Chapter 7

General discussion
The past two decades have shown an intensification of research into the pathophysiological processes and etiological mechanisms of major depressive disorder (MDD), with a specific interest in treatment resistant depression (TRD). Accumulating evidence has been presented for an important role of inflammatory processes and the immune system in depression, but findings were not always unequivocal. In this context, we aimed to explore the role of a dysregulated inflammatory system in the pathophysiology of MDD/TRD by 1) reviewing the role of inflammatory markers in MDD subtyping, 2) reviewing the predictive validity of inflammatory marker levels for TRD treatment outcome, and 3) investigating the involvement of variations within the *FKBP5* and *CNR1* genes in antidepressant treatment resistance. Moreover, a randomized double-blind placebo-controlled antidepressant augmentation trial with N-acetylcysteine (NAC) was initiated to investigate its antidepressant efficacy and safety in TRD patients with increased peripheral inflammatory activity. This ongoing trial is expected to shed more light on the relationship between inflammation and treatment resistance, and whether outcome can specifically be improved with anti-inflammatory medication. The results of this trial will be analyzed as soon as the number of inclusions has been reached, but are not part of this thesis. In this general discussion, the main findings will be discussed, followed by methodological considerations, a discussion of the relationship between inflammatory dysregulation and MDD/TRD, the potential for anti-inflammatory treatment, clinical implications, directions for future research, and closing remarks.

**Summary of main findings**

**Part 1. Inflammatory markers for treatment response and MDD subtypes**

Chapter 2 is a systematic review of studies investigating the relationship between baseline levels of inflammatory cytokines and MDD subtypes. It shows that increased values of serum interleukin-6 (IL-6) and 1β (IL-1β) are more common in the melancholic subtype compared to the non-melancholic subtype or healthy controls, while C-reactive protein (CRP) may be a pointer for the non-melancholic subtype.

Chapter 3 is a systematic review of studies investigating the usefulness of inflammatory markers for predicting treatment response in TRD. The conclusion is that IL-6 and CRP/hsCRP are promising markers for predicting treatment response in TRD. Other markers such as IL-2, IL-10 and tumor necrosis factor-α (TNF-α) were far less promising in this respect. This came rather unexpected especially for the latter marker, because TNF-α and IL-6 have similar effects on the production of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone and cortisol by acting directly on hypothalamic and pituitary cells [1]. This could hint at a less important role of stress in TRD compared to MDD. But the
consistent results of chapter 2 and 3 for IL-6/1β and CRP/hsCRP indicate that these are promising inflammatory markers for MDD subtypes and TRD treatment response.

Part 2. Association study of MDD susceptibility and treatment response phenotypes with FKBP5 and CNR1 genes in Han Chinese people: a case-control pilot study

The high prevalence of MDD and insufficient treatment responses cause a heavy burden for both the individual and society. The identification of single nucleotide polymorphisms (SNPs) in genes putatively related to pathophysiological processes in MDD might improve both diagnosis and antidepressant treatment prediction. Based on biological function, their role in the pathophysiology of MDD and a minimum allele frequency (MAF) in the Han Chinese population, the FKBP5 and CNR1 genes were selected as markers for MDD (chapter 4 and 5, respectively). We have investigated the association of gene variants (allele, genotype, and haplotype) with MDD susceptibility and treatment response phenotypes in 181 Han Chinese with MDD and 80 healthy controls. We could not demonstrate significant differences in the distributions of alleles, genotypes and haplotypes between MDD cases and healthy controls or between patients with TRD and depressed patients without TRD (chapter 4). However, several SNPs and haplotypes of the CNR1 gene were associated with an increased risk for MDD as well as with antidepressant treatment resistance. These gene variants included rs806367, rs6454674 and the haplotype C-T-T-C of rs806366, rs806367, rs806368, and rs806370, although some of these significant findings did not survive multiple testing. (chapter 5). It is clear that follow-up studies will need much larger, better specified and more homogeneous samples to draw a definitive conclusion regarding the involvement of these gene variants in MDD.

Part 3. Clinical trial in patients with TRD and increased inflammatory activity

With the background that antidepressant treatment resistance is associated with increased inflammatory activity and with earlier studies suggesting that anti-inflammatory treatment can benefit depressed patients with increased inflammatory activity particularly, we aimed to investigate the efficacy of NAC supplementation in patients with TRD and increased inflammatory activity, and to explore potential roles of inflammation involved in the alleged pathophysiological processes of TRD, through supplying NAC to ongoing antidepressant therapy. Importantly, we only included depressed patients with increased CRP levels, that for this population were between 0.85 and 10 mg/L. This ongoing clinical trial takes both TRD and increased inflammatory activity as inclusion criteria, which will provide reliable evidence for the efficacy of NAC in patients with TRD and increased inflammatory activity, and also will help explore further the roles of inflammation involved in the alleged pathophysiological processes of TRD.
Methodological considerations

For the review studies (chapter 2 and 3) we used systematic methods collecting secondary data and synthesizing findings to critically appraise the role of peripheral inflammatory biomarkers in subtyping MDD and predicting TRD treatment outcomes, respectively. Systematic review is a method designed to provide a complete, exhaustive summary of current evidence relevant to a research question, which provides important methodological support for evidence-based medicine. But a reliable review study requires qualified data. In eligible articles of our review studies, there were many methodological deficiencies, altogether contributing to a limited generalization of findings. For example, in chapter 2, there was a lack of consensus in MDD subtype definitions across studies. Some eligible studies made a distinction between melancholic and non-melancholic depression while others used the terms typical and atypical depression. We had to make a compromise, categorizing atypical depression under the umbrella term non-melancholic depression, to allow comparisons between the studies. In line with this, divergent definitions also undermined the reliability of findings in chapter 3. Furthermore, different evaluation tools, emphasizing different aspects of symptom presentation, were used. For instance, in chapter 2, some studies used sign-based methods such as Latent Class Analysis (LCA) instead of symptom-based DSM criteria in diagnosing depression; and different rating scales were used in evaluating severity of depressive symptoms, such as Hamilton Rating Scale of Depression (HAMD)-7/17/24 items and Montgomery-Åsberg Depression Rating Scale (MADRS). Finally, both review studies included limited number of articles, which could amplify the negative effects of methodological deficiency.

Secondly, two genetic association studies focused on Han Chinese population from Tianjin municipality; and we stratified participants into three groups, including TRD patients with hyperactive inflammation, normal MDD patients, and healthy controls. Both actions increased the homogeneity of samples, which is an important determinant for genetic association studies on depression. Chapter 5 observed significant distribution for the SNP rs6454674 only in the TRD patients with increased inflammatory activity, but not in the normal MDD patients or MDD cases. Furthermore, we have adjusted for multiple testing to avoid type I errors, which prompted the power of possible positive findings in chapter 5. But there are also some deficiencies in methodology. First, the samples used came from two separate studies, lacking detailed information for adjusting findings. For example, we had no information on negative life events, which was an important covariate adjusting the association between CNRI genotype and depression [2]. Second, we had a small sample size, only recruiting 181 depressed patients and 80 healthy controls. The sample size is one of the most important determinants for discovering reliable genetic associations [3]. Nevertheless, post-hoc power analysis showed sufficient power (>0.9) for detecting the significant effect of CNRI SNPs.
Finally, the ongoing clinical trial (chapter 6) will be important to explore the role and malleability of inflammation in TRD. As such, the study design holds several strengths. First, the combined approach of biomarker measurements and neuroimaging can provide a deeper insight into how various neuronal systems involved in the pathophysiology of MDD/TRD function separately and jointly, as well as their relation with treatment outcome. Second, it recruits a relatively homogeneous group of TRD patients, which will increase the reliability of the findings. Last, despite the fact that this has been suggested by several authors, to the best of our knowledge this is the first study that includes TRD patients on the basis of increased inflammatory activity. Nevertheless, there are also some limitations in the design, such as the definition of TRD used, the setting of cutoff value for “inflammatory depression” and so on. These will be discussed in more detail subsequently.

**Definition of TRD in different studies**

As yet there is no consensus on the definition of TRD, which likely contributes to the mixed findings in TRD studies. The definition of TRD used in our NAC study (chapter 6) is an insufficient response to one or more antidepressants given for at least six weeks and in an adequate dose during the current depressive episode. This is a relatively mild version of TRD, chosen because we have a strict inclusion criterion for recruiting participants in terms of CRP levels, which is also associated with treatment resistance [4]. Furthermore, patients can benefit more from timely and more personalized treatment than when depression has become chronic, in term of cost-utility perspective. Theoretically, the possibility exists that levels of TRD could vary between study arms, which would increase the risk for a significant imbalance in the degree of refractory depression between the NAC and control group. As a countermeasure, we have recorded detailed information on antidepressants taken, which can in part correct the effects of significant imbalance in the degree of refractory depression.

**What is the accurate cutoff value for ‘inflammatory depression’?**

The NAC study (chapter 6) involves TRD patients with increased inflammatory activity with serum CRP values between 0.85~10 mg/L. Several lines of evidence indicated that increased levels of inflammatory cytokines contribute to treatment resistance [5], but this phenomenon is likely restricted to a subgroup of patients [6]. Accordingly, the accuracy of the CRP range is particularly important. The lower cutoff values for the NAC study were derived from the upper one third of CRP level distribution in a pilot study with 62 MDD patients without severe medical illness and immune disorders that were treated in the Tianjin Anding Hospital where the study was later run. This idea of the upper 30% was based on the findings from Raison’s study in which the infliximab, a monoclonal antibody of TNF-α, exerted antidepressant effects in patients with hs-CRP value >5mg/L, taking one third of all participants [7]. Furthermore, a large, population-based study aimed to evaluate the relationship between elevated plasma levels of CRP and depression in the Danish population,
and illustrated that one third of patients with a history of hospitalization had increased inflammatory activity defined as CRP > 3mg/L [8]. A second study showed that low-grade inflammation was found in 21-42% of acutely unwell patients admitted to a mental hospital with a range of different mental disorders [9]. This is in line with the idea that low-grade inflammation is relevant for around 1/3 of patients, and fits the design we choose for the NAC study. The upper limit of 10 mg/L was considered as a cutoff to exclude patients having a comorbid medical illness, like active infection. Regarding treatment specifically focused on this subgroup of patients, very few studies have actually been able to do so, with mixed results [10]. In line with our hypothesis, the research group of prof. Khandaker in the UK recently started a randomized controlled trial (RCT) with tocilizumab, specifically focusing on patients with depression and low-grade inflammation [11].

**Pragmatic versus rigid inclusion criteria**

In the NAC study, antidepressants were restricted to specific selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenalin reuptake inhibitors (SNRIs) within a certain dosage range instead of a fixed one. Benzodiazepines were allowed when needed, but SSRIs and SNRIs with known anti-inflammatory effects were excluded. This strategy made the study population more representative of patients in regular treatment settings and also prompted patients’ adherence to the study. Yet, considering the diverse biological functions of NAC, the various types of medications will likely complicate their interactions with NAC beyond the immune system. For example, olanzapine has the ability to modulate brain and serum glutamatergic concentrations as well as anti-inflammatory effects [12, 13], which could mask the actions of NAC. It is also important to note that several included SSRIs and SNRIs could still have anti-inflammatory properties although such data was not available at the time the study was started. This may likely affect the associations between NAC and the immune system. On the other hand, this relatively naturalistic design will make it easier to generalize the findings to other TRD patients in regular mental healthcare.

**Peripheral markers**

We aimed to explore the role of the immune system and inflammatory cytokines in the pathophysiology of TRD by combining antidepressant treatment with NAC and by investigating the distributions of SNPs in two genes putatively related to TRD (chapter 4, 5, 6). In the NAC study (chapter 6) blood and morning urine samples will be taken at different time points. Part of the patients will also undergo functional-magnetic resonance image (f-MRI) and diffusion tensor imaging (DTI). It is important to note that in comparison with fMRI, components of blood and urine can only remotely inform on pathophysiological processes in the brain, among others because of the existence of the blood brain barrier (BBB). Yet several peripheral biomarkers such as inflammatory cytokines can selectively pass the
BBB [14]. Using a biomarker panel with high sensitivity (>95%) and specificity (>95%) for MDD we will also investigate the involvement of other pathological processes such as monoamine dysfunction, magnesium deficiency, oxidative stress and endocrine dysfunction. The latter is important because many depressed patients have an impaired resilience to cope with stressful life events, which likely relates to an impaired endocrine and immune system function [15-17]. It is clear that measuring biomarkers in body fluids has distinct advantages such as easy sampling, low invasiveness and relatively low costs but they may also be more sensitive to environmental factors such as diet [18] and stress which could compromise their usefulness for studying pathophysiological processes in the brain.

**Relationship between inflammatory dysregulation and MDD/TRD**

The macrophage theory of depression connecting MDD with inflammatory dysregulation was firstly proposed by Smith et al. [19]. This idea was based on the notion that macrophage-derived cytokines induced depressive symptoms in healthy volunteers [20]. Afterwards, many studies have suggested a key role for a dysregulated inflammatory system in the pathophysiology of MDD [21, 22].

The relationship between inflammatory dysregulation and MDD is far-ranging. Firstly, in response to stress, infection or other inflammatory conditions, both peripheral and central cytokines can induce affective and behavioral symptoms [5]. For instance, systemic exposure to lipopolysaccharide (LPS) induces an elevation of peripheral cytokines, resulting in sickness behavior [23]. In addition, activated microglia-derived cytokines following exposure to stress can induce symptoms of depression by damaging neurons directly or through interacting with the hypothalamic-pituitary-adrenal (HPA) axis [1]. Secondly, cytokine profiles contribute to clinical presentations of MDD by influencing several mechanisms related to monoamine/glutamate neurotransmission and hippocampal functioning/neurogenesis [24, 25]. Many studies have tried to clarify whether MDD subtypes are connected with different cytokine profiles with the aim to advance both diagnosis and treatment strategy of depression. We have reviewed these studies in chapter 2 and found that serum levels of IL-6 and IL-1β were promising measures to differentiate between the melancholic MDD subtype and both healthy controls and the non-melancholic MDD subtype. We also found support for CRP as an indicator for the non-melancholic MDD (chapter 2). However, some caution is warranted given the paucity of eligible studies and the diverse definitions of MDD subtypes. Thirdly, a dysregulated inflammatory system is related to treatment response of MDD. It has been reported that an insufficient response to antidepressant treatments is associated with increased inflammatory activity [5], while improvements by antidepressant treatment were accompanied by reductions of inflammatory markers [5]. In line with this, we found that peripheral IL-6 and CRP/hsCRP could be promising markers for predicting treatment response in TRD (chapter 3). In conclusion several lines of evidence indicate an involvement of dysregulated inflammatory processes in the pathophysiology of MDD. It must be mentioned, however, that
inflammation-driven MDD is likely restricted to a subgroup of patients and might only involve specific symptoms of depression [6, 26].

**Anti-inflammatory treatment in MDD/TRD**

Under normal conditions the immune system primarily performs as a safeguard of the body through its involvement in the surveillance of peripheral and central tissues and the protection against pathogens and various forms of injury [14]. Furthermore, inflammatory cytokines are essential for brain development and brain function through multiple mechanisms, including the support of neurogenesis/neuronal plasticity and the maintenance of brain homeostasis [27].

The immune/inflammation hypothesis was purported as a pathophysiological mechanism for MDD. Many studies have indeed shown a dysregulated inflammatory system in patients with MDD [28, 29]. Interactions with a wide range of processes involving neurotransmitter systems, neuroendocrine systems, oxidative stress and neurotrophic factors contribute to the inflammatory dysregulation in MDD. First, cytokines influence the functioning of neurotransmitters such as serotonin, dopamine, and glutamate by altering their production, transport and metabolism [30]. For instance, cytokines stimulate indoleamine 2,3-dioxygenase (IDO), an enzyme essential for tryptophan metabolism. The increased IDO activity by pro-inflammatory cytokines stimulates the breakdown of tryptophan via the kynurenine pathway [31]. Because tryptophan is an essential amino-acid the production of neuronal serotonin may become in danger. Moreover, several breakdown products from the kynurenine pathway have neurotoxic effects. It has been proposed that both factors contribute to the development of depressive symptoms [32]. Second, under normal conditions a negative feedback loop between the HPA-axis and inflammatory system will prevent hyperactivity of the inflammatory system [33]. However, during inflammation this feedback loop is disturbed and the increased levels of cytokines may induce glucocorticoid resistance by disrupting glucocorticoid receptor expression and function, which in turn may lead to an unrestrained inflammatory response [34, 35]. Finally, cytokines can promote reactive oxygen species which can directly damage neurons and glia cells in brain regions involved in MDD [36]. This may cause disruptions in cellular signaling mechanisms [37] with clear negative consequences such as symptoms of depression. In addition, cytokines can also increase the extracellular levels of excitotoxic glutamate which eventually will reduce the production of neurotrophic factors. Both brain-derived neurotrophic factor (BDNF) and glia-derived neurotrophic factor (GDNF) [38, 39] support neuronal plasticity and neurogenesis. Because these processes are important for maintaining neuronal health [40], the increased cytokine levels may also hamper this protective mechanism in MDD.

Theoretically anti-inflammatory agents might reduce inflammatory responses and alleviate symptoms of depression. Actually, several lines of evidence have indicated that relief
of depression by an anti-inflammatory intervention is accompanied by a normalization of cytokine levels. For example, Taraxacum officinale, an important medicinal herb with anti-inflammatory activity [41], exerted antidepressant effects in a mouse model of depression through a modulation of BDNF gene expression and by reducing corticosterone levels [42]. Furthermore, increased depression-like behavior and HPA axis hyperactivity were both reversed by fluoxetine [43], an antidepressant with intrinsic anti-inflammatory properties [44]. In addition to its suppression of IL-1β and TNF-α it would improve cognitive function by up-regulating the glutamate transporter 1 (GLT-1) thus decreasing glutamate levels in hippocampus [45]. This suggests that the antidepressant effect is partly mediated through the glutamate system. In this respect, it is important to mention that inflammatory dysregulation might be restricted to specific symptoms and subtypes of depression (chapter 2) [26, 46]. Accordingly, not all depressed patients would benefit from anti-inflammatory treatment. For example, a study by Raison et. al illustrated that only depressed patients with baseline CRP levels ≥ 5mg/L significantly responded to an anti-inflammatory intervention [7]. Thus anti-inflammatory agents are promising adjuvants in antidepressant treatment of TRD patients with increased inflammatory activity. This is corroborated by a clinical study demonstrating that depressed patients with a hyperactive inflammatory system benefit less from antidepressants [47]. Moreover, our review study (chapter 3) indicated that certain inflammatory cytokines hold some promise as predictors of treatment response in TRD, also implicating that TRD patients with higher levels of IL-6 or CRP/hsCRP would benefit the most from anti-inflammatory treatment.

Finally, one may ask the question why anti-inflammatory intervention has little or no efficacy in MDD patients with relatively lower level of inflammatory cytokines. Firstly, MDD is highly heterogeneous and thus not all types of depressions are inflammatory driven [48]. Secondly, as pointed out before, inflammatory cytokines execute important physiological effects at normal levels such as support of neurogenesis and neuronal plasticity. Lastly, one study by Warner-Schmidt et al. demonstrated that anti-inflammatory agents may weaken the efficacy of serotonergic antidepressants both in mice and humans [49]. Thus application of anti-inflammatory treatment in depressed patients without increased inflammation is probably non-effective or even pernicious.

In conclusion, we propose that anti-inflammatory treatment could benefit MDD/TRD patients with increased inflammatory activity through rectifying aberrant biological processes in neurotransmitter systems, neuroendocrine systems, neurotrophic factors and following oxidative stress. In line with the illustration in the general introduction, exploration of new antidepressant agents targeting diverse biological systems definitely deserves more attention, including drugs affecting the neurotrophic system, glutamate system, opioid system, and dopamine system, all of which may interact with the immune system directly or indirectly [50-53].
Clinical implications

Our systematic reviews indicated that IL-6/1β and CRP are promising markers for MDD subtyping while IL-6 and CRP/hsCRP might be useful for predicting antidepressant treatment response. Given the quality and paucity of eligible studies it is clear that generalization to all patients with TRD remains difficult. Yet some of these biomarker measurements might help to improve both the accuracy of diagnosis and treatment. It is clear that this field of research is far from mature, and more work is needed on levels of inflammatory markers to accurately discriminate inflammatory subtype of depression from others. For example, many conditions, like lifestyle (smoking) and physical activity, can influence the levels of inflammatory markers [8], which could fluctuate within a certain range. So, for TRD patients who plan to receive anti-inflammatory treatment, it would be helpful to test the levels of sensitive inflammatory markers multiple times before starting and monitor their levels during the treatment. Alternatively, systematically collecting such information in clinical settings (e.g. in combination with clinical data from routine outcome monitoring) could be helpful to get a better idea of these patterns in regular treatment settings. The study of Osimo et al. that has looked at these levels in patients who were acutely admitted is an example of such work [9]. Relating such cross-sectional levels of inflammation to treatment outcome would provide relevant information to advance this field, in combination with regular RCT.

It should be noted, that not all types of depression are inflammatory driven [48] and one would probably not intervene in the normal functioning of the immune system because cytokines play a key role in the maintenance of brain function through regulation of neural integrity, neurogenesis and synaptic pruning [54]. In this regard, IL-6/1β and CRP/hsCRP could be helpful in MDD subtyping and decision making of a sensible treatment strategy.

Directions for future research

After many efforts, the relationship between inflammatory dysregulation and the pathophysiology of MDD is becoming increasingly clear. Yet the sometimes mixed findings require more well-designed studies. To increase the generalization of findings for depressed patients more homogeneous patient samples are needed. For example, the eligible studies in chapter 2 used a dichotomic method to make a distinction between melancholic and non-melancholic depression or between typical and atypical depression, but as a result a subgroup of patients could be classified as non-melancholic depression in one study and typical depression in other studies. In this respect, more explicit and unified definitions for MDD subtypes are required. In line with this, a consensus on the definition of TRD is also important for future studies. In chapter 3, the recruited studies determined the degree of treatment resistance using various standards and rating tools. This indicates that the severity and resistance spectrum varies among the studies which complicates comparisons between the included studies considerably. As stressed before the pivotal role of inflammatory cytokines in...
sustaining brain physiology must be taken into consideration [54] especially because not all types of depression are inflammatory driven [48]. Finally, considering the extensive interactions between the inflammatory system and other neuronal systems [50-52, 55], it would be wise to extend measurement of inflammatory cytokines with markers for other neuronal functions.

Closing remarks
Depression causes a substantial burden for the individual and society. Moreover, excess depression-related mortality has been documented for patients with many other diseases [56]. The role of inflammation in MDD is still far from clear but the relationship between inflammatory dysregulation and MDD might partly explain why around one third of the depressive patients do not sufficiently respond to conventional therapy. The levels of inflammatory biomarkers, especially peripheral biomarkers such as IL-1β, IL-6, and CRP/hs-CRP of MDD patients could be useful to improve both diagnosis and treatment of depression, as illustrated by emerging clinical studies [7, 57]. Besides agents with anti-inflammatory properties, conventional antidepressants such as SSRIs and TCAs may also have anti-inflammatory effects [58, 59], which could be an adequate choice for MDD patients with increased inflammatory activity. However, even though considerable lines of evidence support the immune/inflammation hypothesis of depression, treatment strategies are mainly targeted at monoaminergic systems. There is still a long way to go before therapy guidelines will be adapted, and it is clear that many well-designed studies based on a general consensus of TRD and MDD subtype definitions are needed to advance the applicability of anti-inflammatory treatments in MDD. Finally, one may wonder if mankind will ever be capable of developing a vaccine for the inflammatory driven type of depression.
References

32. Dantzer, R., et al., Inflammation-associated depression: from serotonin to kynurenine. Psychoneuroendocrinology,
59.58.55.54.53.52.51.50.49.48.47.46.45.42.40.38.37.36.33.328.327.326.325.324.323.322.321.320.221.220.219.218.217.216.215.214.213.212.211.210.29.28.27.26.25.24.23.22.21.20.19.18.17.16.15.14.13.12.11.10.9.8.7.6.5.4.3.2.1.0.
