-6 and INF-γ in PTSD (93). The meta-study suggested a role of IL-1β and IL-6 as biomarkers of PTSD duration and severity, respectively (93). A recent meta-study emphasized that the evidence has shown significant differences in inflammatory levels indicated by levels of C-reactive protein (CRP), between patients with PTSD on the one hand, and patients with anxiety disorders, or obsessive-compulsive disorders (OCD) and healthy controls on the other hand (94), again supporting our results.

### 6.2.5 Inflammation, mental distress and anti-inflammatory drugs

We found that IL-1RA and MCP-1 were associated with the overall level of GSI. Patients with depression diagnosis with increased inflammatory markers at baseline are known not to respond to anti-depressive treatment, which has led to the proposition of a relationship between inflammation and resistance to treatment (242). That relationship is in line with our

findings which suggest that IL-1RA and MCP-1 levels are associated with the overall level of GSI.

In other words, these longitudinal analyses give the same results as commonly reported in cross-sectional studies, an inflammatory state being present in psychiatric patients. In addition, when stratifying on the use of anti-inflammatory drugs, a possible moderating effect of such drugs was found, i.e. those who used such drugs did not exhibit any relationship between cytokines and mental distress. This suggests that anti-inflammatory drugs could be utilized as adjuvant treatment in psychiatric inpatient treatment, playing an anti-depressant role, a finding which is in line with a recent meta-study (243).

Several bodies of evidence have previously proposed that NSAIDs may have clinical use in treatment of depression, either as monotherapy or as adjuvant treatment together with anti-depressants (244). However, our sample size was small, and one should not draw conclusions on how these factors are related. The research so far is limited, and the results are mixed. It has been suggested that the main problem is that research has been mainly conducted in acute depression (245). The results from this thesis should be further assessed in future studies, using larger cohorts with various psychiatric disorders and for instance by categorizing the patients by the type of drugs in use.

The non-varying levels of IL-1RA and TNF-α in patients with depression, anxiety and eating disorders are not in accordance with two papers by Dahl and colleagues, which assessed depression patients in treatment and found IL-1RA and TNF-α to decline during treatment (100, 101). The non-varying levels in TNF-α in the current study is more in line with findings in a recent review, which analyzed 43 meta-studies, concluding with inconsistent results on whether TNF-α increases or decreases (by estimating and comparing effect sizes) in depression patients compared to controls (119). The same study found MCP-1 to increase in depression patients, a finding which was not supported by the findings in this thesis. However, that 2019 review study also stated that longitudinal studies are still rare and thus it is difficult to speculate in whether cytokines are trait or state in the various disorders (119).

# 7 Conclusions

This thesis was built upon a desire to illuminate cytokine levels and symptom severity across time in a broad patient sample in an inpatient treatment setting. The major conclusions of this endeavor were the following:

- Severely depressed patients with trauma history had higher levels of TNF-α and IL-1RA than severely depressed patients without trauma history (paper I)
- AUDIT score was not associated with BDI-II depression level (paper I)
- For IL-1RA, this difference was also present in patients classified as having mild/moderate depression (paper I)
- Levels of IL-1β, TNF-α and MCP-1 developed differently across time in PTSD patients compared to patients without PTSD (paper II)
- Overall level of IL-1RA was higher in PTSD patients compared to those without PTSD (paper II)
- Patients with higher levels of IL-1RA and MCP-1 had higher psychiatric symptom severity (paper III)
- We found no associations between IL-1RA and MCP-1 and symptom severity across time when assessing all patients. However, we found an association between the levels of IL-1RA, MCP-1 and PTSD disorder in relation to overall level of symptom severity (GSI) in the entire patient sample and in the stratum which did not use anti-inflammatory drugs. In the stratum which used such drugs, we found no relationships between cytokines and symptom severity (paper III)

# 8 Implications and future research

The implications of this study are elaborated in the following:

## 8.1 Clinical implications

The current study supports the notion of an inflammatory state characterized by elevated cytokine levels in patients with mental disorders. The association between cytokines and mental distress was not seen in the patients who used anti-inflammatory drugs. Our results suggest that patients in treatment not currently using anti-inflammatory medications could possibly benefit from taking such drugs as adjuvant treatment to psychotherapeutic treatment. That finding is somewhat in line with a recent systematic review and meta-analysis on randomized controlled trials which showed that anti-inflammatory agents reduce depressive symptoms in patients with MDD (243). However, those patients were not currently receiving psychological treatment, thus not directly comparable to the current study.

Nevertheless, a systematic review concluded that targeting cytokines might have some clinical benefit in treatment of patients with MDD, but it was also suggested that the immune response in MDD must be better characterized before attenuating cytokine production has clinical implications (246). It may be important to assess inflammatory biomarkers in patients eligible for psychiatric treatment to rule out any ongoing chronic infection. Elevated inflammatory markers have been found to predict a worse response to antidepressants, and those who do not respond to antidepressants have shown persistently increased inflammation (247).

As well, it has been suggested that PTSD patients could possibly benefit from intervention targeting inflammation (162). In light of the exaggerated neuroinflammatory response occurring in parallel with reduced symptoms of mental distress throughout the treatment period in the current study, the concept of treating the immune system together with psychological treatment seems promising. Immune re-balancing in PTSD patients has been called for, an intervention which already has its use in treatment of e.g. cancer, rheumatoid artritis and type 1 diabetes (162).

The immune response in PTSD could be related to the debate on trait and state of the inflammatory status, in the sense that years of suffering from psychiatric diseases have given rise to a chronic inflammation as a trait in these patients, and not a fluctuating state. This alteration might explain why combat veterans diagnosed with PTSD do not respond particularly well to treatment (248). PTSD patients could possibly have achieved psychological recovery to a higher extent if the immune system had been re-directioned, suggesting an additional pharmacological intervention alongside existing treatment (162). Such intervention could possibly have reduced their symptom load to a greater extent. Exactly what kind of pharmaco-immunological approach one might consider for reduction of inflammation in PTSD patients have yet to be described, but there are some preliminary suggestions. Vitamin D metabolites have been found to restore immune balance in various clinical conditions and has been proposed in regard to PTSD (162). Anti-depressives have been tested and been found to reduce PTSD symptoms, but the evidence from RCTs is limited due to small number of participants and other methodological problems, making it premature to draw conclusion (249).

Targeting inflammation in patients undergoing psychiatric treatment may be a sound approach, but we would like to emphasize that more longitudinal studies should be conducted, including the use of anti-inflammatory drugs in combination with treatment as usual. This study does not have any direct implications on clinical intervention. Rather, it describes neuroimmune mechanisms in patients receiving inpatient treatment. No further implications should be extracted from this study.

## 8.2 Research implications

This study contributes with information regarding cytokine levels in relation to alcohol consumption and in various categories of depression level, the longitudinal associations between IL-1RA and MCP-1 on average level of psychiatric symptoms, and with information on cytokines in PTSD patients developing different across time compared to non-PTSD patients. Such information might be relevant to the growing knowledge base on using inflammatory markers to identify risk and to predict treatment response (250). Cytokines have been proposed as useful biomarkers for disease activity (251) and progression (252) in

psychiatric disorders. However, a major issue in the field of psychoneuroimmunology is that the main articles describe cross-sectional studies which compare biomarkers in a patient sample with a healthy control sample. In addition, the studies often focus on only one disorder (161). Indeed, this was a good starting point for research in this field. This approach gave important leads on which neuroimmune biomarkers are associated with psychiatric diseases, especially depression. In the future, the field should include more longitudinal studies of patients with various disorders and take into account a broad range of neuroimmune biomarkers and examine how they vary across time. Also, examine how the biomarkers correlate with psychiatric symptoms. Many psychiatric disorders are episodic, and patients experience remission and relapse (253). This underscores the need for measuring biomarkers multiple times to see how they reflect the patient's current status at any given stage.

Future researchers should use our results as leads for further studies, taking more biomarkers into account, further scrutinizing PTSD patients in order to illuminate the pattern of their immunological response over time and simultaneously assess mental distress. Also, future studies should be designed with a follow-up period of more than 12 weeks to enhance the possibility of capturing changes. It will also be interesting to see what other longitudinal studies will reveal when assessing cytokines in samples of patients with different characteristics than the Modum Bad sample, as well as similar characteristics.

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# Appendix

Appendix 1: Consent form

Appendix 2: Information brochure

Appendix 3: REK approval

Appendix 4: Supplementary tables for paper III

# SAMTYKKEERKLÆRING

***Jeg har lest gjennom og forstått innholdet i informasjonsskrivet om DARCY-prosjektet.***

1. Jeg er villig til å delta i studien Immunologi knyttet til psykiske helse-lidelser med eller uten bruk av alkohol ved Modum Bad i regi av Sykehuset Innlandet. Jeg er også villig til å avlegge blodprøver tre ganger under oppholdet på Modum Bad.

☐ Ja ☐ Nei

2. Jeg er villig til at det gjøres analyse av utvalgte gener som har betydning for alkoholmisbrukslidelse, eventuelt angst/depresjonslidelser i blodprøvene som tas.

☐ Ja ☐ Nei

3. Jeg er villig til at det gjøres oppfølginger i helseregistre og/eller at dataene kobles sammen med andre tilsvarende studier.

☐ Ja ☐ Nei

4. Selv om jeg har sagt ja til deltakelse, kan jeg når som helst og uten å oppgi noen grunn trekke meg fra studien.

..... *Dato:* .....

*Signatur prosjektdeltaker*

Jeg bekrefter å ha gitt informasjon om studien:

..... *Dato:* .....

*Signatur prosjektmedarbeider/personale*

Koden din kobles til ditt navn og oppbevares på et avlåst sted der kun godkjente prosjektmedarbeidere har tilgang. Grunnen til at vi trenger tilgang til ditt navn er fordi vi ønsker å ta kontakt med deg for noen spørsmål seks måneder og tolv måneder etter avsluttet opphold på Modum Bad.

## Oppbevaring av data:

- Oppbevaring av data er godkjent av personvernombudet ved Modum Bad, som er Datatilsynets stedlige representant.
- De vil inngå i en database sammen med data fra alle andre deltakere i studien
- Det vil bli gjort DNA-analyse av utvalgte gener som har betydning for alkoholmisbrukslidelse, eventuelt angst/depresjonslidelser i blodprøvene som tas.
- Data vil bli analysert på gruppenivå, og resultatene vil bli publisert gjennom forskningsartikler i internasjonale fagtidsskrifter. Dette innebærer at deltakerne ALDRI vil kunne bli identifisert ut fra hva som publiseres.

Vi ønsker også tillatelse til å innhente informasjon om deg fra helseregistre og/ eller at dataene kobles sammen med andre tilsvarende studier.

## Finansiering

Studien finansieres av Sykehuset Innlandet og Modum Bad.

## Ja, jeg vil delta

Dersom du ønsker å delta, undertegner du samtykkeerklæringen som ligger ved denne folderen og leverer det til personalet ved Modum Bad.
Dersom du senere ønsker å trekke deg eller har spørsmål til studien, kontakt:

**Terje Tilden, Forskningsinstituttet, Modum Bad**
**tlf. 32 74 98 69.**

### Forskere:


Helge Toft


Lars Lien


Terje Tilden


Jørgen Bramness

EN FORSKNINGSSTUDIE

# DARCY-prosjektet

**Immunologi knyttet til psykiske helselidelser med eller uten bruk av alkohol**


MODUMBAD
- en kilde til liv

## DARCY-prosjektet:

- **DARCY er en forkortelse for depresjon, alkohol, rus og cytokiner.**
- **Cytokiner er en type hormoner som kan måles i blodet for å kartlegge immunforsvaret.**
- **Forskning viser at mennesker med depressive lidelser og/eller alkoholproblemer ofte har problemer med et svekket immunforsvar.**
- **Mange av disse personene har også erfaring med tidlige traumer.**

# Mer kunnskap - bedre behandling

Vi vet at det er et stort antall mennesker som sliter med denne kombinasjonen av problemer som nevnt ovenfor.

For lite kunnskap om hvilke sammenhenger det er mellom depresjon, alkoholforbruk og redusert immunforsvar, koblet opp mot traume-erfaringer, fører til at mange av disse ikke opplever å få tilstrekkelig hjelp i behandlingsapparatet.

**Mer kunnskap om disse faktorenes innvirkning på hverandre, vil kunne :**

- **Gi mer tilpasset behandling for den enkelte pasient**
- **Gi raskere og bedre hjelp**
- **Hjelpe flere**

## Prosjektet ved Modum Bad

Denne studien er en del av et større prosjekt som styres fra Sykehuset Innlandet under ledelse av professor Lars Lien, leder av Nasjonal kompetansetjeneste for samtidig rusmisbruk og psykisk lidelse.

Prosjektet har flere samarbeidspartnere, herunder professor Jørgen Bramness ved Senter for rus og avhengighetsforskning (SERAF), laboratorietjenesten ved Oslo Universitetssykehus, Norges Teknisk Naturvitenskapelige Universitet i Trondheim, samt fagmiljøer i USA, Nepal og Uganda.

Det er ansatt en doktorgradsstipendiat som heter Helge Toft. Han skal sørge for gjennomføring av studien i samarbeid med Forskningsinstituttet og de enkelte kliniske avdelinger ved Modum Bad.

DARCY- prosjektet - en forskningsstudie

## Oppstart februar 2015

Studien igangsettes i februar 2015. Datainnsamlingen vil pågå minst ett år. Studien gjennomføres i regi av Sykehuset Innlandet i samarbeid med Modum Bad ved Forskningsinstituttet. Databehandlingsansvarlig for studien er professor Lars Lien.

# Vil du delta?

Vi henvender oss til alle voksne som innkalles til et opphold ved Modum Bad, uavhengig av om du har noen av de nevnte problemområdene.

Det betyr at du ikke er nødt til å ha (eller hatt) et alkoholproblem for å kunne delta i studien. Din deltagelse er like viktig for oss hvis du for eksempel føler deg deprimert og/eller har en angstlidelse eller en traumelidelse. Grunnen er at vi ønsker en bredest mulig kartlegging, slik at vi kan sammenlikne de som har slike problemer opp mot de som ikke har slike problemer.

## Frivillig deltakelse

Deltakelse i studien er frivillig. Det vil si at uansett om du sier ja eller nei til deltakelse, vil ikke ditt valg ha noen innvirkning på type terapi eller innhold i terapien du får på Modum Bad.

Dersom du sier ja til deltakelse, vil du bli bedt om å svare på noen spørreskjemaer i tillegg til de som inngår som standard ved Modum Bad. Fordi immunforsvar best kan måles ved å kartlegge et hormon i blodet, trenger vi å ta blodprøver av deg tre ganger i løpet av oppholdet. Dette skjer på laboratoriet ved Modum Bad.

**Mulige ulemper:**

- Du må besvare noen flere spørreskjemaer enn hva som er standard ved et opphold på Modum Bad
- Du må avgi blodprøve tre ganger i løpet av oppholdet.

**Mulig fordel:**

- Ditt bidrag til prosjektet vil styrke kunnskapen om disse problemene, og øke mulighetene for at du og andre kan få bedre og raskere hjelp.

Som nevnt er det frivillig å delta i studien. Selv om du har sagt ja til deltakelse, kan du når som helst og uten å oppgi noen grunn trekke ditt samtykke til å delta i studien.

## Hva skjer med informasjonen om deg?

Alle data vil bli avidentifisert. Det vil si at dine data merkes med en tallkode som nedtegnes i en kodebok.

Fortsettelse neste side

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

| Region: | Saksbehandler: | Telefon: | Vår dato: | Vår referanse: |
|---|---|---|---|---|
| REK sør-øst | Silje U. Lauvrak | 22845520 | 09.03.2015 | 2014/2189/REK sør-øst D |
| | | | **Deres dato:** | **Deres referanse:** |
| | | | 12.02.2015 | |

Vår referanse må oppgis ved alle henvendelser

Lars Lien
Nasjonal kompetansetjeneste for samtidig rusmisbruk og psykisk lidelse

**2014/2189 Immunologi, depresjon og alkoholproblemer**

Vi viser til tilbakemelding fra prosjektleder, mottatt 12.02.2015, samt revidert informasjonsskriv mottatt 06.03.2015, i forbindelse med ovennevnte søknad. Tilbakemeldingen ble behandlet av komiteens leder på delegert fullmakt.

**Forskningsansvarlig:** Sykehuset Innlandet HF
**Prosjektleder:** Lars Lien

***Prosjektleders prosjektbeskrivelse***
*Forskningen vil belyse cytokin-nivåer i blodprøver tatt av 150 pasienter med alkohollidelse og depresjon. I tillegg vil psykometriske tester besvares. Datamaterialet innhentes fra pasienter ved Modum Bad. Datainnhenting vil foregå ved inntak, jevnlig gjennom innleggelsen og inntil et år etter utskrivelse. Aktivering av immunapparatet via økning av cytokin-nivåer fører til lavere serotonin-nivåer og økning av tryptophan-katabolitter. Dette medfører en oppstart eller forverring av depresjon. Det er i denne sammenhengen alkoholens påvirkning på immunapparatet blir relevant, ettersom alkoholkonsum kan øke forekomsten av betennelsesfremmende cytokiner. Det pågår en debatt om hvorvidt den observerte forsterkningen av betennelsesnivået oppstår samtidig som, eller er grunnlaget for, en depressiv tilstand, og over hvorvidt cytokiner er en grunnsten i depresjonens patofysiologi. Forandringer i cytokin-nivåer er relatert til depresjon, og kan derfor best belyses av en longitudinell studie.*

**Saksgang**
Søknaden ble behandlet i møtet 14.01.15, hvor komiteen utsatte å fatte vedtak i saken. Komiteen etterspurte en nærmere redegjørelse for hovedhypotese og styrkeberegning. Komiteen hadde også spørsmål knyttet til biobank og informasjonsskriv.

Prosjektleders tilbakemelding ble mottatt 12.02.2015.

**Komiteens vurdering**
I tilbakemeldingen redegjør prosjektleder for hovedhypotese og styrkeberegning. Prosjektleder klargjør også at det skal opprettes en spesifikk forskningsbiobank hvor blodprøvene lagres en kort periode inntil de sendes for analyse og deretter destrueres, og at prosjektet ikke innebærer utveksling av data/materiale til utlandet.

Komiteens spørsmål er tilfredsstillende besvart, og komiteen har ingen innvendinger til at prosjektet gjennomføres slik det er beskrevet i søknad, protokoll og tilbakemelding fra prosjektleder. Komiteen ber imidlertid om at det i informasjonsskrivet beskrives hvilke DNA-analyser som skal gjøres (i søknaden oppgis analyser på serotonin transportgener), og at navn på ansvarshavende for biobanken inkluderes.

På denne bakgrunn setter komiteen som vilkår for godkjenning at informasjonsskrivet revideres i tråd med

komiteens kommentarer og ettersendes til orientering.

**Vedtak**
Med hjemmel i helseforskningsloven § 9 jf. 33 godkjenner komiteen at prosjektet gjennomføres under forutsetning av at ovennevnte vilkår oppfylles.

I tillegg til vilkår som fremgår av dette vedtaket, er godkjenningen gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i søknad, protokoll, tilbakemelding fra prosjektleder og de bestemmelser som følger av helseforskningsloven med forskrifter.

Komiteen godkjenner opprettelse av en spesifikk forskningsbiobank, i tråd med det som er oppgitt i søknaden. Biobankregisteret vil få kopi av dette brev. Hvis forskningsbiobanken opphører, nedlegges eller overtas av andre, skal det søkes REK om tillatelse, jf. helseforskningsloven § 30.

Tillatelsen gjelder til 31.01.2019. Av dokumentasjonshensyn skal opplysningene likevel bevares inntil 31.01.2024. Forskningsfilen skal oppbevares avidentifisert, dvs. atskilt i en nøkkel- og en opplysningsfil. Opplysningene skal deretter slettes eller anonymiseres, senest innen et halvt år fra denne dato.

Forskningsprosjektets data skal oppbevares forsvarlig, se personopplysningsforskriften kapittel 2, og Helsedirektoratets veileder for «Personvern og informasjonssikkerhet i forskningsprosjekter innenfor helse og omsorgssektoren».

Dersom det skal gjøres vesentlige endringer i prosjektet i forhold til de opplysninger som er gitt i søknaden, må prosjektleder sende endringsmelding til REK.

Prosjektet skal sende sluttmelding på eget skjema, senest et halvt år etter prosjektslutt.

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningslovens § 28 flg. Klagen sendes til REK sør-øst D. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK sør-øst D, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Vi ber om at alle henvendelser sendes inn på korrekt skjema via vår saksportal: http://helseforskning.etikkom.no. Dersom det ikke finnes passende skjema kan henvendelsen rettes på e-post til: post@helseforskning.etikkom.no.

Vennligst oppgi vårt referansenummer i korrespondansen.

Med vennlig hilsen

Finn Wisløff
Professor em. dr. med.
Leder

Silje U. Lauvrak
Rådgiver

**Kopi til:** ola.dahl@sykehuset-innlandet.no;
Sykehuset Innlandet HF ved øverste administrative ledelse: kari.lillehaug@sykehuset-innlandet.no;
Biobankregisteret ved Nina Hovland: nina.hovland@fhi.no

Paper III. Supplementary table 1. Distribution of main diagnoses (n = 113).

| ICD-10 code [a] | Main diagnosis | Frequency |
|---|---|---|
| F30.0 | Hypomania | 1 |
| F31.3 | Bipolar affective disorder, current episode mild or moderate depression | 2 |
| F31.8 | Other bipolar affective disorders | 3 |
| F32.1 | Moderate depressive episode | 9 |
| F32.2 | Severe depressive episode without psychotic symptoms | 1 |
| F33.0 | Major depressive disorder, recurrent, mild | 1 |
| F33.1 | Recurrent depressive disorder, current episode moderate | 7 |
| F33.2 | Recurrent depressive disorder, current episode severe without psychotic symptoms | 2 |
| F33.4 | Recurrent depressive disorder, currently in remission | 1 |
| F34.1 | Dysthymia | 5 |
| F40.0 | Agoraphobia | 1 |
| F40.01 | Agoraphobia with panic disorder | 1 |
| F40.1 | Social phobias | 8 |
| F40.2 | Specific (isolated) phobias | 1 |
| F41.0 | Panic disorder (episodic paroxysmal anxiety) | 3 |
| F41.1 | Generalized anxiety disorder | 1 |
| F43.1 | Post-traumatic stress disorder | 32 |
| F43.21 | Adjustment disorder with depressed mood | 2 |
| F44.9 | Dissociative (conversion) disorder, unspecified | 6 |
| F50.0 | Anorexia nervosa | 4 |
| F50.1 | Atypical anorexia nervosa | 6 |
| F50.2 | Bulimia nervosa | 9 |
| F50.3 | Atypical bulimia nervosa | 2 |
| F60.3 | Emotionally unstable personality disorder | 1 |
| F60.5 | Anankastic personality disorder | 2 |
| F60.6 | Anxious (avoidant) personality disorder | 1 |
| F61.0 | Mixed and other personality disorders | 1 |

Notes: [a] International Statistical Classification of Diseases and Related Health Problems 10th revision.

Paper III. Supplementary table 2. Distribution of drugs categorized as anti-inflammatory (n = 28).

| Generic name | Frequency [a] |
|---|---|
| Pulmicort | 1 |
| Prednisolon | 1 |
| Piroksikam | 1 |
| Diclofenacnatrium | 8 |
| Ibuprofen | 13 |
| Etorikoksib | 2 |
| Adapalen | 1 |
| Klobetasolpropionat | 1 |
| Naproxen | 1 |
| Mesalazin | 1 |
| Hydrocortisone | 2 |
| Metotreksat | 1 |
| Pimecrolimus | 1 |
| Tibolon | 1 |
| Azatioprin | 1 |

Notes: [a] Some patients may have used more than one kind of drug.

Paper III. Supplementary table 3. Fixed and random effects of GSI across therapy.

The fixed intercept shows that the average of the GSI scores in all patients at treatment initiation was 1.5, and this score was significantly different from zero. The fixed effect of time showed that GSI score in all patients declined at -0.027 across time, and this effect was significant. The random intercept variation showed that the patients differed significantly from each other at treatment initiation by a GSI score of 0.336, and this score was different from zero.

| | **GSI [a]** |
|---|---|
| **Fixed effects** | β(SE) [b] |
| Intercept | 1.505 (0.059)*** |
| Slope (time) | -0.027 (0.004)*** |
| **Random effects** | |
| Intercept | 0.336 (0.049)*** |
| **Fit index [c]** | |
| -2 LL | -292 |
| BIC | 608 |

Notes: [a] GSI = Global Severity Index. [b] β = Regression coefficient. SE = Standard Error. [c] -2 LL = -2 Log Likelihood. BIC = Bayes Information Criterion. *** $p < 0.001$.

Paper III. Supplementary table 4. HSCL-90R subscales

| Variable | Coefficient | SE | P-value |
|---|---|---|---|
| MCP-1 | 0.004 | 0.003 | 0.173 |
| IL-1RA | 0.005 | 0.002 | **0.008** |

Notes: Somatization subscale. MCP-1 and IL-1RA analyzed separately. Significant p-values in boldface. SE: Standard error.

| Variable | Coefficient | SE | P-value |
|---|---|---|---|
| MCP-1 | 0.005 | 0.003 | **0.050** |
| IL-1RA | 0.004 | 0.002 | **0.046** |

Notes: Depressive subscale. MCP-1 and IL-1RA analyzed separately. Significant p-values in boldface. SE: Standard error.

| Variable | Coefficient | SE | P-value |
|---|---|---|---|
| MCP-1 | 0.008 | 0.003 | **0.005** |
| IL-1RA | 0.006 | 0.002 | **0.000** |

Notes: Anxiety subscale. MCP-1 and IL-1RA analyzed separately. Significant p-values in boldface. SE: Standard error.

Paper III. Supplementary table 5a. Linear mixed effects models of IL-1RA, adjusted for age and sex, on GSI

| | Main effects | | | Interaction with time | | |
|---|---|---|---|---|---|---|
| **Parameters** | **β (SE)** | **95 % CI** | | **β (SE)** | **95 % CI** | |
| **Fixed effects** | | **Lower** | **Upper** | | **Lower** | **Upper** |
| **Level 1** | | | | | | |
| Within effect | <0.001 (<0.001) | -0.001 | 0.001 | <0.001 (<0.001) | -0.001 | 0.001 |
| **Level 2** | | | | | | |
| Between effect | 0.004 (0.001)* | 0.001 | 0.007 | 0.004 (0.002)* | <0.001 | 0.007 |
| Between effect x time | | | | <0.001 (<0.001) | <-0.001 | <0.001 |
| Intercept | 1.639 (0.273)*** | -0.0133 | 0.007 | 1.634 (0.273)*** | 1.094 | 2.175 |
| **Covariates** | | | | | | |
| Time | -0.027 (0.004)*** | -0.036 | -0.185 | -0.026 (0.005)*** | -0.036 | -0.016 |
| Age | -0.003 (0.005) | -0.013 | 0.007 | -0.003 (0.005) | -0.013 | 0.007 |
| Sex | 0.136 (0.130) | -0.120 | 0.392 | 0.136 (0.129) | -0.120 | 0.392 |
| **Random effects** | | | | | | |
| Intercept | 0.317 (0.047)*** | 0.236 | 0.425 | 0.316 (0.047)*** | 0.316 | 0.047 |
| Residuals | 0.138 (0.013)*** | 0.115 | 0.166 | 0.139 (0.013)*** | 0.139 | 0.013 |
| **Fit index** | | | | | | |
| -2 LL | -302 | | | -310 | | |
| BIC | 651 | | | 673 | | |

Notes: *** Significant at the <0.001 level. ** Significant at the <0.01 level. * Significant at the <0.05 level. β (SE): Regression coefficient (Standard Error). -2 LL: Log likelihood. BIC: Bayes Information Criterion.

Paper III. Supplementary table 5b. Linear mixed effects models of MCP-1, adjusted for age and sex, on GSI

| | Main effects | | | Interaction with time | | |
|---|---|---|---|---|---|---|
| **Parameters** | **β (SE)** | **95 % CI** | | **β (SE)** | **95 % CI** | |
| **Fixed effects** | | **Lower** | **Upper** | | **Lower** | **Upper** |
| **Level 1** | | | | | | |
| Within effect | <0.001 (0.001) | -0.002 | 0.003 | <0.001 (0.002) | -0.002 | 0.004 |
| **Level 2** | | | | | | |
| Between effect | 0.005 (0.002)* | 0.001 | 0.010 | 0.005 (0.002)* | <0.001 | 0.010 |
| Between effect x time | | | | <0.001 (<0.001) | <-0.001 | <0.001 |
| Intercept | 1.516 (0.270)*** | 0.981 | 2.051 | 1.517 (0.271)*** | 0.981 | 2.052 |
| **Covariates** | | | | | | |
| Time | -0.027 (0.004)*** | -0.036 | -0.019 | -0.027 (0.005)*** | -0.037 | -0.018 |
| Age | -0.002 (0.005) | -0.012 | 0.008 | -0.002 (0.005) | -0.012 | 0.008 |
| Sex | 0.186 (0.131) | -0.073 | 0.444 | 0.186 (0.131) | -0.073 | 0.444 |
| **Random effects** | | | | | | |
| Intercept | 0.322 (0.048)*** | 0.024 | 0.431 | 0.321 (0.048)*** | 0.240 | 0.431 |
| Residuals | 0.138 (0.013)*** | 0.115 | 0.167 | 0.139 (0.013)*** | 0.116 | 0.167 |
| **Fit index** | | | | | | |
| -2 LL | -302 | | | -309 | | |
| BIC | 650 | | | 671 | | |

Notes: *** Significant at the <0.001 level. ** Significant at the <0.01 level. * Significant at the <0.05 level. β (SE): Regression coefficient (Standard Error). -2 LL: Log likelihood. BIC: Bayes Information Criterion.

## Papers I-III

# I

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BMC Psychiatry

RESEARCH ARTICLE Open Access

# The effect of trauma and alcohol on the relationship between level of cytokines and depression among patients entering psychiatric treatment

Helge Toft[1,2*], Sudan Prasad Neupane[1,3], Jørgen G. Bramness[1,4], Terje Tilden[5], Bruce E. Wampold[5,6] and Lars Lien[1,7]

## Abstract

**Background:** Depression is associated with immunological responses as reflected by altered levels of circulating cytokines. Alcohol use and trauma may modulate immune activity, and few studies have investigated these factors in depressed patients. We aimed to explore the association between circulating peripheral cytokine levels and degree of depressive symptoms, taking trauma and alcohol into account.

**Methods:** The study was a cross-sectional assessment of patients at admission to a specialized psychiatric center in Norway. A total of 128 patients were included. Information was gathered using the self-administered questionnaires Beck Depression Inventory-II (BDI-II) and the Alcohol Use Disorders Identification Test (AUDIT), in addition to clinical interviews recording childhood or adult life trauma. Serum levels of the cytokines Interleukin-1β (IL-1β), Interleukin-1 Receptor Antagonist (IL-1RA), Tumor Necrosis Factor-α (TNF-α) and the chemokine Monocyte Chemoattractant Protein-1 (MCP-1) were assessed. A Luminex bead-based multiplex assay was used for cytokine measurements. Patient cytokine levels were compared to those of healthy volunteers by the Mann-Whitney *U* test.

**Results:** Levels of cytokines did not differ across patients with mild, moderate and severe depression. AUDIT score was not related to cytokine levels, but to level of depression. A history of trauma was related to higher levels of IL-1RA and TNF-α ($p = 0.048$ and $p = 0.033$, respectively), especially among the severely depressed. Serum levels of MCP-1 and TNF-α were significantly higher among psychiatric patients than in healthy volunteers.

**Conclusions:** Findings indicate that depression was not related to levels of circulating cytokines among patients in treatment, but that traumatized patients had higher levels of IL-1RA and TNF-α than patients without trauma experience. The lack of relationship between cytokine level and depression was evident both in those without and with trauma.

**Keywords:** Cytokines, Trauma, Depression, Alcohol, Comorbidity, Immune activation

## Background

Patients with major depressive disorder (MDD) are found to have significantly elevated levels of circulating pro-inflammatory cytokines, such as IL-6, TNF-α and IL-1RA [1–3]. There is also some evidence showing that recovery from depressive disorder correlates with return of the inflammatory state to normality [4]. A relationship between immune responses and depression is illustrated by the fact that up to 50% of patients develop clinical depression following high doses of immune therapy with interferon-α (IFN-α) [5]. Also, there is some evidence that elevated cytokine levels may predict depressive illness, suggesting a causal relationship between elevated immune activation and MDD [6, 7].

In addition to MDD, pro-inflammatory cytokines have been found to accompany anxiety disorders such as general anxiety disorder [8, 9], panic disorder and the spectrum of phobias [10] in addition to post-traumatic stress disorder (PTSD) [11]. This may be due to activation of central and peripheral

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Full list of author information is available at the end of the article

immune cells releasing cytokines, as well as activation of the stress response system of the hypothalamic-pituitary-adrenal (HPA) axis by pro-inflammatory cytokines [12].

One possible confounder for the relationship between cytokines and mental health disorders might be the use of anti-inflammatory drugs, where non-users have higher levels of IL-1β, IL-6, and TNF-α [11]. Other factors that might cause variations are sample characteristics like comorbidity, type of trauma experienced, and time elapsed since the trauma [10, 13]. Such inconsistencies also apply to eating disorders, where previous results are conflicting [14, 15]. This suggests that the relationship between immunological functioning and the broad spectrum of psychiatric disorders should be further examined.

Alcohol use disorder is comorbid in about 30% of patients with major depression, which makes it important to consider alcohol use in investigations of immune changes in depression [16]. Although acute alcohol consumption inhibits immune response, resulting in suppression of the pro-inflammatory cytokines, chronic alcohol use is associated with increased pro-inflammatory cytokine production due to sensitization of immune cells [17, 18]. The resulting dysregulation of the innate immune system may lead to the development of depression [19], and the response may lead to development and progression of depressive disorders among alcoholics [20]. Assessing alcohol dependence to evaluate immune changes in depression patients is therefore critical [21].

Elucidating the role of inflammation in depressed patients in light of previous alcohol use and traumatic life events might provide further insight into the mechanisms of how depressive disorders develop. This could have important translational and clinical implications, such as the development of new therapeutic agents which target the immune system in treatment of major depression. Immunological biomarkers could be used to identify the kind of treatment an individual is likely to benefit from. We aimed to explore the association between circulating peripheral cytokine levels and degree of depressive symptoms, taking trauma and alcohol into account.

## Methods

### Study participants and recruitment procedure

Patients were recruited at admission to Modum Bad, a specialized psychiatric center in Norway. The facility treats patients with long- standing and treatment-resistant trauma, anxiety, eating and depression disorders. Patients with severe self-destructive behavior or psychotic disorders were not eligible for admission. The center does not treat patients with substance abuse disorders (SUD) as such, but many patients have comorbid addiction as part of their problem spectrum. The facility offers group and individual therapies in a 12-week inpatient treatment program. Therapy is paid by public insurance, and patients in work are entitled to sick leave while in treatment. The staff is multidisciplinary, including psychiatrists, psychologists, nurses, art therapists, occupational therapists, social workers and pastoral staff. Data were collected from March 2015 to April 2016. The study was approved by the Norwegian Regional Ethics Committee (REK) prior to data collection (reference number 2014/2189).

Patients were recruited from the following units: Depression, Eating Disorders, Anxiety and Trauma. The patients joined the study in groups of eight at a time. They were given a 15-min presentation about the study during group therapy by the first author on one of the first days of their stay. A written brochure was handed out, explaining the aim of the study and the procedures involved. A written consent form was also distributed to each potential participant. Altogether, 148 (59% of the 249 patients approached) gave their written consent, and one individual withdrew her consent two weeks later. We excluded 19 patients from the data set, due to extreme cytokine levels indicating acute infections. Thus, the present study included baseline data provided by 92 women (72%, mean age 39.04, SD 11.26) and 36 men (28%, mean age 49.06, SD 9.36), giving a total of 128 patients. The 102 patients who did not participate consisted of 82 women (80%, mean age 35.77, SD 11.83) and 21 men (21%, mean age 44.52, SD 8.58).

### Healthy volunteers group

As a control group, we utilized data from an experimental study with healthy volunteers performed by our group [22]. Sampling procedures are detailed in the original study. In short, healthy volunteers were recruited through an advertisement in the Correctional Service Staff Academy in Oslo, Norway. The volunteers were 20 males aged from 20 to 45 (mean age 28.84, SD 5.3) who all had previous experience of high-dose alcohol drinking. Exclusion criteria were having any significant medical illness, alcohol or other substance use disorders, or metabolic disorders. Demographic information was recorded, the AUDIT questionnaire was answered, and cytokines were analyzed by venous blood collection. The blood was collected at 7 am following an overnight fasting. One volunteer was excluded due to an extreme blood level of TNF-α. For the experimental study, an inclusion criterion was some experience of high-dose alcohol drinking but current non-dependence on alcohol. Thus, an inclusion maximum was set to 15 on the AUDIT. As this volunteer group differed in age and gender from the patients in our clinical study, we selected the youngest male patients from our study to be compared to this healthy volunteer sample with regard to cytokine levels.

### Methods

All patients were interviewed by trained psychologists or psychiatrists using the Mini-International Neuropsychiatric

Interview (MINI) [23]. The measurements were conducted during the first week of admission. The MINI interview results in a diagnosis in the 10th revision of the International Classification of Diseases and Related Health Problems (ICD-10). A combination of psychometric questionnaires and clinical judgment was also taken into account in assessing disorders. There were 54 patients with only one disorder, and 59 with two or more disorders. There were 15 patients with missing diagnosis and 14 with missing trauma status due to staff failing to record the patient's disorder. Disorders within F30–39 were treated as one variable of mood disorders. Disorders within the range of F40–49 were merged to one variable of anxiety disorders. Disorders within F50.0-F50.9 were merged to one variable of eating disorders.

In addition, the patients completed various self-report questionnaires on a computer or digital tablet. The following questionnaires were included:

The 21-item Beck Depression Inventory (BDI-II). The BDI-II [24] was administered by the therapists to assess the level of depressive symptoms during the two weeks prior to the interview. The Norwegian validated version was used [25, 26]. Each of the 21 items is scored from 0 to 3. Based on the average score, we categorized the answers into three levels of depression severity: Minimal and mild depression (score 0–18), moderate (score 19–29), and severe (score 30–63).

The Alcohol Use Disorder Identification Test (AUDIT). This is a 10-item screening test designed to identify harmful, hazardous or possible alcohol dependence the last 12 months. The AUDIT has been proven able to detect DSM-IV alcohol dependence and DSM-IV alcohol use disorder (AUD) when compared with semi-structured clinical interviews [27]. Some examples of questions are: "How often do you have a drink containing alcohol?" and "How many drinks containing alcohol do you have on a typical day when you are drinking?" The scores range from 0 to 4. In these examples, a score of 0 refers to "never" and "1–2 drinks", respectively. A higher score refers to a more severe drinking pattern, and in these two examples, a score of 4 means "4 times a week" and "10 or more drinks". The cutoff scores for harmful or hazardous drinking were set at 8 for men and 6 for women [28].

Trauma history was recorded by therapists at the facility as part of clinical history taking. There were five questions on trauma exposure: 1) Has the patient been exposed to sexual assaults in childhood? 2) Has the patient been exposed to physical abuse in childhood? 3) Has the patient during childhood experienced other traumatic events which have led to serious problems later in life? 4) Has the patient been exposed to sexual assaults or abuse in adulthood (after 18 years of age)? 5) Has the patient in adulthood experienced other traumatic events which led to serious problems later?

### Blood collection and serum preparation

The blood samples were taken in the laboratory at Modum Bad between 8 and 9 am. One of the groups from the Depression Unit had their blood samples taken between 12 and 3 pm. The blood samples were collected in Vacuette 8 ml serum tubes, which were immediately turned upside down about 8–10 times. They were then set to rest in a blood tube stand for between 30 min and one hour before being centrifuged in a Kubota 2420 swing-out centrifuge at room temperature. The centrifuging procedure was set to 10 min, and the centrifuge achieved a rotation power of 1917 g. The separated serum was drawn from the Vacuette tubes with a 1 ml single use pipette into two 2 ml Nunc tubes. The samples were then stored in a − 80 degrees Celsius freezer until assay.

### Cytokine measurements

All samples were thawed on ice, vortexed, and then spun down a tube with 250 μl serum at 14,000×g for 10 min at 4 °C, before dilution (1 + 4) and further processing. The following cytokines were assessed: IL-1β, IL-1RA, IL-6, IL-10, IL-17A, IFN-γ, MCP-1 and TNF-α. These cytokines were selected based on the available literature on the neuroimmune correlates of psychiatric disorders. We present cytokines that were within the detectable range, which were IL-1β, IL-1RA, MCP-1 and TNF-α. Cytokine measurements were performed using Bio-Plex xMAP technology (Bio-Rad, Austin, Texas, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA), powered using Bio-Plex Manager (version 6.0. 1) software. Multiplex bead-based technologies such as Luminex allow detection and quantification of multiple cytokines with good efficiency, speed and dynamic range at reasonable cost. The assay was performed according to the manufacturer's instructions, but an additional standard point was included. To achieve a more reliable result, individual sets of samples from patients were run in the same assay, all samples were assayed in duplicate and a magnetic plate washer was used during assay set up. The StatLIA software package (version 3.2, Brendan Scientific, Carlsbad, California, USA), incorporating a weighted, five-parameter logistic curve-fitting method, was used to calculate sample cytokine concentrations. Longitudinal controls were used in order to validate inter-assay variation: IL-1β (18.1), IL-1RA (10.2), MCP-1 (6.7) and TNF-α (7.4). Numbers in parentheses are inter-assay coefficients of variability (CV), where a lower number is better. Any number below 21 is considered acceptable. The mean inter-assay CV for all blood sample plates was 10.4%. The serum levels were measured in picograms per milliliter (pg/ml). The minimum detectable values were 0.01 pg/ml for IL-1β, 3 pg/ml for IL-1RA, 0.76 pg/ml for MCP-1, and 0.02 pg/ml for TNF-α.

### Statistical analysis

The statistical package SPSS version 23 for Windows (SPSS Inc., Chicago IL, USA) was used for the statistical analysis. One patient failed to fill out the AUDIT questionnaire, and was treated as missing and excluded from the analysis. Descriptive statistics were used to present the cytokine values. The values were rather skewed, and therefore presented by medians and 25/75 percentiles. We attempted to normalize the skewed cytokine data by log transformation. However, the Kolmogorov-Smirnov test of normality remained significant, and we therefore did not explore this approach further. Nonetheless, we explored the analyses with and without log-transformed data, which remained unchanged. Undetectable cytokine levels were imputed with 1% of the mean value. In the patients, 64 (50.39%) imputations of the cytokine IL-1β were conducted, 1 (0.9%) of IL-1RA, 9 (7.09%) of MCP-1, and 53 (41.73% of TNF-α. In the healthy volunteers, 6 (31.6%) imputations of IL-1β were conducted, none of IL-1RA, 5 (26.3%) of MCP-1, and 15 (79%) of TNF-α. These undetectable cytokine levels were above zero, but under the detectable limit, leaving imputation as a way of presenting undetectable levels in close resemblance to their actual levels. Patients with cytokine levels above the 95th percentile were defined as outliers, and removed from the material, thus reducing the potential distortion from any somatic inflammatory diseases. For the continuous variable of age, Spearman's rho correlation coefficient was used. The significance level between the cytokines and categorical variables was calculated by the Mann-Whitney *U*-test and by the Kruskal-Wallis one-way ANOVA. Pearson's chi-square was used for calculating the significance level between the categorical variables. Finally, a comparison of mean cytokine values between patients and healthy volunteers was made. Group differences in the levels of cytokines between the patients and healthy volunteers were calculated using the Mann-Whitney *U* test, and *p*-values for the tests are reported. All tests were two-tailed with statistical significance set at the 5% level. Some of the group-wise comparisons may have lacked statistical power due to small group sizes, raising the possibility of type II error.

## Results

Table 1 presents demography, medication, trauma history, and diagnosis across the BDI-II scores categorized into three different levels. The only difference between these three levels of depression was the AUDIT scores, where the higher score was in those with moderate depression ($p = 0.009$). The use of anti-inflammatory and anti-depressive medication was positively associated with depression severity. A positive history of trauma was most frequent in the severely depressed patients. Patients classified under the main diagnostic groups (mood/anxiety/eating/trauma) had a uniform distribution of depression severity.

Table 2 shows the distribution of cytokine values according to demography, distress, medication, trauma and diagnostic groups. Levels of IL-1β were significantly higher in the group with mood disorder and anxiety disorder than in those without ($p = 0.037$ and $p = 0.008$, respectively). IL-1RA and TNF-α were significantly higher in patients with a history of trauma than in those without trauma experience ($p = 0.048$ and $p = 0.033$, respectively). Participants scoring above cutoff in the AUDIT had higher values of all measured cytokines; however, the differences were not statistically significant.

Table 3 displays mean cytokine values from the 19 male, healthy volunteers and 19 matched patients from our clinical study. All levels were higher in the patient group than in the healthy volunteers, with MCP-1 and TNF-α reaching statistical significance ($p = 0.012$ and $p < 0.001$, respectively). The selected patient group was older than the healthy volunteers group ($p < 0.001$). We also performed analyses of a patient cohort consisting of all patients compared to the healthy volunteers, and of all 36 male patients compared to the healthy volunteers. However, these different approaches did not change the results significantly, the findings being consistent across male and female patients, as well as between the whole sample and the healthy volunteers sample.

To investigate possible effects of trauma history or alcohol use on the relationship between cytokine levels and level of depression as quantified by the BDI-II, we stratified the material into non-traumatized and traumatized patients (Fig. 1) and those scoring below or above clinical cutoff on AUDIT (Fig. 2). As depicted in Fig. 1, patients who were categorized in the minimal or mild depression group, as well as in the severe depression group, had significantly different levels of cytokine IL-1RA when stratified on trauma and no trauma experience ($p = 0.046$ and $p = 0.047$, respectively). This was also true for levels of TNF-α with the same strata in the severe depression group ($p = 0.029$). There were no significant relationships between cytokine levels and depression levels in the two strata of patients below and above AUDIT score.

## Discussion

Patients with mood disorder and anxiety disorder had higher levels of IL-1β, but we found no systematic relationship between level of depression and level of the measured cytokines. Patients reporting trauma had higher levels of the cytokines IL-1RA and TNF-α. We found no significant relationship between cytokines and AUDIT scores.

The observed lack of relationship between cytokine levels and level of depression may be because patients at this high-threshold facility all have increased levels of cytokines at admission. They represent a treatment-resistant population that has suffered for years with mental illness and received mental health treatment with

**Table 1** Clinical characteristics of the included patients according to level of depression. Depression severity was measured by the Beck Depression Inventory-II (BDI-II)

| Variable | | Minimal or mild depression[a] | | Moderate depression | | Severe depression | | |
|---|---|---|---|---|---|---|---|---|
| | | *n* = 28 (21.9%) | % / SD | *n* = 42 (32.8%) | % / SD | *n* = 58 (45.3%) | % / SD | Sig.[b] |
| Demography | | | | | | | | |
| Women | n (%) | 20 | 71.4 | 28 | 66.7 | 44 | 75.9 | 0.600 |
| Age (years) | Mean (SD) | 39.4 | 10.8 | 44.2 | 12.0 | 41.3 | 11.7 | 0.210 |
| Alcohol use | | | | | | | | |
| AUDIT score[c] | Mean (SD) | 4.70 | 4.1 | 6.7 | 5.4 | 3.7 | 4.3 | *0.009*[d] |
| Medication | | | | | | | | |
| Anti-inflammatory drug (any) | n (%) | 5 | 17.9 | 5 | 11.9 | 9 | 15.5 | 0.775 |
| Antidepressants (any) | n (%) | 9 | 32.1 | 12 | 28.6 | 22 | 37.9 | 0.609 |
| History of trauma | | | | | | | | |
| Childhood trauma | n (%) | 15 | 62.5 | 23 | 62.2 | 25 | 48.1 | 0.316 |
| Adult trauma | n (%) | 16 | 66.7 | 16 | 43.2 | 28 | 53.8 | 0.199 |
| Any trauma | n (%) | 19 | 79.2 | 26 | 70.3 | 39 | 73.6 | 0.743 |
| Main diagnosis[e] | | | | | | | | |
| Mood disorder | n (%) | 8 | 34.8 | 14 | 36.8 | 10 | 19.2 | 0.139 |
| Anxiety disorder | n (%) | 12 | 52.2 | 17 | 44.7 | 26 | 50 | 0.825 |
| Eating disorder | n (%) | 3 | 13.6 | 5 | 13.2 | 14 | 26.9 | 0.181 |
| MDD (ICD-10, F33)[f] | n (%) | 3 | 10.7 | 5 | 11.9 | 3 | 5.2 | 0.447 |

Notes: [a]14 of these patients scored 13 or less in the BDI-II, indicating that they were not depressed, and were classified with minimal depression. [b]Significance for categorical variables was tested with Pearson's chi-square test, and for continuous variables with Kruskal-Wallis one-way ANOVA. [c]AUDIT: Alcohol Use Disorder Identification Test. [d]AUDIT score was different across the levels of BDI-II. [e]Main diagnosis: Mood disorders: F30-F34, Anxiety disorders: F40-F44, Eating disorders: F50.0-F50.3. [f]MDD: Major Depression Disorder, as classified by the ICD-10

no or little effect. A consequence may be that the immune system is chronically activated. However, despite not reaching statistical significance, mean and median cytokine levels were generally higher in groups who scored high on depression. The use of both antidepressants and anti-inflammatory drugs has been reported to distort the immune response, which may have contributed to the non-significant findings [29].

We found a relationship between patients suffering childhood or adulthood trauma and higher cytokine levels, represented by IL-1RA and TNF-α being significantly elevated, but we found no links between childhood trauma or adulthood trauma and depression level. This led us to perform a stratified analysis investigating non-traumatized and traumatized patients separately. In these strata, we found a difference in levels of IL-1RA in the minimal to mild depression group, and in levels of IL-1RA and TNF-α in the severe depression group. Stress-induced depression may follow inhibited glucocorticoid release as a result of a chronically activated HPA axis, a sign of inflammation [30]. In traumatized or maltreated children, insufficient glucocorticoid signaling may potentially lead to an unrestrained state of inflammation as adults, rendering them vulnerable to depression [31]. Studies report that childhood maltreatment is a predictor of elevated levels of inflammatory markers in adulthood, and such individuals are more susceptible to developing depression [32]. Also, adult experiences of trauma may lead to the development of chronic stress and thus elevated pro-inflammatory cytokines [33].

The elevated IL-1RA level in the traumatized patients may be a result of the initial inflammatory response being countered by a down-regulated inflammatory response [34, 35], limiting the pro-inflammatory effects of IL-1 [36]. Previous studies found elevated levels of IL-1β [11, 37, 38] and TNF-α [11, 39], while others found no such association of IL-1β [39], TNF-α [40] or other neuroimmune biomarkers. One possible explanation for these mixed results may be that different kinds of trauma yield different immunologic responses. For instance, whether the trauma was experienced as a child or as an adult is a relevant factor [13]. The HPA axis and brain in childhood are immature and developing, and the adaptive response to maltreatment has long-lasting effects on the stress response system. As a consequence, some individuals develop a persistent vulnerability to stressors. This maladaptive vulnerability accompanies them in adult life, potentially making such individuals more susceptible to developing depression or anxiety disorders [41, 42].

Score above cutoff on the AUDIT questionnaire, possibly indicating alcohol use problems, was related to level of

**Table 2** Median levels (25th and 75th percentile) of peripheral circulating cytokines according to clinical characteristics of the included patients

| Variable | | IL-1β (pg/mL) | | IL-1RA (pg/mL) | | TNF-α (pg/mL) | | MCP-1 (pg/mL) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Median (25th, 75th percentile) | Sig.[a] | Median (25th, 75th percentile) | Sig.[a] | Median (25th, 75th percentile) | Sig.[a] | Median (25th, 75th percentile) | Sig.[a] |
| Demography | | | | | | | | | |
| Men | *n* = 36 | 0.003 (0.003, 0.130) | 0.604 | 26.2 (17.8, 46.1) | 0.804 | 0.080 (0.046, 1.390) | 0.346 | 27.7 (15.3, 43.7) | 0.069 |
| Women | *n* = 91 | 0.030 (0.003, 0.160) | | 27.5 (17.4, 40.3) | | 0.420 (0.046, 2.450) | | 21.1 (11.5, 32.6) | |
| Age | | 0.033 | 0.716 | 0.141 | 0.114 | 0.068 | 0.450 | 0.027 | 0.762 |
| Psychometrics | | | | | | | | | |
| AUDIT (under cutoff score) | *n* = 95 | 0.003 (0.003, 0.160) | 0.730 | 24.8 (16.5, 42.5) | 0.342 | 0.420 (0.046, 1.930) | 0.768 | 20.8 (11.4, 33.1) | 0.497 |
| AUDIT (over cutoff score) | *n* = 32 | 0.050 (0.003, 0.128) | | 28.1 (20.5, 39.0) | | 0.420 (0.046, 2.393) | | 24.8 (10.4, 40.3) | |
| BDI-2 (minimal and mild depression) | *n* = 27 | 0.003 (0.003, 0.200) | 0.840 | 24.0 (15.4, 41.1) | 0.564 | 0.420 (0.046, 2.450) | 0.973 | 23.7 (14.4, 40.4) | 0.277 |
| BDI-2 (moderate and severe depression) | *n* = 100 | 0.025 (0.003, 0.130) | | 27.5 (17.5, 42.9) | | 0.245 (0.046, 2.108) | | 21.9 (12.4, 34.1) | |
| Medication | | | | | | | | | |
| Anti-inflammatory drugs (no) | *n* = 108 | 0.003 (0.003, 0.130) | *0.050*[b] | 27.5 (17.4, 42.8) | 0.634 | 0.180 (0.046, 2.008) | 0.818 | 22.9 (13.2, 34.3) | 0.095 |
| Anti-inflammatory drugs (yes) | *n* = 19 | 0.090 (0.003, 0.300) | | 24.8 (17.5, 34.3) | | 1.020 (0.060, 3.410) | | 22.2 (10.7, 38.6) | |
| Antidepressants (no) | *n* = 85 | 0.040 (0.003, 0.160) | 0.510 | 27.5 (17.0, 42.5) | 0.654 | 0.420 (0.046, 2.455) | 0.370 | 24.3 (14.8, 37.9) | 0.126 |
| Antidepressants (yes) | n = 42 | 0.003 (0.003, 0.133) | | 26.7 (19.6, 41.0) | | 0.185 (0.046, 1.250) | | 19.3 (10.6, 33.1) | |
| History of trauma | | | | | | | | | |
| Childhood trauma (no) | *n* = 50 | 0.003 (0.003, 0.123) | 0.379 | 24.5 (16.3, 39.4) | 0.215 | 0.090 (0.046, 1.060) | 0.060 | 23.8 (14.7, 37.7) | 0.576 |
| Childhood trauma (yes) | *n* = 62 | 0.012 (0.003, 0.218) | | 28.1 (19.9, 41.3) | | 0.920 (0.046, 3.068) | | 22.9 (10.4, 36.9) | |
| Adult trauma (no) | *n* = 53 | 0.003 (0.003, 0.100) | 0.235 | 22.8 (17.0, 35.5) | 0.097 | 0.310 (0.046, 2.095) | 0.786 | 23.3 (13.4, 33.1) | 0.438 |
| Adult trauma (yes) | *n* = 59 | 0.060 (0.003, 0.170) | | 28.3 (18.7, 42.3) | | 0.420 (0.046, 2.450) | | 24.9 (10.7, 43.0) | |
| Any trauma (no) | *n* = 30 | 0.003 (0.003, 0.100) | 0.154 | 21.7 (14.8, 30.7) | *0.048*[c] | 0.070 (0.046, 0.775) | *0.033*[d] | 23.5 (16.9, 32.7) | 0.838 |
| Any trauma (yes) | *n* = 83 | 0.040 (0.003, 0.190) | | 28.4 (18.7, 42.1) | | 0.920 (0.046, 2.900) | | 23.7 (10.6, 38.6) | |
| Main diagnosis[e] | | | | | | | | | |
| Mood disorder (no) | *n* = 80 | 0.060 (0.003, 0.200) | *0.037*[f] | 27.1 (17.3, 41.7) | 0.982 | 0.390 (0.046, 2.450) | 0.634 | 22.8 (11.6, 38.4) | 0.829 |
| Mood disorder (yes) | n = 32 | 0.003 (0.003, 0.060) | | 26.9 (20.4, 39.3) | | 0.310 (0.046, 2.045) | | 24.1 (14.1, 33.4) | |
| Anxiety disorder (no) | *n* = 55 | 0.003 (0.003, 0.090) | *0.008*[g] | 25.5 (17.5, 37.6) | 0.325 | 0.060 (0.046, 1.780) | 0.111 | 23.8 (13.9, 33.1) | 0.740 |
| Anxiety disorder (yes) | *n* = 57 | 0.090 (0.003, 0.270) | | 29.3 (18.7, 44.1) | | 0.920 (0.046, 3.340) | | 23.3 (10.0, 43.0) | |
| Eating disorder (no) | n = 91 | 0.003 (0.003, 0.160) | 0.429 | 28.1 (18.7, 42.1) | 0.171 | 0.420 (0.046, 2.460) | 0.561 | 23.7 (13.2, 38.6) | 0.414 |
| Eating disorder (yes) | *n* = 21 | 0.003 (0.003, 0.115) | | 22.3 (15.7, 36.0) | | 0.100 (0.046, 1.905) | | 22.2 (11.8, 31.3) | |

Notes: [a]Significance levels for categorical variables were tested with the Mann-Whitney *U* test and for continuous variables with Spearman's rho correlation. [b]Patients using anti-inflammatory drugs had higher levels of IL-1β. Exact p-value: 0.0498. [c + d]Patients with trauma history had higher levels of IL-1RA and TNF-α. [e]Main diagnosis: Mood disorders: F30-F34, Anxiety disorders: F40-F44, Eating disorders: F50.0-F50.3. [f]Patients with mood disorders had higher levels of IL-1β. [g]Patients with anxiety disorders had higher levels of IL-1β

depression. However, we found no relationship between levels of cytokines and AUDIT. This differed from our previous cross-sectional study in Nepal, where we found increased pro-inflammatory cytokines in AUD (alcohol use disorder) patients with comorbid MDD, but not with PTSD [43, 44]. In line with the findings in that study, we investigated whether there were different relationships between cytokine levels and levels of depression in those with or without an alcohol problem, but analyzing the data in this way by stratifying according to AUDIT score did not reveal any such effect in the present study. The literature suggests that low levels of drinking might dampen an immunological response, while high levels may increase circulating cytokines [18]. In the current study, patients scored quite low on the AUDIT, indicating low levels of alcohol problems. This might explain the lack of relationship between cytokines and AUDIT score.

When healthy volunteers were compared to a group of matched patients, the patients were found to have higher levels of the cytokines TNF-α and MCP-1. Although some

**Table 3** Mean age (SD) and mean levels (SD) of peripheral circulating cytokines in healthy volunteers and a subgroup of matched patients

| Variable | | Matched patients | Healthy volunteers | Sig.[a] |
|---|---|---|---|---|
| | | n = 19 | n = 19 | |
| Age | (years) | 42.5 (7.9) | 28.8 (5.4) | *<0.001*[b] |
| Il-1β | (picograms/ml) | 0.15 (0.31) | 0.13 (0.19) | 0.740 |
| Il-1RA | (picograms/ml) | 36.3 (33.0) | 35.4 (19.5) | 0.385 |
| TNF-α | (picograms/ml) | 2.67 (6.67) | 0.82 (2.75) | *< 0.001*[c] |
| MCP-1 | (picograms/ml) | 26.8 (21.4) | 15.8 (15.5) | *0.012*[d] |

Note: [a]Significance levels were tested by the Mann-Whitney *U* test. [b]Patients were older than the healthy volunteers. [c+d] Levels of TNF-α and MCP-1 were higher in patients Both healthy volunteers and matched patients were male

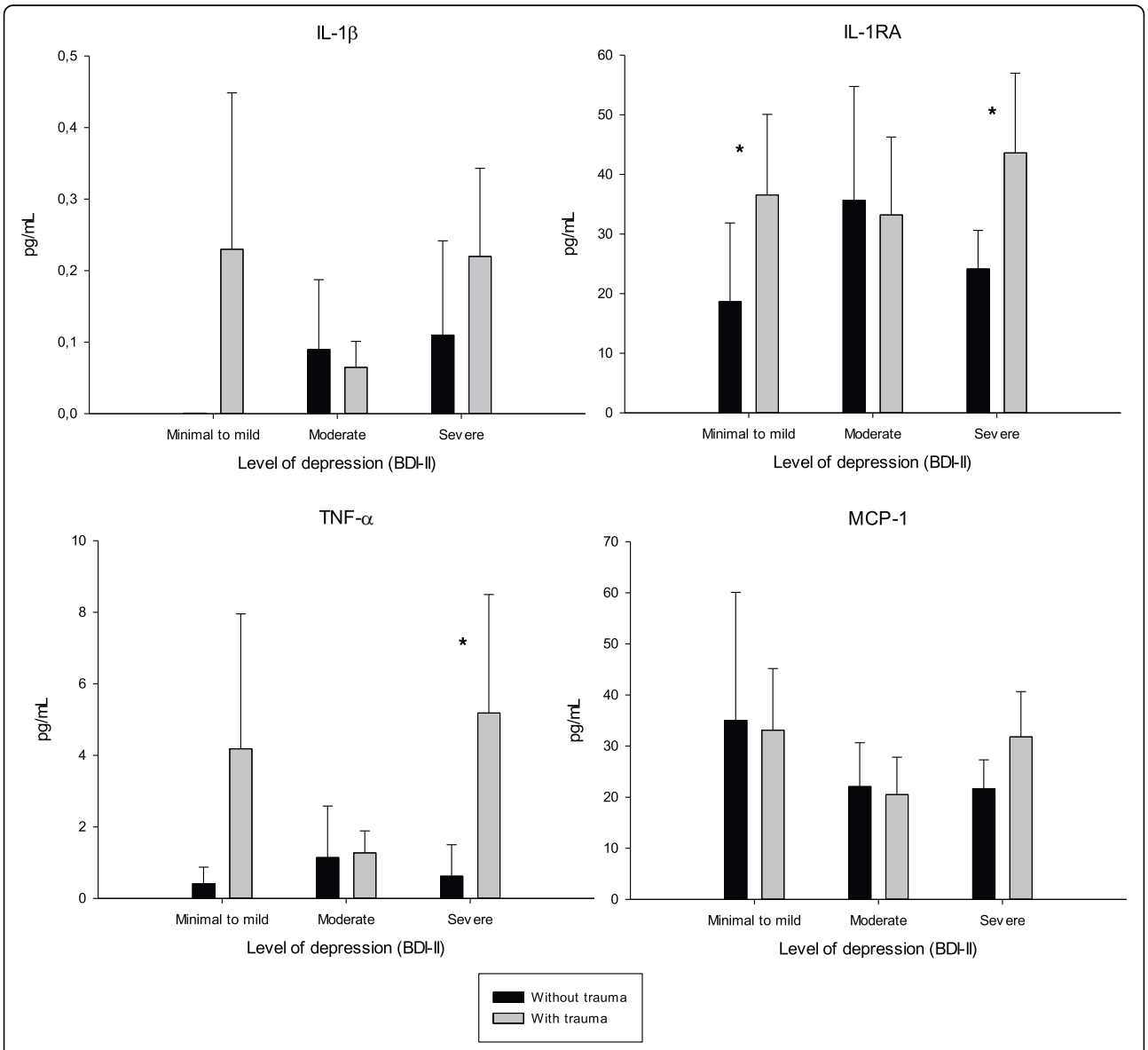


**Fig. 1** Distribution of cytokine levels (mean, upper bound of 95% CI) in depression groups for non-traumatized vs. traumatized patients (any trauma; childhood or adult) grouped by the Beck Depression Inventory-II. An asterisk (*) denotes a significant difference between groups (Mann-Whitney *U*-test) ($p < 0.05$)

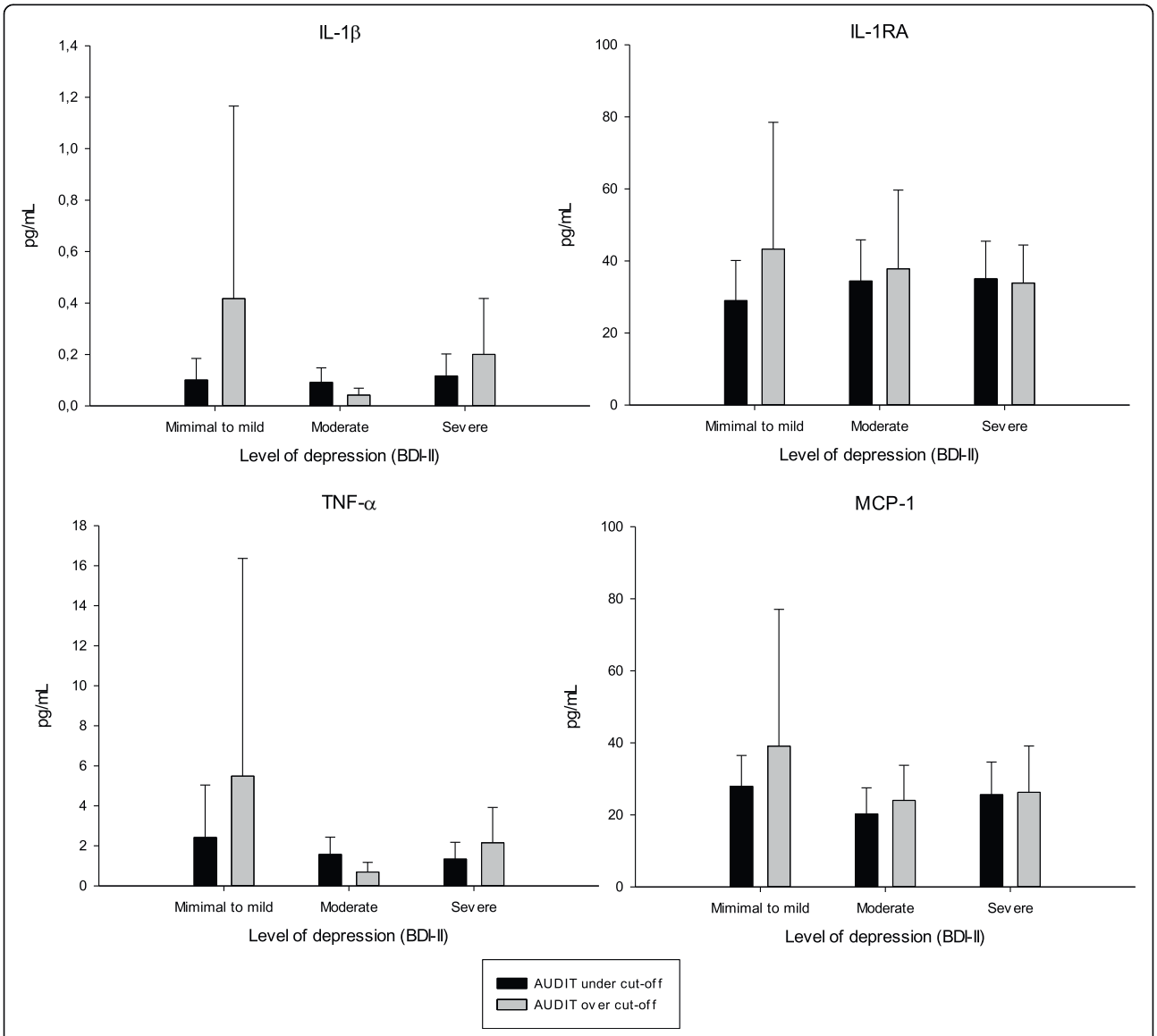


**Fig. 2** Distribution of cytokine levels (mean, upper bound of 95% CI) in depression groups for patients scoring below vs. above clinical cutoff for AUDIT, grouped by Beck Depression Inventory-II

studies have found MCP-1 to be lower in patients with MDD [45], our findings of higher levels of TNF-α and MCP-1 in the patient group add to several studies showing a correlation between psychiatric disorders and elevated pro-inflammatory cytokines [46]. This general heightened cytokine level among the patients could contribute to masking differences in cytokines between different levels of depression in the patients.

There are limitations to this study. The 19 healthy volunteers were younger than the 19 youngest matched male patients. Aging is associated with higher risk of comorbidity, and these factors may result in increased levels of TNF-α and IL-1 [47]. The healthy volunteers were male only, which was unfortunate since the majority of the patients were female. Unlike the healthy volunteers, the patients were not fasting when the blood samples were taken. Eating three meals a day versus one meal a day has been shown to elevate levels of TNF-α and MCP-1, suggesting eating habits could mediate inflammatory pathways [48]. The Body Mass Index (BMI) of the patients was not assessed in this study. Studies have shown the levels of pro-inflammatory cytokines to be higher in obese people (BMI > 30 kg/m2). Adipocytes are known to release circulating cytokines, notably TNF-α, IL-1β and IL-6 [49], and one cannot exclude the possibility of BMI confounding the cytokine levels. Trauma history was recorded during clinical interviews using five standardized questions regarding child and/or adult physical or sexual

abuse. These questions are less valid than validated trauma questionnaires, but provide face validity since they were discussed with each patient by trained staff. Furthermore, the study is cross-sectional and should be followed by longitudinal studies. We decided to report the results with uncorrected *p*-values. Using Bonferroni correction or similar would be an overly conservative approach when conducting multiple tests in an exploratory study. Consequently, there is also a risk of type I errors. Against this background, one should interpret the results with caution. Despite these limitations, this study fills gaps in the literature by elucidating cytokine levels across trauma and depression symptoms. Finally, there is a risk of type II errors when analyzing such small samples.

## Conclusions

Patients with a history of trauma had higher levels of the cytokines IL-1RA and TNF-α. There were no associations between cytokine levels and depression severity, even when analyzing in sub-groups stratified according to variables that themselves were related to cytokine level like trauma and alcohol use.

#### Abbreviations

AUD: Alcohol Use Disorder; AUDIT: Alcohol Use Disorders Identification Test; BDI-II: Beck Depression Inventory-II; HPA axis: Hypothalamic-pituitary-adrenal axis; ICD-10: International Classification of Diseases and Related Health Problems 10th revision; IL-1RA: Interleukin-1 Receptor Antagonist; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; MCP-1: Monocyte Chemoattractant Protein-1; MDD: Major Depression Disorder; MINI: Mini-international Neuropsychiatric Interview; PTSD: Post-traumatic Stress Disorder; TNF-α: Tumor Necrosis Factor-alpha

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#### Availability of data and materials

The data and material in this study is not publicly available due to confidentiality reasons. It is however available on reasonable request.

#### Authors' contributions

The study was designed and planned by LL, JGB, SPN, BW and TT. HT recruited the participants, collected blood samples, and was involved in collecting the psychometric questionnaires. All authors have been involved in interpreting data, and in writing and reviewing the manuscript. The final manuscript was read and approved by all authors.

#### Ethics approval and consent to participate

All participants gave their written, informed consent before enrolment in the study. The study was approved by the South-Eastern Norway Regional Committee for Medical and Health Research Ethics (Reference number 2014/2189). The Declaration of Helsinki was followed when this study was conducted.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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II

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ORIGINAL RESEARCH

# PTSD patients show increasing cytokine levels during treatment despite reduced psychological distress

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**Background:** A reciprocal relationship between activated innate immune system and changes in mood and behavior has been established. There is still a paucity of knowledge on how the immune system responds during psychiatric treatment. We aimed to explore circulating cytokines and assess psychiatric symptom severity scores during 12 weeks of inpatient psychiatric treatment.

**Methods:** The study was a longitudinal assessment of 124 patients (88 women and 36 men) in treatment at Modum Psychiatric Center, Norway. The patient sample comprised a mixed psychiatric population of whom 39 were diagnosed with posttraumatic stress disorder (PTSD). Serum blood samples for cytokine analysis and measures of mental distress using Global Severity Index were collected at admission ($T_0$), halfway ($T_1$), and before discharge ($T_2$). Other factors assessed were age, gender, and the use of antidepressants and anti-inflammatory drugs. Multilevel modeling was used for longitudinal analyses to assess the repeated cytokine samples within each patient.

**Results:** Overall level of IL-1RA was higher in PTSD patients when compared to those without PTSD ($P$=0.021). The level of IL-1β, MCP-1, and TNF-α increased over time in PTSD compared to non-PTSD patients ($P$=0.025, $P$=0.011 and $P$=0.008, respectively). All patients experienced reduced mental distress as measured by self-reported Global Severity Index scores. Stratified analysis showed that PTSD patients who used anti-inflammatory drugs had higher levels of IL-1β ($P$=0.007) and TNF-α ($P$=0.049) than PTSD patients who did not use such drugs.

**Conclusion:** The study indicates that traumatized patients may have a distinct neuroimmune development during recovery. Their activated immune system shows even further activation during their rehabilitation despite symptom reduction.

**Keywords:** cytokines, trauma, inflammation, PTSD, immune activation

## Introduction

Patients with various psychiatric disorders have been found to exhibit a low-grade inflammatory state characterized by elevated levels of pro- and anti-inflammatory cytokines.[1,2] In particular, major depression disorder (MDD) and inflammation has been extensively studied in the recent years. Cytokine levels have been found to be elevated in depression[3] and to be reduced over time following treatment.[4]

The large body of evidence on heightened circulating cytokines in MDD patients contrasts the conflicting and lesser evidence on immune response in patients with PTSD.[5] Previous research has shown both altered immune response and no difference from healthy controls in cross-sectional assessments.[6] Further, some studies have reported that recovery from PTSD and MDD is accompanied by reduction in levels



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of various pro- and anti-inflammatory cytokines, even to the levels of healthy controls, regardless of treatment modality or outcome.[7,8] Such correlations highlight the possibility that specific immune markers may be useful in monitoring and intervening in the involved neuroimmune dysregulation.[9] Treatment of patients with PTSD could improve not only symptoms but also rebalance the chronic inflammatory state, as measured by levels of circulating inflammatory cytokines.

Inconsistent findings in previous research could be due to various confounders like the use of anti-inflammatory drugs,[10] which might alter inflammatory activity by inhibiting production of enzymes COX-1 and COX-2.[11] Such drugs have been reported to relieve depression symptoms by reducing levels of IL-6,[12] and also attenuate effects of the antidepressive selective serotonin reuptake inhibitors drugs when used in combination.[13]

We have earlier found bivariate associations between trauma and IL-1RA and TNF-α in a cross-sectional assessment,[14] and we decided to explore these relationships longitudinally. Research suggests that the immunological response in PTSD patients differs from the response in psychiatric patients without PTSD. On this background, we hypothesized that patients with PTSD might have higher cytokine levels and possibly increasing trajectories over time, and thus exhibit an inflammatory pattern different from other patients. The aim was to explore levels and development of cytokines in PTSD patients vs patients without PTSD, and see this in light of their self-reported levels of mental distress, taking the use of anti-inflammatory drugs into account.

# Materials and methods

## Study participants and recruitment procedure

The study is part of a larger research project and some methods have been presented elsewhere.[14] The patients were recruited from a high-threshold psychiatric center in Norway treating patients with depression, anxiety, and eating or trauma disorders. Enrolled patients have previously tried treatment elsewhere with no or little success, many of them for years. They were offered a 12 weeks inpatient treatment program where a multidisciplinary staff provided psychotherapy treatment and psychoeducation both in groups and individually. Psychopharmacological treatment was used to some extent, but hospital policy restricted the use of such drugs to a minimum. Patients with self-destructive behavior, severe substance abuse, or psychotic disorders were not treated here, but some patients may have had comorbid addiction. Data were collected from March 2015 until April 2016 by the first author. The patients received written and oral information about the study during one of their first group sessions. Of the 249 patients approached, 148 (59%) gave their written consent. One patient chose to withdraw her consent. We identified 19 patients to have extreme cytokine levels above the 95th percentile, and they were excluded due to suspected ongoing infections. One additional female patient was excluded from cytokine analyses due to failed venipuncture, and lastly 4 patients were identified as outliers and excluded after residual diagnostics was conducted. The study thus consisted of 88 women (71%, mean age 38.98 years, SD 11.31) and 36 men (29%, mean age 49.06 years, SD 9.36), giving a total of 124 patients. The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics prior to data collection (reference number 2014/2189).

## Methods

A psychiatrist or psychologist at the facility performed the MINI clinical interview,[15] giving diagnosis according to the 10th Revision of the International Classification of Diseases and Related Health Problems (ICD-10). Thirty-one patients met the criteria for PTSD diagnosis as their primary disorder, with an additional 8 with PTSD as secondary diagnosis, 31 patients with mood disorders, 54 with anxiety disorders, and 20 with eating disorders. There were 53 patients diagnosed with 1 disorder, 56 with 2 disorders or more, and 15 patients with no registered disorder due to missing data. These 15 patients were excluded from analyses involving diagnoses. The symptom severity was assessed by the patients themselves using the Hopkins Symptom Checklist (HSCL-90R) questionnaire. The patients filled out the digital questionnaire on a computer or tablet 3 times during their stay during one of the first days ($T_0$), the second at halfway ($T_1$), and the last just before discharge ($T_2$). The 90 questions measure the level of general distress for the last 7 days. The answers to each question range from 0 to 4 and corresponds to "not at all," "a little bit," "moderately," "quite a bit," and "extremely." Mean score is calculated for the HSCL-90R, referred to as the Global Severity Index Index (GSI).[16] The HSCL-90R has been found to provide valid evaluation of the severity of symptoms in a broad range of psychiatric patients.[17] Based on previous literature, we set the cutoff score for caseness at 0.85.[18] The drugs that the patients used during the stay were recorded by the first author by looking into their medical charts.

## Blood collection and serum preparation

The blood samples were also drawn at $T_0$, $T_1$ and $T_2$. The blood samples were collected between 08.00 am and

09.00 am, except for one of the groups from the department of depression, who had their blood drawn between 12.00 am and 3.00 pm. Vacuette 8 mL serum containers were used for blood collection. These were turned upside-down for approximately 8–10 times immediately after the blood was drawn, and set to rest in a blood tube stand for a minimum of 30 minutes and a maximum of 1 hour. They were then centrifuged in a Kubota 2420 swing-out centrifuge set at 10 minutes. The centrifugation power reached 1,917 *g*, and the centrifugal process was conducted at room temperature. Finally, the blood was drawn with 1 mL single-use pipettes into Nunc tubes before they were set to rest in −80°C until assay.

## Cytokine and chemokine measurements

We analyzed 7 cytokines and 1 chemokine based on the available literature on the neuroimmune correlates of psychiatric disorders. The cytokines were IL-1β, IL-1RA, IL-6, IL-10, IL-17A, IFN-y, and TNF-α and the chemokine was MCP-1. Four cytokines had too many values under the limit of detection (LOD) (>55%) and were excluded from the study. The following markers were taken into consideration: IL-1β, IL-IRA, MCP-1 and TNF-α. All blood samples were thawed on ice, vortexed, and then spun down a tube with 250 µL serum at 14,000× *g* for 10 minutes at 4°C, before before a 4 fold dilution and further processing. Cytokine measurements were performed using Bio-Plex xMAP technology (Bio-Rad, Austin, TX, USA) with a Luminex IS 100 instrument (Bio-Rad), powered using Bio-Plex Manager (version 6.0.1) software (Bio-Rad). The assay was performed according to the manufacturer's instructions, but an additional standard point was included. To achieve more reliable results, individual sets of samples from patients were run in the same assay, all samples were assayed in duplicate and a magnetic plate washer was used during assay set up. The StatLIA software package (ver. 3.2, Brendan Scientific, Carlsbad, CA, USA), incorporating a weighted, 5-parameter logistic curve-fitting method, was used to calculate sample cytokine concentrations. Longitudinal controls were used in order to validate interassay variation; IL-1β (18.1), IL-1RA (10.2), MCP-1 (6.7), and TNF-α (7.4). The interassay percent coefficient of variability (CV) in parentheses is a measure of variation between plates, where a lower figure is better. Any figure below 21% is considered acceptable. The mean interassay percent CV for all blood sample plates was 10.4%. The unit of measurement was picograms per milliliter (pg/mL). The LODs were 0.01 pg/mL for IL-1β, 3 pg/mL for IL-1RA, 0.76 pg/mL for MCP-1, and 0.02 pg/mL for TNF-α.

## Imputation

Cytokine levels below LOD were imputed with 1% of the mean value. At $T_0$ we performed 64 (50.39%) imputations for IL-1β, 1 (0.8%) for IL-1RA, 9 (7.1%) for MCP-1, and 53 (41.7%) for TNF-α. One patient did not have the first blood sample collected. At $T_1$, there were 62 imputations (51.2%) for IL-1β, no imputations for IL-1RA, 5 (5.2%) for MCP-1, and 7 (5.8%) for TNF-α. Seven patients did not show up for blood sampling at $T_1$. At $T_2$, we had 64 imputations (53.8%) for IL-1β, no imputations for IL-1RA, 8 (7.2%) for MCP-1, and 45 (37.8%) for TNF-α. Eleven patients did not show up for blood sampling at $T_2$.

## Statistical analyses

The Mann–Whitney *U* Test, the Wilcoxon signed rank test and the Kruskal–Wallis equality-of-populations rank test were used for bivariate analyses at $T_0$ and to assess differences in GSI scores between groups at $T_0$, $T_1$, and $T_2$. Multilevel modeling was conducted for longitudinal data analyses.[19] The material comprised 3 observations of each patient, which gave a 2-level structure with cytokine measurements nested within patients across time. Due to the dependency of cytokine measurements within patients, multilevel modeling was chosen as the statistical method for the longitudinal results. In multilevel modeling, all available data is used. Thus, a patient lacking data from one measurement is still included and contributes to estimation of model parameters. We used a stepwise modeling approach, where the multilevel models were initially run as empty models including only the dependent variable and random intercept. Next, a random slope was added in models. The −2 Log Likelihood and Bayes Information Criterion (BIC) were performed to assess model fit. A model with random intercept and slope gave a better fit for all cytokines than a model with random intercept only. Patients differed at cytokine levels at treatment initiation, and allowing the patients to have different slopes of cytokines during treatment improved model fit. The following likelihood ratio tests were performed: IL-1β: $\chi^2(2) = 17.96$, $P \leq 0.001$, IL-1RA: $\chi^2(2) = 15.09$, $P \leq 0.001$, MCP-1: $\chi^2(2) = 12.90$, $P \leq 0.001$, and TNF-α: $\chi^2(2) = 55.85$, $P \leq 0.001$. The fixed and random effects are presented in Table S1. The assumption of homoscedastic residual variance and normally distributed residuals was inspected by plotting residual distribution in histograms and QQ-plots with Gauss curves. It was formally confirmed with likelihood ratio tests that the residual variance differed over time for IL-1β ($\chi^2[2] = 14.01$, $P < 0.001$), for TNF-α ($\chi^2[2] = 7.20$, $P = 0.027$), and for IL-1RA ($\chi^2[2] = 55.02$, $P < 0.001$). Chemokine MCP-1 did not improve model fit when we allowed for heteroscedastic residuals

($\chi^2$[2] =3.27, *P*=0.195), and thus the assumption of homoscedastic residuals was considered met for MCP-1. Consequently, we allowed for heteroscedastic residual distribution for IL-1β, IL-1RA, and TNF-α, which gave a block diagonal structure in the covariance matrix for the residuals, allowing the estimates in each block to differ. For all four markers, we applied robust standard errors to account for nonnormally distributed errors. The assumption of linearity was confirmed by visually inspecting spaghetti plots of cytokine development. Unstructured covariance was chosen due to the low number of repeated measurements. All multilevel models were in addition adjusted for age and gender, which did not significantly alter the *P*-values. Consequently, we chose to present the results without this adjustment. Some patient groups had different lengths of inpatient stay due to holiday seasons and due to minor differences between departments. Because of this inequality, the average length of stay for all patient groups was calculated. This gave a time variable encoded as 0 ($T_0$), 5 weeks ($T_1$), and 11 weeks ($T_2$) of stay. For bivariate analyses, we attempted to normalize the skewed cytokine data by log transformation. The Kolmogorov–Smirnov test of normality remained significant after log transformation. We also explored bivariate analyses with and without log transformed data and found no significant changes. We then decided to run all analyses with nontransformed data. Those who did not attend every blood sample collection were defined as missing completely at random (MCAR), which indicated no specific pattern of missing data. Consequently, there was no increased variability which could bias the regression coefficients. MCAR was confirmed by Little's MCAR test[20] with $\chi^2$=9.73 (*P*=0.973). All tests were 2-sided, and *P*-values below 0.05 were considered statistically significant. No correction for multiple hypothesis testing was implemented as we considered the study to be exploratory. The statistical package STATA (StataCorp. 2015, Stata Statistical Software: Release 15, StataCorp LP, College Station, TX, USA) was used for all statistical analyses.

## Results

Table 1 shows the mean cytokine levels across demographic variables and sample characteristics at $T_0$. Male patients had higher levels of chemokine MCP-1 (*P*=0.046). Patients who used anti-inflammatory drugs were found to have higher levels of cytokine IL-1β (*P*=0.038), and almost reached significance in levels of TNF-α (*P*=0.062). Having PTSD diagnosis was almost related to higher levels of cytokine IL-1RA (*P*=0.074).

When analyzing the main effect of PTSD and the effect of PTSD in interaction with time on cytokines (Table 2), we found that the overall level of IL-1RA was higher in PTSD than in non-PTSD patients (*P*=0.021). Patients with PTSD showed increasing development of IL-1β (*P*=0.025), MCP-1

**Table 1** The association between sample characteristics and distribution of cytokine levels at $T_0$

| Variables | n | IL-1β, mean (SD) | *P*-value[a] | IL-1RA, mean (SD) | *P*-value | MCP-1, mean (SD) | *P*-value | TNF-α, mean (SD) | *P*-value |
|---|---|---|---|---|---|---|---|---|---|
| Gender | | | | | | | | | |
| Female | 87 | 0.121 (0.032) | 0.698 | 33.894 (2.913) | 0.728 | 23.488 (1.900) | **0.046** | 2.287 (0.548) | 0.487 |
| Male | 36 | 0.153 (0.052) | | 37.971 (5.821) | | 31.735 (3.997) | | 2.581 (1.254) | |
| Age | | | | | | | | | |
| 18–29 | 25 | 0.124 (0.045) | 0.537 | 27.822 (4.104) | 0.256 | 28.716 (4.310) | 0.599 | 1.358 (1.358) | 0.394 |
| 30–49 | 62 | 0.151 (0.045) | | 36.567 (3.757) | | 23.639 (2.164) | | 2.331 (0.830) | |
| 50–66 | 36 | 0.145 (0.042) | | 37.585 (5.735) | | 27.844 (3.920) | | 3.152 (1.071) | |
| GSI | | | | | | | | | |
| Under 0.85 | 25 | 0.091 (0.030) | 0.554 | 34.643 (6.336) | 0.945 | 29.084 (4.673) | 0.642 | 2.062 (0.904) | 0.935 |
| Above 0.85 | 98 | 0.157 (0.033) | | 35.200 (2.946) | | 25.090 (1.931) | | 2.453 (0.627) | |
| Anti-inflammatory drugs | | | | | | | | | |
| No | 104 | 0.123 (0.026) | **0.038** | 36.425 (3.088) | 0.687 | 25.701 (1.981) | 0.732 | 2.160 (0.557) | 0.062 |
| Yes | 19 | 0.255 (0.101) | | 27.766 (3.083) | | 27.002 (4.459) | | 3.541 (1.606) | |
| Antidepressants | | | | | | | | | |
| No | 83 | 0.137 (0.0287) | 0.490 | 33.598 (2.862) | 0.602 | 27.660 (2.356) | 0.121 | 2.840 (0.716) | 0.276 |
| Yes | 40 | 0.157 (0.059) | | 38.179 (5.678) | | 22.253 (2.570) | | 1.405 (0.666) | |
| PTSD diagnosis | | | | | | | | | |
| No | 69 | 0.101 (0.021) | 0.524 | 31.691 (3.046) | 0.074 | 26.065 (2.106) | 0.613 | 2.221 (0.749) | 0.492 |
| Yes | 39 | 0.213 (0.069) | | 41.563 (5.481) | | 24.847 (3.162) | | 3.251 (1.007) | |

**Notes:** [a]*P*-values for dichotomous variables calculated with the Mann–Whitney *U* test. *P*-values for age variable calculated with the Kruskal–Wallis equality-of-populations rank test. Statistically significant *P*-value <0.05 shown in bold.
**Abbreviations:** GSI, Global Severity Index; PTSD, posttraumatic stress disorder.

**Table 2** The effect of PTSD disorder on cytokine level and development

| Variables | IL-1β | | IL-1RA | | MCP-1 | | TNF-α | |
|---|---|---|---|---|---|---|---|---|
| | Main effect of PTSD | PTSD interaction with time | Main effect of PTSD | PTSD interaction with time | Main effect of PTSD | PTSD interaction with time | Main effect of PTSD | PTSD interaction with time |
| | β (SE)[a] | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) |
| Fixed effects | | | | | | | | |
| Intercept | 0.106 (0.0208)[b] | 0.102 (0.020)[b] | 29.754 (2.456)[b] | 31.059 (2.491)[b] | 24.942 (2.013)[b] | 25.746 (2.052)[b] | 2.075 (0.741)[b] | 2.221 (0.747)[c] |
| Slope (time) | 0.004 (0.002) | −0.0007 (0.002) | −0.101 (0.132) | −0.285 (0.139)[d] | −0.102 (0.115) | −0.325 (0.135)[d] | 0.064 (0.034) | −0.016 (0.023) |
| PTSD | 0.101 (0.0706) | 0.111 (0.071) | 12.332 (5.344)[d] | 8.780 (5.725) | 2.217 (3.683) | 0.013 (3.723) | 1.436 (1.263) | 1.030 (1.247) |
| Slope × PTSD | | 0.014 (0.006)[d] | | 0.494 (0.293) | | 0.599 (0.236)[d] | | 0.219 (0.082)[c] |
| Random effects | | | | | | | | |
| Intercept | 0.080 (0.032)[d] | 0.080 (0.032)[d] | 535.628 (174.583)[b] | 533.579 (177.964)[b] | 285.666 (60.745) | 284.685 (59.958) | 38.319 (15.841) | 38.282 (15.694)[d] |
| Slope (time) | <0.001 (<0.001) | <0.001 (<0.001) | 0.189 (0.818) | 0.155 (0.791) | 0.683 (0.2638) | 0.598 (0.239) | 0.104 (0.053) | 0.093 (0.046)[d] |
| Intercept × slope | <0.001 (0.001) | <0.001 (0.001) | −1.221 (5.536) | −0.982 (5.698) | −1.426 (2.800) | −1.145 (2.471) | −0.229 (0.401) | −0.206 (0.334) |
| Fit index[e] | | | | | | | | |
| −2 LL | −41 | −38 | −1,334 | −1,332 | −1,245 | −1,242 | −845 | −840 |
| BIC | 135 | 133 | 2,719 | 2,727 | 2,531 | 2,530 | 1,743 | 1,738 |

**Notes:** [a]Regression coefficient. [b]$P<0.001$. [c]$P<0.01$. [d]$P<0.05$. [e]−2 LL.
**Abbreviations:** −2 LL, −2 log likelihood; BIC, Bayes information criterion; PTSD, posttraumatic stress disorder; SE, robust standard error.

($P$=0.011), and TNF-α ($P$=0.008). The initial levels and decreasing GSI score in all patients are visualized in Figure 1. Bivariate analysis of GSI score differed between PTSD and non-PTSD at $T_0$ ($P$=0.007) and $T_2$ ($P$=0.001). Patients without PTSD decreased from $T_0$ to $T_1$ ($P$=0.017), from $T_1$ to $T_2$ ($P$=0.003), and from $T_0$ to $T_2$ ($P \leq 0.001$). Patients with PTSD decreased from $T_0$ to $T_1$ ($P$=0.024) and from $T_0$ to $T_2$ ($P$=0.051). The level and development of cytokines IL-1β, IL-1RA, and TNF-α and chemokine MCP-1 are illustrated in Figure 2A–D.

Those of the general patient sample who used anti-inflammatory drugs during treatment (n=27) were found to have higher overall levels of IL-1β ($P<0.05$) than those who did not (n=97) use such drugs (Table 3). To disentangle the relationship between cytokines, PTSD, and anti-inflammatory drugs, we stratified PTSD disorder and ran the anti-inflammatory drugs variable as a predictor and in interaction with time. The patients with PTSD (Table S2) who used anti-inflammatory drugs (n=12) had a higher overall level of IL-1β ($P$=0.007) as compared to PTSD patients who did not use such drugs (n=28). We also found a higher overall level of TNF-α in this strata ($P$=0.049). The patients without PTSD (Table S3) who used anti-inflammatory drugs (n=13) had an overall level of MCP-1 which was higher ($P$=0.025) than in those who did not use such drugs (n=56).

## Discussion

In this study, having PTSD disorder was related to higher overall level of anti-inflammatory cytokine IL-1RA when compared to patients without PTSD diagnosis. An increasing development of proinflammatory cytokines IL-1β and TNF-α and of proinflammatory chemokine MCP-1 was found. The increasing development during treatment occurred in parallel

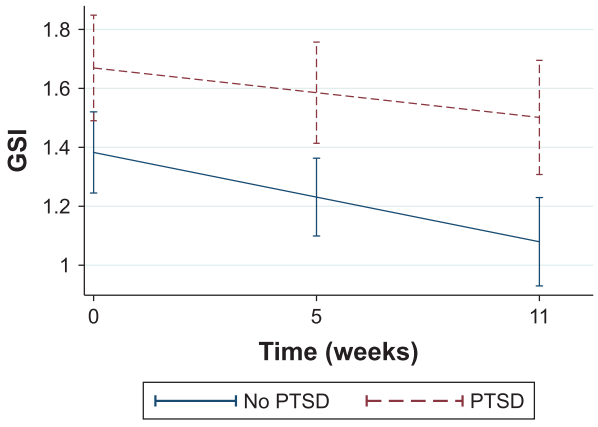


**Figure 1** Levels and development (95% CI) of GSI stratified on PTSD diagnosis.
**Abbreviations:** GSI, Global Severity Index; PTSD, posttraumatic stress disorder.

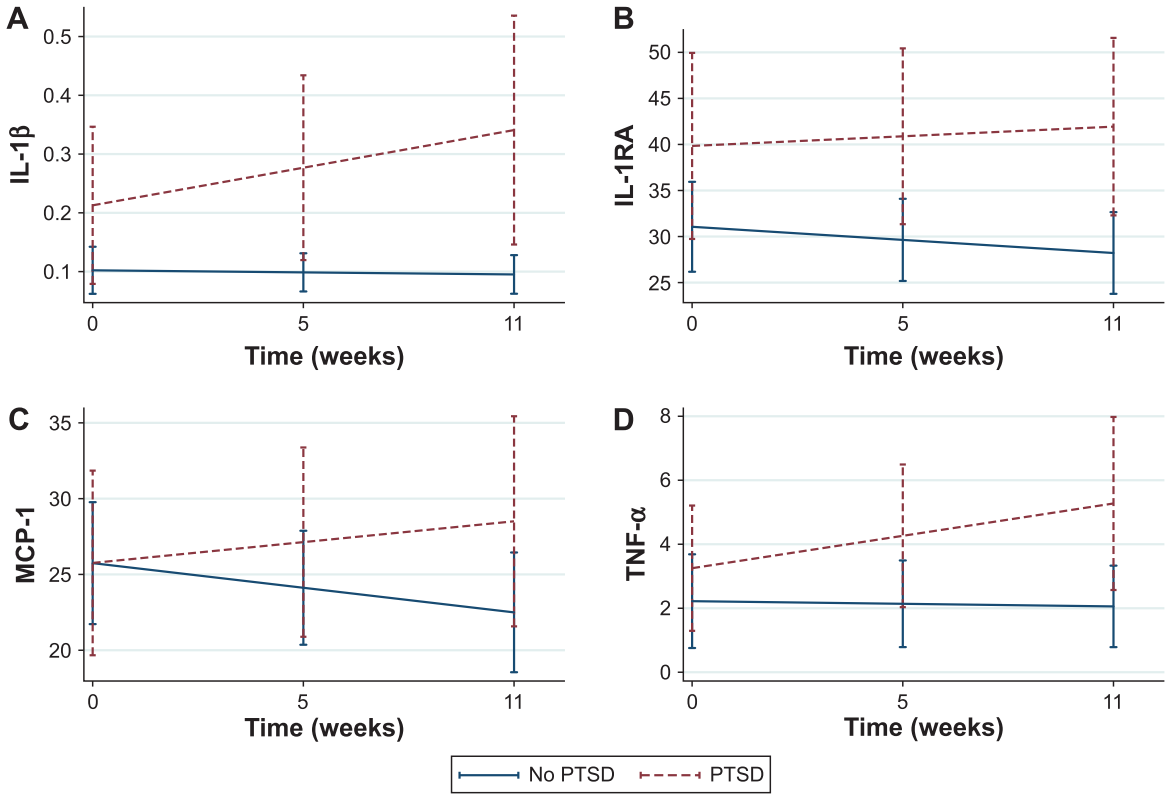


**Figure 2** Levels and development (95% CI) of IL-1β, IL-1RA, MCP-1, and TNF-α stratified on PTSD diagnosis.
**Notes:** (**A**) The initial level and development (95% CI) over time for cytokine IL-1β stratified on PTSD diagnosis. (**B**) The initial level and development (95% CI) over time for cytokine IL-1RA stratified on PTSD diagnosis. (**C**) The initial level and development (95% CI) over time for chemokine MCP-1 stratified on PTSD diagnosis. (**D**) The initial level and development (95% CI) over time for cytokine TNF-α stratified on PTSD diagnosis.
**Abbreviation:** PTSD, posttraumatic stress disorder.

with a decreasing level of symptom severity, as indicated by the self-reported GSI score.

Our findings are in accordance with some, but not all previous research. Studies have found PTSD symptoms to decrease over time,[10] with increasing IL-1β levels,[21] but also with decreasing IL-1β levels.[22] Increasing levels of TNF-α have also been found despite decreasing symptom severity.[23] The patients who received inpatient psychotherapy, as our patients did, showed increasing cytokine levels during treatment. Possibly, PTSD patients in inpatient intensive treatment reexperience previous trauma through talk therapy or by exposure therapy, while nontraumatized individuals may not have such an experience in their therapy sessions. Also, patients suffering from PTSD are known to have disruption in their hypothalamic-pituitary-adrenal (HPA) axis vs immune system feedback and may have adapted to prolonged cortisol release[24] resulting from years of chronic stress, inflammation, and cortisol production. In the short term, these responses would be desirable in attempts to protect the individual, but the chronic inflammatory activation in PTSD patients ultimately results in an impaired feedback regulation due to glucocorticoid resistance.[25]

Cytokine TNF-α is regarded as a key inflammatory cytokine of the immune response,[26] but elevated levels of proinflammatory cytokines TNF-α and IL-1β have simultaneously been found in PTSD patients when compared to controls,[27] and it has been postulated that a combination of several elevated cytokines may be an indicator of PTSD.[26] We also found chemokine MCP-1 and cytokine IL-1β to increase over time in PTSD patients. It may be that increased TNF-α level accompanied by elevated IL-1β and MCP-1 could be viewed as biomarkers in PTSD. The increasing inflammatory response and the declining GSI score over 12 weeks in the current study suggests that PTSD patients who experience reduced psychiatric symptoms do not reflect their psychological improvement at the inflammatory level. Psychiatric symptoms were reduced, but this effect could possibly have been improved to a greater extent if immune rebalancing had occurred in parallel through pharmacotherapy targeting the immune system.[28]

Anti-inflammatory agents have been suggested to reduce IL-1β and play a role in future treatment of PTSD.[10,29] We analyzed anti-inflammatory drug use in the patient sample in

**Table 3** The effect of anti-inflammatory drugs during treatment on cytokine level and development

| Variables | IL-1β | | IL-1RA | | MCP-1 | | TNF-α | |
|---|---|---|---|---|---|---|---|---|
| | Main effect of anti-inflammatory drugs | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time |
| | β (SE)[a] | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) |
| Fixed effects | | | | | | | | |
| Intercept | 0.103 (0.020)[b] | 0.105 (0.021)[b] | 33.581 (2.661)[b] | 33.070 (2.697)[b] | 24.310 (1.998)[b] | 24.712 (2.015)[b] | 1.881 (0.556) | 1.910 (0.558)[b] |
| Slope (time) | 0.003 (0.003) | 0.001 (0.003) | −0.134 (0.123) | −0.068 (0.142) | −0.104 (0.106) | −0.196 (0.113) | 0.060 (0.031) | 0.039 (0.033) |
| Anti-inflammatory | 0.204 (0.096)[c] | 0.196 (0.095)[c] | 2.513 (5.490) | 4.817 (6.361) | 7.569 (4.237) | 5.739 (4.229) | 2.244 (1.471) | 2.112 (1.451) |
| Slope × anti-inflammatory | | 0.007 (0.005) | | −0.291 (0.280) | | 0.407 (0.280) | | 0.091 (0.081) |
| Random effects | | | | | | | | |
| Intercept | 0.061 (0.025)[d] | 0.061 (0.025)[d] | 626.850 (198.956)[b] | 626.202 (196.943)[b] | 349.855 (107.841)[b] | 349.490 (106.831)[b] | 33.657 (13.913)[b] | 33.655 (13.904)[b] |
| Slope (time) | <0.001 (<0.001) | <0.001 (<0.001) | 0.494 (0.896) | 0.494 (0.907) | 0.632 (0.245)[d] | 0.599 (0.230)[d] | 0.095 (0.048)[c] | 0.095 (0.049)[c] |
| Intercept × slope | <0.001 (<0.001) | 0.001 (<0.001) | −3.552 (6.370) | −3.501 (6.163) | −2.415 (2.6142) | −2.318 (2.444) | −0.173 (0.325) | −0.175 (0.319) |
| Fit index[e] | | | | | | | | |
| −2 LL | −51 | −51 | −1,492 | −1,492 | −1,398 | −1,397 | −928 | −928 |
| BIC | 156 | 160 | 3,038 | 3,042 | 2,838 | 2,841 | 1,910 | 1,914 |

**Notes:** [a]Regression coefficient. Twelve patients used anti-inflammatory drugs. [b]$P<0.001$. [c]$P<0.05$. [d]$P<0.01$. [e]−2 LL.
**Abbreviations:** −2 LL, −2 log likelihood; BIC, Bayes information criterion; SE, robust standard error.

general to get an impression of overall levels and development of inflammatory markers. We found a higher level of IL-1β in those using anti-inflammatory drugs. As many studies suggest that PTSD patients differ in immunological response,[30,31] we stratified the sample according to PTSD diagnosis. The overall levels of IL-1β, MCP-1, and TNF-α were higher in PTSD patients who used such drugs. In the strata of patients without PTSD who used anti-inflammatory drugs, there was a significant relationship with level of chemokine MCP-1. This could suggest that patients using anti-inflammatory drugs have a reduced immunological response, but PTSD patients do not seem to benefit from using such drugs. Anti-inflammatory drugs have been postulated as a future remedy in PTSD treatment in a systematic review,[10] and could potentially have contributed to declining cytokine slopes over time if the observation period had been longer than 12 weeks. However, the current results do not lend support to this hypothesis. It should be noted that patients who used anti-inflammatory drugs at any time point during the stay were coded as users regardless of frequency of use. Cytokine levels are likely elevated in such patients due to infections and not due to the use of anti-inflammatory drugs, which makes cytokine levels in this group conditional to confounding by indication. In some respect, our results challenge the therapeutic use of pharmaceuticals with immunomodulating capabilities in treatment of PTSD patients, which has been suggested.[6,10,29] Nevertheless, antidepressive drugs are hypothesized to partly act on mood disorders through anti-inflammatory effects,[12,32] and have been found to have clinical effect on PTSD symptoms.[28,33]

A limitation to the present study is that the patients were not fasting at blood collection time. Meal frequency has been found to be associated with increased cytokine production.[34] Further, body mass index was not measured. Both low and high amount of body fat has been reported to affect production of proinflammatory cytokines.[35] Furthermore, we did not assess smoking status. Smokers have been found to have a higher basal level of cytokines when compared to nonsmokers.[36] It is also important to take into consideration that the patients in the current study had their maladies for many years, many since their childhood, without improving from other psychiatric treatment. As such, they could be regarded as a treatment-resistant population with mental and biological status more trait-like than state-like. This suggests that 12 weeks of observation is a rather short amount of time for observing significant changes. This limits the conclusions that can be drawn. The reader should bear in mind that there is a risk of type 2 errors due to small sample size. The strength of this study is that we utilized a longitudinal design and analyzed

cytokines over time in a mixed psychiatric population with a comparison between PTSD and non-PTSD patients.

## Conclusion

Patients with PTSD had higher levels of some inflammatory markers, and their levels increased during treatment despite a decreasing symptom load.

## Acknowledgment

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## Disclosure

The authors report no conflicts of interest in this work.

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# Supplementary materials

**Table S1** Fixed- and random-effect estimates for cytokines during treatment

| Parameter | IL-1β | IL-1RA | MCP-1 | TNF-α |
|---|---|---|---|---|
| | β (SE)[a] | β (SE) | β (SE) | β (SE) |
| Fixed effects | | | | |
| Intercept | 0.147 (0.027)[b] | 34.143 (2.462)[b] | 25.971 (1.783)[b] | 2.373 (0.531)[b] |
| Slope (time) | 0.003 (0.003) | −0.134 (0.123) | −0.104 (0.106) | 0.060 (0.031)[c] |
| Random effects | | | | |
| Intercept | 0.070 (0.031) | 629.551 (200.348)[b] | 354.747 (104.564)[b] | 34.412 (13.905)[b] |
| Slope (time) | <0.001 (<0.001) | 0.538 (0.899) | 0.633 (0.245)[c] | 0.095 (0.048)[c] |
| Intercept × slope | 0.001 (0.001) | −3.783 (6.295) | −1.833 (2.526) | −0.139 (0.322) |
| Fit index | | | | |
| −2 LL | −56 | −1,492 | −1,400 | −930 |
| BIC | 160 | 3,032 | 2,835 | 1,907 |

**Notes:** [a]Regression coefficient. [b]$P<0.001$. [c]$P<0.05$. Table S1 shows that the whole patient sample at treatment initiation had an overall level (ie, fixed intercept) of cytokine IL-1β at 0.147 pg/mL, of IL-1RA at 37.143 pg/mL, of MCP-1 at 25.971 pg/mL, and of TNF-α at 2.781 pg/mL. These levels were different from zero ($P<0.001$). The significant random intercept variance of IL-1RA, MCP-1, and TNF-α shows that the patients differed significantly from each other at treatment initiation ($P<0.001$). The significant random slope for MCP-1 and TNF-α shows that the development varied between patients over time ($P<0.05$).

**Abbreviations:** −2 LL, −2 log likelihood; BIC, Bayes information criterion; SE, robust standard error.

**Table S2** The effect of anti-inflammatory drugs during treatment on cytokine level and development for patients with PTSD

| Variables | IL-1β | | IL-1RA | | MCP-1 | | TNF-α | |
|---|---|---|---|---|---|---|---|---|
| | Main effect of anti-inflammatory drugs | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time |
| | β (SE)[a] | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) |
| Fixed effects | | | | | | | | |
| Intercept | 0.068 (0.025)[b] | 0.062 (0.028)[b] | 38.907 (5.407)[c] | 38.477 (5.373)[c] | 24.387 (3.634) | 24.459 (3.642)[c] | 1.638 (0.587)[d] | 1.551 (0.596)[c] |
| Slope (time) | 0.012 (0.006)[d] | 0.01 (0.008) | 0.168 (0.255) | 0.231 (0.313) | 0.275 (0.195) | 0.219 (0.216) | 0.202 (0.080)[b] | 0.151 (0.094) |
| Anti-inflammatory | 0.472 (0.175)[d] | 0.489 (0.180)[d] | 3.577 (11.305) | 4.938 (12.888) | 4.459 (7.000)[c] | 4.223 (6.980) | 5.270 (2.677)[b] | 5.550 (2.680)[b] |
| Slope × anti-inflammatory | | 0.006 (0.010) | | −0.200 (0.536) | | 0.181 (0.458) | | 0.164 (0.173) |
| Random effects | | | | | | | | |
| Intercept | 0.129 (0.051)[d] | 0.129 (0.050)[d] | 922.953 (422.806) | 922.826 (417.926)[d] | 325.464 (63.951)[c] | 325.452 (63.838)[c] | 28.807 (11.361)[d] | 28.902 (11.265)[d] |
| Slope (time) | <0.001 (<0.001) | <0.001 (0.001) | 0.406 (1.933) | 0.411 (1.922) | 0.543 (0.353) | 0.536 (0.340) | 0.171 (0.196) | 0.176 (0.203) |
| Intercept × slope | 0.003 (0.002) | <0.001 (<0.001) | −4.401 (13.741) | −4.389 (13.195) | 2.921 (4.335) | 2.930 (4.201) | 0.673 (0.652) | 0.639 (0.585) |
| Fit index[e] | | | | | | | | |
| −2 LL | −23 | −23 | −503 | −503 | −468 | −468 | −334 | −334 |
| BIC | 90 | 94 | 1,049 | 1,053 | 969 | 973 | 711 | 715 |

**Notes:** [a]Regression coefficient. [b]$P<0.05$. [c]$P<0.001$. [d]$P<0.01$. [e]−2 LL.
**Abbreviations:** −2 LL, −2 log likelihood; BIC, Bayes information criterion; PTSD, posttraumatic stress disorder; SE, robust standard error.

**Table S3** The effect of anti-inflammatory drugs during treatment on cytokine level and development for patients without PTSD

| Variables | IL-1β | | IL-1RA | | MCP-1 | | TNF-α | |
|---|---|---|---|---|---|---|---|---|
| | Main effect of anti-inflammatory drugs | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time | Anti-inflammatory as main effect | Anti-inflammatory interaction with time |
| | β (SE)[a] | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) | β (SE) |
| Fixed effects | | | | | | | | |
| Intercept | 0.120 (0.023)[b] | 0.109 (0.023)[b] | 31.268 (2.806)[b] | 30.441 (2.864)[b] | 23.424 (2.152)[b] | 23.998 (2.184)[b] | 2.350 (0.880) | 2.364 (0.912)[c] |
| Slope (time) | −0.002 (0.002) | −0.002 (0.002) | −0.279 (0.139)[d] | −0.177 (0.160) | −0.329 (0.136)[d] | −0.413 (0.141)[c] | −0.015 (0.022) | −0.016 (0.025) |
| Anti-inflammatory | 0.021 (0.065) | −0.017 (0.051) | −1.166 (4.605) | 3.146 (5.687) | 12.362 (5.506)[d] | 9.306 (5.628) | −0.685 (1.443) | −0.757 (1.084) |
| Slope × anti-inflammatory | | <0.001 (0.002) | | −0.539 (0.271)[d] | | 0.450 (0.401) | | 0.004 (0.057) |
| Random effects | | | | | | | | |
| Intercept | 0.018 (0.007)[c] | 0.017 (0.008) | 313.404 (121.226)[c] | 312.002 (123.424)[c] | 251.305 (91.85898)[c] | 250.402 (90.675)[c] | 38.051 (22.540) | 38.051 (22.522) |
| Slope (time) | <0.001 (<0.001) | <0.001 (<0.001) | 0.020 (0.729) | 0.019 (0.709) | 0.639 (0.320)[d] | 0.610 (0.300)[d] | 0.026 (0.010)[c] | 0.026 (0.009)[c] |
| Intercept × slope | −0.0003 (<0.0002) | −0.0004 (<0.001) | 0.722 (3.856) | 0.878 (3.342) | −5.480 (3.255) | −5.436 (3.064) | −0.596 (0.435) | −0.597 (0.435) |
| Fit index[e] | | | | | | | | |
| −2 LL | 29 | 66 | −818 | −817 | −767 | −766 | −444 | −444 |
| BIC | −20 | −80 | 1,683 | 1,896 | 1,571 | 1,575 | 936 | 941 |

**Notes:** [a]Regression coefficient. [b]$P<0.001$. [c]$P<0.01$. [d]$P<0.05$. [e]−2 LL.
**Abbreviations:** −2 LL, −2 log likelihood; BIC, Bayes information criterion; PTSD, posttraumatic stress disorder; SE, robust standard error.

III

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# Cytokine concentrations are related to level of mental distress in inpatients not using anti-inflammatory drugs

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## Original Article

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## Abstract

*Objective:* Cross-sectional data show elevated levels of circulating cytokines in psychiatric patients. The literature is divided concerning anti-inflammatory drugs' ability to relieve symptoms, questioning a causal link between inflammatory pathways and psychiatric conditions. We hypothesised that the development of circulating cytokine levels is related to mental distress, and that this relationship is affected by the use of anti-inflammatory drugs. *Methods:* The study was a longitudinal assessment of 12-week inpatient treatment at Modum Bad Psychiatric Center, Norway. Sera and self-reported Global Severity Index (GSI) scores, which measure psychological distress, were collected at admission ($T_0$), halfway ($T_1$) and before discharge ($T_2$). Other variables known to distort the neuroimmune interplay were included. These were age, gender, diagnosis of PTSD, antidepressants and anti-inflammatory drugs. A total of 128 patients (92 women and 36 men) were included, and 28 were using anti-inflammatory medication. Multilevel modelling was used for data analysis. *Results:* Patients with higher levels of IL-1RA and MCP-1 had higher GSI scores ($p = 0.005$ and $p = 0.020$). PTSD patients scored higher on GSI than non-PTSD patients ($p = 0.002$). These relationships were mostly present among those not using anti-inflammatory drugs ($n = 99$), with higher levels of IL-1RA and MCP-1 being related to higher GSI score ($p = 0.023$ and 0.018, respectively). Again, PTSD patients showed higher GSI levels than non-PTSD patients ($p = 0.014$). *Conclusions:* Cytokine levels were associated with level of mental distress as measured by the GSI scores, but this relationship was not present among those using anti-inflammatory drugs. We found no association between cytokine levels and development of GSI score over time.

## Significant outcomes

- Cytokines were related to level of mental distress in psychiatric inpatients.
- Cytokines were not related to the progression of mental distress in treatment over time.
- Anti-inflammatory drugs seemed to modify the relationship between cytokines and mental distress.

## Limitations

- Body mass index and smoking status were not assessed.
- The sample size was small and raises the question of type II error.
- Caution should be used in interpreting the results, as some patients had serum levels under detectable limit.
- Patients were not fasting before blood collection.



## Introduction

Several studies have found an association between cytokine levels and severity of mental distress (Maes, 1995; Furtado & Katzman, 2015; Miller & Raison, 2016; Dalton *et al.*, 2018). Meta-analyses and systematic reviews of cross-sectional studies have shown that circulating pro-inflammatory

cytokines, such as interleukin (IL)-1, IL-6, and tumour necrosis factor-alpha (TNF-α), are significantly elevated in depressed individuals as well as in a broad spectre of other psychiatric diagnoses compared to healthy controls (Dowlati *et al.*, 2010; Passos *et al.*, 2015; Dalton *et al.*, 2018). We have previously found both higher levels and a distinct escalation during treatment of pro-inflammatory and anti-inflammatory cytokines in patients with post-traumatic stress disorder (PTSD), but not for other psychiatric disorders (Toft *et al.*, 2018). There is evidence of a causal pathway from low-grade inflammation to depression, as raised inflammatory markers have been found to precede depressive symptoms in longitudinal studies (Valkanova *et al.*, 2013). There is, however, also evidence of an opposite direction, that is, mental distress preceding an inflammatory response (Wang *et al.*, 2017).

The notion that low-grade inflammation may lead to depression has been supported by a longitudinal study in the general population reporting that elevated levels of IL-6 was associated with psychological distress, and where low levels at the first measurement and follow-up after 6 years were associated with being symptom free (Virtanen *et al.*, 2015). Other studies have shown that patients with inflammatory diseases have greater risk of depression (Maes, 2011; Miller & Raison, 2016), that repeated exposure to systematic inflammation increased the risk of future depressive symptoms among women (Bell *et al.*, 2017), and pro-inflammatory agents are known to induce depression as a side effect (Schiepers *et al.*, 2005; Köhler *et al.*, 2016). This supports the notion of causality. Furthermore, cytokines and psychiatric symptoms in outpatients have been found to decline during treatment (Dahl *et al.*, 2016), and also to play a role in the progression and severity of established depressive disorders in various populations (Young *et al.*, 2014).

The growing understanding of inflammatory processes being related to psychiatric disorders has led to clinical trials using anti-inflammatory drugs in treatment of depressed patients. In such studies, the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with improved antidepressant treatment response, suggesting an antidepressant effect of anti-inflammatory treatment (Köhler *et al.*, 2014; Miller & Raison, 2016). It has been speculated that some patients have low-grade inflammation as a trait and may benefit from anti-inflammatory drugs (Morch *et al.*, 2017). However, anti-inflammatory drugs alone have shown a low to negligible effect on psychopathology (Eyre *et al.*, 2015), and may even contribute to more severe depression symptoms in patients treated with selective serotonin reuptake inhibitors (SSRI), seemingly attenuating the central effect of the SSRI treatment (Warner-Schmidt *et al.*, 2011). Such results underline the necessity that anti-inflammatory drugs are taken into consideration when assessing cytokines and mental health.

Even if the effects of anti-inflammatory drugs on mental distress seem to be ambiguous, it is probable that the use of such drugs may influence the pathomechanism between inflammatory processes and mental distress, as both NSAIDs (Müller *et al.*, 2006) and immuno-suppressants (Köhler *et al.*, 2014) inhibit the production of pro-inflammatory cytokines. The use of anti-viral drugs may also interfere with cytokine levels (Canivet *et al.*, 2015).

A 5-year follow-up study found higher levels of IL-1β, IL-1RA, and TNF-α at the first measurement to be related to depressive symptoms over time (van den Biggelaar *et al.*, 2007), a finding supported by a literature review (Dantzer *et al.*, 2008). There is also evidence on cytokines being related to development of mental disorders once it has already been established (Kim *et al.*, 2019). However, more research is needed as studies on the level of mental distress seen in light of inflammatory biomarkers over time are still scarce, with inconsistent findings (Eyre *et al.*, 2015). In the present study we hypothesised that there is an association between elevated cytokine levels and mental distress in patients undergoing treatment. Thus, we aimed to investigate the relationship between cytokines levels and development of mental distress and the potential moderating role of anti-inflammatory drugs.

## Material and methods

### Study participants and recruitment procedure

Patients were recruited from Modum Bad Psychiatric Center, a specialised psychiatric centre in Norway, treating patients with long-standing or treatment-resistant trauma, anxiety, eating and depressive disorders. Patients with severe self-destructive behaviour or psychotic disorders were not eligible for admission. The facility offered group and individual therapies in a 12-week inpatient treatment programme. Therapy was paid by public insurance, and patients in work were entitled to sick leave while in treatment. Data were collected from March 2015 through April 2016.

Patients were recruited from the Depression, the Eating, the Anxiety, and the Trauma departments. The patients were admitted in groups of eight at a time. They were given a 15 min presentation during group therapy by the first author about the study during one of the first days of their stay. Written information was handed out, explaining the aim of the study and the procedures involved. A consent form was also distributed to each potential participant. Altogether 148 (59% of the total 249 patients approached) gave their written consent. One individual withdrew her consent 2 weeks later. We excluded 19 patients from the data material due to their having extreme cytokine levels above the 95th percentile, indicating possible acute infections. These 95th percentile limits were 436.6 pg/ml for IL-1RA and 216.3 pg/ml for MCP-1. One patient had missing values in cytokine data due to failed venipuncture. Altogether the present study comprised data provided by 92 women (72%, mean age 39.04 years, SD 11.26) and 36 men (28%, mean age 49.06 years, SD 9.36), giving a total of 128 patients. The 102 patients who did not participate comprised 81 women (79%, mean age 35.96, SD 11.77) and 21 men (21%, mean age 44.52, SD 8.58).

The material comprised venous blood samples and psychometric data. The blood samples and Hopkins Symptom Checklist 90 Revised (HSCL-90R) questionnaires were submitted within 1 week after entering treatment ($T_0$), at halfway ($T_1$), and a few days before discharge ($T_2$). The study was approved by the Norwegian Regional Ethics Committee prior to data collection (reference number 2014/2189).

### Methods for clinical data

All patients were interviewed by trained psychologists or psychiatrists using the MINI clinical interview (Sheehan *et al.*, 1998). The MINI interview gives diagnoses within the 10th revision of the *International Classification of Diseases and Related Health Problems* (*ICD-10*). The staff used a combination of psychometric questionnaires and clinical judgement taken into consideration when disorders were assessed. All disorders were in the spectrum of depression, anxiety, and eating disorders. Fifty-four patients had only one disorder, and 59 had two or more disorders. Thirty-two patients had PTSD as primary diagnosis, some with one or even two additional disorders. Fifteen patients had no registered diagnosis due to missing data. An overview of diagnoses and frequencies of the diagnoses are included in Supplementary

Table S1. Patients completed the self-reporting questionnaire HSCL-90R either on a computer or on a digital tablet. The HSCL-90R is a 90-item questionnaire measuring levels of psychological distress during the past 7 days, each item ranging from 0 to 4, giving a mean score of all responses as the Global Severity Index (GSI) (Derogatis *et al*., 1973). The questionnaire assesses symptoms of somatisation, obsession and compulsion, depression, anxiety, and hostility. The HSCL-90R has been shown to provide a psychometrically valid evaluation of psychiatric patients regarding the severity of depression, specific anxiety, and interpersonal sensitivity (Bech *et al*., 2014). A cut-off for caseness was set to a GSI score at 0.85 (Pedersen & Karterud, 2004). In comparison, a previous study has found the GSI to be 0.49 in healthy controls (Rytila-Manninen *et al*., 2016). The GSI is an overall scale taking various symptoms into account, thus tapping into symptoms common to a heterogeneous psychiatric patient sample (Rytila-Manninen *et al*., 2016). At $T_0$, all patients successfully submitted their GSI scores, but at $T_1$, data were missing on 6 patients and at $T_2$, on 13 patients. We also explored three subscales of the HSCL-90R. These were the scales for somatisation, anxiety, and depression. The pattern of missing values was the same as in the GSI.

Anti-inflammatory drugs were NSAIDS, anti-viral drugs, and immuno-suppressants. We do not know the exact indications for which these drugs were given. The dichotomous anti-inflammatory drugs variable was used to stratify the patients in two groups. An overview of the anti-inflammatory drugs is included in Supplementary Table S2. Patients were recorded as users of these drugs if they had used such drugs at three occasions or more during the treatment period. The drugs categorised as anti-depressants were SSRIs, norepinephrine-dopamine reuptake inhibitors (NDRI), tricyclic anti-depressives (TCAs), and serotonin-norepinephrine reuptake inhibitors (SNRIs). The drugs in use were recorded by looking into each patient's medical charts.

### Blood collection and serum preparation

The blood samples were drawn at $T_0$, $T_1$, and $T_2$. They were collected between 08:00 a.m. and 09:00 a.m., except for 16 patients from the depression ward, who had their blood drawn between 12:00 am and 03:00 pm. Patients were not fasting and sat in an upright position when blood was collected. Vacuette 8 ml serum containers were used for blood collection. These were turned upside-down 8–10 times immediately after the blood was collected, and allowed to clot for 30–60 min. The samples were then spun at 1917 g for 10 min at room temperature. Separated serum samples were immediately put to freeze at −80°C until assay.

### Cytokine and chemokine measurements

We analysed seven cytokines and one chemokine based on the available literature on the neuroimmune correlates of psychiatric disorders: IL-1β, IL-1RA, IL-6, IL-10, IL-17A, IFN-y, MCP-1, and TNF-α. Serum samples were thawed on ice, vortexed, and then spun down a tube with 250 µl serum at 14 000 × g for 10 min at 4°C, before dilution (1 : 5) and further processing. The serum levels were measured in picograms per millilitre (pg/ml). The cytokine measurements were performed using Bio-Plex xMAP technology (Bio-Rad, Austin, TX, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, CA, USA), powered using Bio-Plex Manager (version 6.0.1) software. Multiplex bead–based technologies such as Luminex allow detection and quantification of multiple cytokines with good efficiency, speed, and dynamic range at reasonable cost. The assay was performed according to the manufacturer's instructions, but an additional standard point was included. To achieve a more reliable result, individual sets of samples from patients were run in the same assay, all samples were assayed in duplicate, and a magnetic plate washer was used during assay set-up. The StatLIA software package (ver. 3.2; Brendan Scientific, Carlsbad, CA, USA) incorporates a weighted, five-parameter logistic curve-fitting method and was used to calculate sample cytokine concentrations. One longitudinal control from each participant was used for multiple analyses on each plate to define the intra-plate CVs; IL-1RA (3.0%) and MCP-1 (4.0%). Longitudinal controls were also used in order to validate the inter-assay (i.e., between plates) coefficient of variability (CV). The CVs were 10.2% for IL-1RA and 6.7% for MCP-1. An inter-assay per cent CV of 10–12% is common (and acceptable). The mean inter-assay per cent CV for all sample plates was 8.5%. The limit of detection (LOD) was 3 pg/ml for IL-1RA and 0.76 pg/ml for MCP-1.

### Choice of cytokines and imputation

We investigated cytokine IL-1RA and chemokine MCP-1 due to their robustness and very high detectability, a finding in line with other studies (Leemasawatdigul & Gappa-Fahlenkamp, 2011; Holub *et al*., 2013). Also, these cytokines had fewer than 6% below LOD. Cytokine levels below the LOD were replaced with the LOD value. Taking all three blood sampling occasions into consideration, 423 samples were collected. For IL-1RA, we imputed one value (0.2%). For MCP-1, we imputed 25 values (5.9%). We excluded six of the cytokines due to a rather high number of values below LOD. There were 194 (45.9%) values below LOD for IL-1β, 148 (35.0%) for TNF-α, 200 (47.3%) for IL-6, 226 (53.4%) for IL-10, 384 (90.8%) for IL-17, and 332 (78.5%) for interferon-gamma (IFN-γ).

### Statistical analyses

The Mann–Whitney *U*-test and Pearson's chi square were used when analysing demographic data. Non-normally distributed cytokines were attempted normalised by log-transformation. The cytokines were, however, also skewed following log-transformation, and the non-transformed cytokine values were ultimately used. Multilevel models were used to assess the repeated measurements of cytokines and GSI (Rabe-Hesketh & Skrondal, 2016). Main effects of cytokines and cytokines in interaction with time were analysed. We stratified the patients on the use of anti-inflammatory drugs to specifically explore the potential effect of using such drugs. This resulted in two groups ($n = 28$ for users and $n = 99$ for non-users of such drugs). Power analysis was conducted with the software G*Power, release 3.1.9.4, to assess the achieved statistical power with the given sample size. With a standardised mean difference of $d = -0.54$ (Köhler *et al*., 2014), the power was found to be 0.71, giving a 29% chance of not finding an effect even if present. Further, we specifically assessed the relationship between PTSD and GSI since we have previously found the PTSD patients to differ from patients without PTSD (Toft *et al*., 2018). We also assessed the relationship between anti-depressants and GSI in the aforementioned strata in order to distinguish potential effects of different drugs. IL-1RA and MCP-1 were analyzed separately, thus constituting trivariate multilevel analyses with time as predictor variable, the cytokine as explanatory variable, and GSI as dependent variable. The material comprised three measurements of each patient, giving a structure of GSI score nested

**Table 1.** Clinical information of study participants across the treatment period categorised on the use of anti-inflammatory drugs

| Variable | | Not using $n = 100$ | Using $n = 28$ | $p$-value |
|---|---|---|---|---|
| Demography | | | | |
| Women | $n$ (%) | 69 (54%) | 23 (18%) | 0.018* |
| Age | Mean (SD) | 41.89 (11.61) | 41.71 (11.67) | 0.787† |
| Cytokines‡ | | | | |
| IL-1RA | Mean (SD) | 36.96 (50.86) | 43.08 (46.56) | 0.026 |
| MCP-1 | Mean (SD) | 25.53 (29.46) | 34.56 (24.35) | < 0.001 |
| Mental distress | | | | |
| GSI | Mean (SD) | 1.32 (0.67) | 1.53 (0.76) | 0.033 |
| Medication | | | | |
| Anti-depressants (not using) | $n$ (%) | 76 (59%) | 15 (12%) | < 0.001 |
| Anti-depressants (using) | $n$ (%) | 24 (19%) | 13 (10%) | |
| PTSD diagnosis | | | | |
| No PTSD | $n$ (%) | 58 (51%) | 13 (11.5%) | 0.008 |
| PTSD | $n$ (%) | 29 (26%) | 13 (11.5%) | |

GSI, Global Severity Index; IL-1RA, interleukin-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; PTSD, post-traumatic stress disorder.
*Pearson chi square was used for categorical variables.
†The Mann–Whitney $U$-test was used for continuous variables.
‡Cytokine levels in picograms per millilitre (pg/ml).

within patients. We disentangled the between- and within-subjects effects from the total effects. The within-subjects effects represent the expected change in GSI on average for a patient over the 12 weeks of treatment. The between-subjects effects indicate that, on average, higher cytokine levels increase the GSI by the given coefficient (Curran & Bauer, 2011). The process of disentangling the within- and between-subjects effects involved group-mean centering of the cytokine variables. The multilevel modelling was performed in a stepwise approach with fixed effects of cytokines and time, with subject ID as random intercept. A random slope of time was added to allow the slopes to vary across time. A likelihood ratio test was performed to assess model fit. The best model fit was chosen based on the −2 Log Likelihood and Bayes Information Criterion (BIC), and formally confirmed using likelihood ratio test. A model with fixed effects and random intercept gave a better fit than a model which included random slope (likelihood ratio test $\chi^2(2) = 1.93$, $p = 0.381$). The predicted fixed and random effects of time on GSI are shown in Supplementary Table S3. The assumption of linearity in the dependent GSI variable across time was visually inspected with spaghetti plot and considered met. The assumption of homoscedastic residuals was assessed with a likelihood ratio test ($\chi^2(2) = 1.01$, $p = 0.603$). The non-significant test confirmed that the assumption of homoscedastic residuals was met. Normality of residual distribution was assessed with QQ-plot and a histogram with a Gauss curve which showed normally distributed residuals and a nearly perfect normal curve. Restricted maximum likelihood (REML) was used in all multilevel model estimations due to small sample size, and maximum likelihood (ML) was used for model comparison. Those who did not submit all three GSI scores were defined as missing completely at random (MCAR), which indicated no specific pattern of missing data. Consequently, there was no increased variability which could have biased the regression coefficients. MCAR was confirmed by Little's MCAR test (Little, 1988) with $\chi^2 = 0.576$ ($p = 0.902$). All tests were two-sided, and $p$-values below 0.05 were considered statistically significant. No correction for multiple hypothesis testing was implemented as we considered the study to be exploratory. The HSCL-90R subscales anxiety, somatisation, and depression were analysed, but did not provide different results (included in Supplementary Table S4). Also, we explored all longitudinal analyses with adjustment for age and sex, but this did not significantly change any results (adjusted analyses are shown in Supplementary Table S5). Therefore, we chose to present unadjusted results. The statistical package STATA (StataCorp. 2015. Stata Statistical Software: Release 15. College Station, TX: StataCorp LP) was used for all statistical analyses.

## Results

Table 1 shows demographic variables, cytokine levels, use of anti-depressive medications, and PTSD diagnosis in those not using or using anti-inflammatory drugs. There were significantly higher levels of MCP-1 and IL-1RA in those using anti-inflammatory drugs ($p < 0.001$ and $p = 0.026$, respectively). There were also significantly more patients without PTSD and without anti-depressive drugs who also were not using anti-inflammatory drugs ($p < 0.001$ and $p = 0.008$, respectively).

Table 2A and B presents the multilevel models of levels and changes in GSI over time. The table is divided in three, where Table 2A presents the results of all patients ($n = 128$), Table 2B users of anti-inflammatory medication ($n = 28$) and Table 2C non-users ($n = 100$ in total, with 99 of these patients submitted blood samples). In Table 2A, each variable was run as a separate explanatory variable on GSI, first as main effect only and then in interaction with time. There was a significant main effect of IL-1RA ($p = 0.005$), MCP-1 ($p = 0.020$) and having PTSD disorder

**Table 2A.** Trivariate linear mixed effects models of characteristics and mean cytokine levels across treatment on GSI for all patients

| | | Main effect on GSI | | Interaction with time on GSI | |
|---|---|---|---|---|---|
| Variable | *n* | β (SE) | Sig. | β (SE) | Sig. |
| Age | 128 | −0.005 (0.005) | 0.323 | <−0.001 (< 0.001) | **0.025** |
| Women | 92 | 0.175 (0.121) | 0.152 | −0.001 (0.010) | 0.878 |
| IL-1RA | 127 | 0.004 (0.001) | **0.005** | <0.001 (0.001) | 0.680 |
| MCP-1 | 127 | 0.005 (0.002) | **0.020** | <0.001 (0.002) | 0.809 |
| PTSD diagnosis | 42 | 0.356 (0.112) | **0.002** | 0.014 (0.011) | 0.152 |
| Anti-inflam.* | 28 | 0.224 (0.132) | 0.093 | 0.017 (0.011) | 0.108 |
| Anti-depressives | 76 | −0.001 (0.102) | 0.991 | −0.004 (0.010) | 0.676 |

GSI, Global Severity Index; β (SE), regression coefficient (standard error); IL-1RA, interleukin-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; PTSD, post-traumatic stress disorder.
Bold values represent significance value at p = 0.01, p = 0.05 and p = 0.01.
*Anti-inflammatory drugs. All regression models run with time as predictor variable.

**Table 2B.** Trivariate linear mixed effects models of characteristics and mean cytokine levels across treatment on GSI for patients using anti-inflammatory drugs

| | | Main effect on GSI | | Interaction with time on GSI | |
|---|---|---|---|---|---|
| Variable | *n* | β (SE) | Sig. | β (SE) | Sig. |
| Age | 28 | −0.021 (0.011) | 0.061 | −0.002 (0.001) | 0.092 |
| Women | 23 | −0.031 (0.348) | 0.930 | 0.011 (0.025) | 0.659 |
| IL-1RA | 28 | 0.004 (0.003) | 0.186 | <−0.001 (< 0.001) | 0.885 |
| MCP-1 | 28 | 0.001 (0.006) | 0.892 | <−0.001 (< 0.001) | 0.702 |
| PTSD diagnosis | 16 | 0.432 (0.273) | 0.127 | 0.038 (0.021) | 0.077 |
| Anti-depressives | 13 | 0.044 (0.204) | 0.831 | −0.020 (0.021) | 0.359 |

GSI, Global Severity Index; β (SE), regression coefficient (standard error); IL-1RA, interleukin-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; PTSD, post-traumatic stress disorder.
All regression models run with time as predictor variable.

**Table 2C.** Trivariate linear mixed effects models of characteristics and mean cytokine levels across treatment on GSI for patients not using anti-inflammatory drugs

| | | Main effect on GSI | | Interaction with time on GSI | |
|---|---|---|---|---|---|
| Variable | *n* | β (SE) | Sig. | β (SE) | Sig. |
| Age | 100 | <−0.001 (0.005) | 0.992 | <−0.001 (< 0.001) | 0.101 |
| Women | 48 | 0.189 (0.128) | 0.142 | −0.006 (0.010) | 0.577 |
| IL-1RA | 99 | 0.004 (0.002) | **0.023** | <0.001 (0.001) | 0.623 |
| MCP-1 | 99 | 0.006 (0.002) | **0.018** | <0.001 (0.002) | 0.849 |
| PTSD diagnosis | 26 | 0.306 (0.122) | **0.014** | 0.005 (0.011) | 0.669 |
| Anti-depressives | 24 | −0.032 (0.005) | 0.787 | −0.004 (0.012) | 0.760 |

GSI, Global Severity Index; β (SE), regression coefficient (standard error); IL-1RA, interleukin-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; PTSD, post-traumatic stress disorder. IL-1RA, p = 0.023.
Bold values represent significance value at p = 0.05.
All regression models run with time as predictor variable.

($p = 0.002$) on GSI. The only variable related to the slope of GSI over time was age ($p = 0.025$), indicating that older patients improve more in mental distress over time.

Table 2B shows the main effect and interaction with time on GSI in those patients using anti-inflammatory drugs. We found no associations in this stratum. In those not using anti-inflammatory drugs (Table 2C), there were significant main effects of IL-1RA ($p = 0.023$), MCP-1 ($p = 0.018$) and having PTSD disorder ($p = 0.014$) on GSI. Together, Tables 2B and C show that among those not using anti-inflammatory drugs, GSI levels were significantly higher in the PTSD sample compared to non-PTSD patients. This difference was not present in those using anti-inflammatory drugs. Time interaction did not show any significant results in either of the two strata.

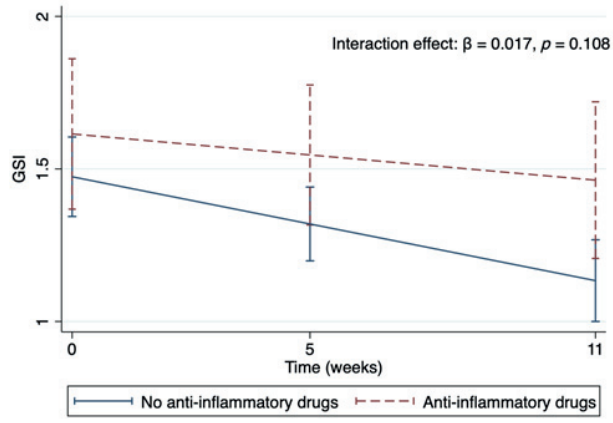


**Fig. 1.** Intercept and slopes in users and non-users of anti-inflammatory drugs depicting the decline levels of GSI (95% CI) during the treatment period.
**Interaction effect of anti-inflammatory drugs with time on GSI. The graph visualises the trajectories of the GSI from the multilevel model found in Table 2A. GSI, GSI, Global Severity Index.**

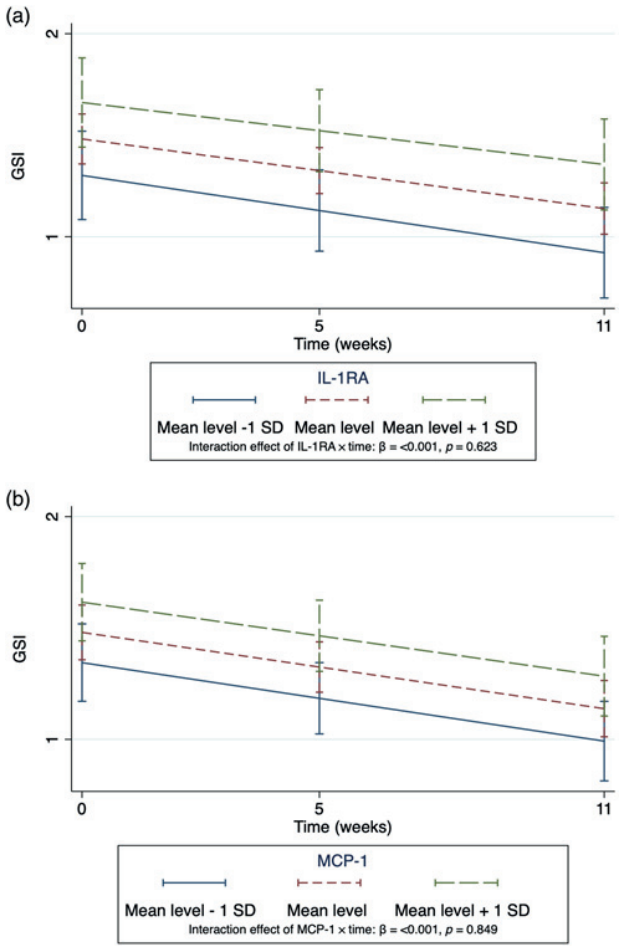


**Fig. 2.** (A and B) Intercepts and slopes of GSI in non-users ($n = 99$) of inflammatory drugs.
**The declining trajectories of GSI are explained by the time variable (as shown in Supplementary Table S5). The GSI is categorised according to level of the inflammatory markers (mean level ± 1 SD). The graphs visualise the trajectories of the GSI from the multilevel models in Table 2C. GSI, Global Severity Index. IL-1RA, interleukin-1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1.**

Fig. 1 shows the intercept and slope of GSI over time in the patients according to their use of anti-inflammatory drugs. There was a tendency that patients who used anti-inflammatory drugs had higher level of GSI, also reflected in Table 2A ($p = 0.093$). The slope of GSI in those who did not use anti-inflammatory drugs was significantly different from zero ($\beta = -0.03$, SE = 0.005, $p < 0.001$), indicating that there was a reduction in symptoms over time. The slope of GSI in the users of anti-inflammatory drugs did not reach significance in decline over time ($\beta = -0.01$, SE = 0.01, $p = 0.149$), and the difference between the two groups was not significant, as shown in Table 2A ($p = 0.108$).

Fig. 2A and B shows the intercepts and slopes of GSI over time for the patients who did not use anti-inflammatory drugs, as shown in Table 2C. The three slopes categorise the patients by the mean level ± 1 SD of IL-1RA and MCP-1 according to their corresponding GSI level. The slopes of the GSI scores declined throughout treatment for all patients, and these slopes were significantly different from zero ($p < 0.001$).

## Discussion

In patients undergoing psychiatric treatment, those with higher levels of IL-1RA and MCP-1 had higher levels of mental distress. There was a decreasing slope of GSI over time for the older patients, indicating that older patients benefited more from treatment. When stratifying according to the use of anti-inflammatory drugs during treatment, we found associations between levels of cytokines and GSI only among those not using anti-inflammatory drugs. There seemed to be an interaction between the use of anti-inflammatory drugs and cytokine levels with the level of mental distress in psychiatric patients in treatment. We did not find a relationship between cytokine levels and the development of GSI scores over time. We still believe our finding underscores the need for taking anti-inflammatory drugs into account in immune-psychiatric investigations and in treatment effect evaluation.

The relationship between cytokine level and degree of mental distress is in line with numerous studies on many different groups of patients with psychiatric diagnosis (Maes *et al.*, 1990; Smith, 1991; Dowlati *et al.*, 2010). The chemokine MCP-1 induces leukocyte infiltration in the CNS and is associated with neuronal damage, which is why it has been studied for its role in depression (Young *et al.*, 2014). Our finding that MCP-1 was related to level of mental distress is in accordance with a series of studies. A review of studies on inflammatory processes in major depressive disorder showed that the synthesis of MCP-1 is involved in both the aetiology and progress of the disorder (Young *et al.*, 2014). The association between elevated MCP-1 and depression and anxiety symptom severity has also been found by others (Vogelzangs *et al.*, 2016), but results are mixed, as serum MCP-1 has also been found to be lowered in patients with MDD (Young *et al.*, 2014). The anti-inflammatory cytokine IL-1RA is also known to be elevated in depression and anxiety patients. The reason for this has been suggested to be due to an increased innate immune response, which involves both pro- and anti-inflammatory cytokine and chemokine production (Maes *et al.*, 1997; Dahl *et al.*, 2014).

We did not find that cytokine levels were related to the progress of mental distress. This discrepancy may have different explanations. An observation period of 12 weeks may not be sufficient to pick up on changes over time. Furthermore, patients enrolled at Modum Bad Psychiatric Center were patients with severe mental disease, often for many years, possibly carrying

high cytokine levels as a trait feature. Also, the variety of diagnoses may explain a wide spread in cytokine levels in our study population. It has been suggested that anti-inflammatory drugs may play a role in outcome of PTSD by altering neuro-immune processes (Miller *et al*., 2018; Waheed *et al*., 2018) and thus contribute to mental recovery. Among those not using anti-inflammatory drugs, the PTSD patients had significantly higher GSI levels than non-PTSD patients. This difference was attenuated in those using anti-inflammatory drugs. This implies that the use of anti-inflammatory drugs affects GSI levels across PTSD diagnosis and suggests other researchers in the field should assess the use of such drugs in future studies of PTSD patients.

The relationship between cytokine level and mental distress was only present among those not using anti-inflammatory drugs. The lack of a relationship between cytokines and mental distress among those using anti-inflammatory drugs may be that both the reason for using these drugs, often an inflammation, and the drugs themselves may influence the level of cytokines much more than mental distress. Furthermore, it has been suggested that anti-inflammatory drugs could be used in the treatment of depression, as it reduces inflammation (Young *et al*., 2014), thus reducing depressive symptoms, especially for the depression patients who are labelled as treatment-resistant due to treatment unresponsiveness (Kornstein & Schneider, 2001). The mechanism is believed to be the inhibition of enzyme cyclooxygenase-1 (COX-1). COX-1 is a major player in modulation of pro-inflammatory microglia activation, and aspirin in particular has been postulated as particularly promising, but still experimental and hypothetical (Baune, 2017). However, the effect of anti-inflammatory medications has been found to exhibit efficacy in treatment-resistant depression patients with high-sensitivity CRP (hs-CRP) concentrations greater than 5 mg/l, but not for treatment-resistant depression with smaller hs-CRP concentrations (Raison *et al*., 2013). This suggests subgroups of depression patients respond differently to drugs according to inflammatory level. Together with our finding on different associations in PTSD patients according to stratification, a screening of inflammatory markers in patients seeking treatment could be warranted.

There are some limitations to mention. The patients were not fasting when blood samples were drawn. Eating has been found to be associated with increased MCP-1 (Dixit *et al*., 2011). Body mass index (BMI) of the patients was not assessed in this study. Adipose tissue produces pro-inflammatory cytokines, meaning that body fat is a possible confounder (Calder *et al*., 2011). We did not assess smoking status. Smokers have been found to have a higher basal level of cytokines when compared to non-smokers (Belchamber *et al*., 2014). Physical exercise might affect the levels of circulating cytokines (Phillips & Fahimi, 2018). We did not record the level of exercise before blood samples were drawn. Conditions causing systemic inflammation, for instance hepatitis, could potentially bias the cytokine levels. The blood samples were collected in the afternoon for 16 patients. During clotting, monocytes may begin releasing MCP-1. However, increase in the protein levels of MCP has been found only after several hours (Campbell *et al*., 2017). Also, future studies will benefit from more sensitive assay techniques as well as inclusion of multiple cytokines of the pro-, anti-, and regulatory classes. The reader should bear in mind that the sample size is small and there is a risk of type 2 errors. The study showed that the levels of cytokine IL-1RA, chemokine MCP-1 and PTSD diagnosis were related to level of GSI. The use of anti-inflammatory drugs appeared to have an immunomodulating effect on this relationship.

**Supplementary material.** To view supplementary material for this article, please visit https://doi.org/10.1017/neu.2019.36

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**Author contributions.** HT was responsible for recruiting patients, collecting the blood samples, and drafting and revising the manuscript. HT also performed the statistical analyses. LL was responsible for the design and planning of the study and was involved in writing and revision of the manuscript. SPN was involved in designing and planning of the study and in writing and revision of the manuscript. DSA was involved in revision of the manuscript and supervision of the statistical analyses. TT was involved in planning of the study and in revision of the manuscript. BEW was involved in planning of the study and in revision of the manuscript. JGB was, together with LL, responsible for the design and planning of the study, and was involved in writing and revision of the manuscript. All authors revised the final version and approved for submission.

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**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 (Williams, 2008).

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