Inflammatory Monocytes in Bipolar Disorder and Related Endocrine Autoimmune Diseases

Inflammatoire Monocyten in de Bipolaire Stoornis en Gerelateerde Endocriene Autoimmuun Ziekten

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I

General Introduction

1.1 BIPOLAR DISORDER

Bipolar disorder (also called manic-depressive illness) is one of the major mood disorders. The term manic-depressive illness was introduced by Emil Kraepelin (1856-1926) in the late nineteenth century.[1] It is in most patients a chronic illness with recurrent manic and depressive episodes, usually alternated with periods with normal mood between the episodes. A manic episode is characterised by an elevated, expansive or irritable mood which can be accompanied by a high self-esteem, decreased need of sleep, flight of ideas or racing thoughts, increased speech, distractibility, psychomotor agitation and excessive involvement in activities with painful consequences. A hypomanic episode meets the criteria for mania but is not associated with social or occupational impairment as is the case with a manic episode. A patient with a depressed episode has a depressed mood together with the possible following symptoms: sleep disturbances, psychomotor retardation or agitation, fatigue, feelings of worthlessness or guilt, impaired thinking or concentration, change of appetite or weight and suicidal thoughts.[2, 3] With its manic episodes bipolar disorder differs from (unipolar) depression, which is characterized by one or more depressive episodes, but never a manic (or hypomanic) episode.

Definition and subtypes

According to the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders)[2] bipolar disorder can be distinguished in various forms based on the type and severity of mood episodes experienced. Bipolar disorder I is the classic form of manic-depressive illness. It is characterized by one or more manic episodes or mixed episodes (in which standards are met for both depression and mania), usually accompanied by major depressive episodes. It is the most severe form of this mental illness. Bipolar disorder II is characterized by one or more major depressive episodes accompanied by at least one hypomanic but never a full manic episode. The third form of bipolar disorder is cyclothymic disorder. This disorder involves more or less chronic alternating hypomanic and minor depressive episodes. The mood cycles between highs and lows, but it will never reach the full criteria of mania or major depression. Finally, the term bipolar disorder Not Otherwise Specified is used for patients who clearly have certain symptoms of bipolar disorder but do not meet the criteria for one of the subtypes described above.

Under-diagnosis

Bipolar disorder is under-diagnosed. Up to 70% of individuals with bipolar disorder reported being misdiagnosed at least once, most commonly with unipolar depression,[4] while the time to receive a correct diagnosis can be more than ten years. Several factors can be hold responsible for this delay in diagnosis.[3] The first has to do with factors inherent to the illness such as the dual nature of the disorder. When patients are (hypo)manic, they are “high on life” and seldom seek treatment. By contrast, if they develop a depression, they do seek treatment, and do not mention the previous (hypo)manic episodes.

Thus, the (hypo)manic episodes remain unrecognized and the patient is incorrectly diagnosed with unipolar depression instead of bipolar disorder. A related factor is that even when asked about it, they may have difficulties in remembering a (hypo)manic episode during a depression. A third factor is that bipolar disorder can go together with or can be complicated by the signs and symptoms of other psychiatric illnesses such as psychotic disorders, anxiety disorders and substance use disorders, further complicating diagnosis. A last reason for under-diagnosis that needs mentioning is the lack of biological diagnostic markers that make it possible to distinguish patients with bipolar disorders from healthy individuals and from patients with other psychiatric diseases.

Epidemiology

Bipolar disorder is a major global health problem. The life time prevalence of bipolar disorder is estimated at around 2%.[5] However, given the matter of under-diagnosis and misdiagnosis this number will probably be an underestimation. The Netherlands Mental Health Survey and Incidence Study, a prospective epidemiological survey in the Dutch general population, calculated a lifetime prevalence for the total bipolar spectrum of 5.2% (95% CI: 2.2-8.1%), including 2% (95% CI: 0.1-4.1%) for bipolar I disorder.[6] Together with unipolar depression bipolar disorder belongs to the ten leading causes of disability worldwide[7] and is associated with a suicide risk of at least 5%.[5]

Treatment and under-treatment

To achieve stabilisation of their mood episodes, patients with bipolar disorder need long-term treatment with so-called mood stabilizers. Lithium is the oldest among these mood stabilizers and is still considered the first choice.[5, 8] Other options are the anticonvulsants valproate, carbamazepine and lamotrigine and several atypical antipsychotics. Often other medications are needed as well, especially to treat the acute manic episodes (with the same medications) or depressive episodes (also with antidepressants). However, even with the available treatment, up to 40% of patients relapse.[9] Besides medication not being sufficient enough, bipolar patients are also at risk for under-treatment. The Netherlands Mental Health Survey and Incidence Study showed that of 136 bipolar patients studied only 15% received adequate treatment,[10] the main reason for this is the above discussed under-diagnosis. Therefore one can assume that better diagnostic tools will substantially improve treatment and consequently outcome in patients with bipolar disorder.

Etiology

The etiology of bipolar disorder is far from unravelled. It is thought to be a multifactorial disease but researchers have not yet developed a single hypothesis that unifies all the genetic, biochemical, pharmacological and anatomical data on the etiology of bipolar disorder. In the following section several aspects regarding the etiology of bipolar disorder will be discussed.

First, epidemiological studies imply that this mental illness is heritable (see also Chapter 4) but specific genes for bipolar disorder have not yet been identified. This could be due to bipolar disorder being a polygenic disorder in which not one gene but several genes determine the vulnerability to develop the illness. Also, it could be that bipolar disorder is not one illness with one etiology but consists of a complex of various illnesses with various etiologies. An indication for this is the heterogeneity of symptom presentation of the bipolar patient population.

Second, various environmental factors have been implicated in bipolar disorder such as stress,[11, 12] infections[13] and diet.[14]

Third, biochemical and pharmacological studies have led to various hypotheses involving several neurotransmitter systems. The monoamine hypothesis suggests a deficiency in monoamine neurotransmitters (especially noradrenalin) to be responsible for depression[15] whilst a increased function is associated with mania.[15-18] Another etiologic theory was introduced by Janowsky as the cholinergic-aminergic balance hypothesis.[19] Here an increased ratio of cholinergic to adrenergic activity is postulated to lead to depression, whereas the reverse might occur in mania. The third hypothesis involves serotonin.[15] It is thought that low serotonin levels could result in both depression and mania through deficient regulation of other neurotransmitters. More recently a decreased activity of γ-aminobutyric acid (GABA, the major inhibitory neurotransmitter) and an increased activity of glutamate (the major excitatory neurotransmitter) are mentioned as possible contributors to the pathofysiology of bipolar disorder.[15]

Fourth, second messenger systems have been mentioned as possible contributors to the etiology of bipolar disorder. Second messengers transduce signals from stimulated receptors on the outside of the membrane to target molecules in the cytosol and/or nucleus inducing in this way a cellular response. Second messengers are thus important *intracellular* signalling molecules while neurotransmitters are important *extracellular* signalling molecules of the brain. In bipolar disorder changes have been described in second messenger systems such as G-proteins, phosphatidylinositol, protein kinase C, cAMP generating system and Ca^{2+} activity.[15] Furthermore, the mood stabilizers lithium and valproate are reported to have an effect on these systems.[20]

Fifth, changes in synaptic plasticity and neuronal survival have been implicated in the pathogenesis of bipolar disorder. Various factors are important for the plasticity of brain, but neurotrophic factors are seen as the key mediators, especially brain derived neurotrophic factor (BDNF). It was demonstrated that stress and depression led to cell loss, neuronal atrophy and down-regulation of BDNF in brain limbic regions that regulate emotion and cognition, whereas anti-depressive drugs blocked or reversed these effects. These observations led to the neurotrophic hypothesis of mood disorders. With regard to bipolar disorder, it is thought that mood episodes and stress change brain structures (via their effect on neurotrophic factors) in such a way that it makes patients more vulnerable to stress and less capable of adopting adequate coping strategies. Consequently, this is thought to lead to a higher susceptibility to develop further mood episodes and cognitive

impairment. That indeed neurotrophins could play a role in the pathogenesis of bipolar disorder, has been shown by the low serum levels of BDNF in manic and depressed patients, the association of the Val66met BDNF polymorphism with bipolar disorder and the up-regulation of BDNF in brain regions by lithium and valproate. In addition, changes in brain plasticity have been demonstrated in bipolar disorder.[21, 22]

Sixth, anatomical and functional studies play a role in disentangling the pathofysiology of bipolar disorder. Post-mortem brain studies reported abnormalities in morphology of neurons[23] and decreased glial densities in prefrontal brain regions.[24, 25] Magnetic resonance imaging (MRI) studies showed a diffuse gray matter loss, regional cell loss in the prefrontal cortex, enlargement of striatum, thalamus and amygdala, atrophy of cerebellum and volume increase of ventricles.[26-28] Some of these structural deficits are present at disease onset and are therefore seen as possible vulnerability factors for disease development.[24] Others correlate with the number of endured episodes, suggesting these brain damages occur as a result of disease.[24 29] It must be noted though that regarding the above mentioned aberrancies also negative and even contradictory results have been published.[26] Furthermore, specificity is uncertain, since brain structure abnormalities have also been reported in other psychiatric diseases as unipolar depression and schizophrenia.[29]

With the use of newer techniques such as magnetic resonance spectroscopy (MRS), it became possible to study *in vivo* brain chemistry. Using this technique in bipolar patients various abnormalities have been reported in choline metabolism, membrane phospholipid metabolism and mitochondrial metabolism.[29]

Finally, there are etiological theories involving the endocrine and immune system. The latter system will extensively be described in the next chapter of this thesis. The relation of the endocrine system with mood disturbances has been an early observation in biological psychiatry. A main system that seems to be involved is the hypothalamic-pituitary-thyroid (HPT) axis. Bipolar patients are frequently troubled with HPT-axis abnormalities.[30, 31] Similarly, patients with thyroid problems can develop psychiatric symptoms such as a depressed mood and cognitive dysfunction.[32, 33] There is also ample evidence implicating the hypothalamic-pituitary-adrenal (HPA) axis in bipolar disorder. Patients with Cushing's syndrome (thus patients with high cortisol levels) often encounter psychiatric problems such as depression, mania and cognitive dysfunction.[34, 35] And HPA-axis abnormalities are frequently reported in bipolar disorder. They are mentioned in Chapter 1.3.

1.2 THE IMMUNE SYSTEM

Before the role of the immune system in mood disorders is discussed, the immune system itself will be briefly introduced here[36] (Figure 1 and Table 1).The immune system is divided in the innate immune system and the adaptive immune system.

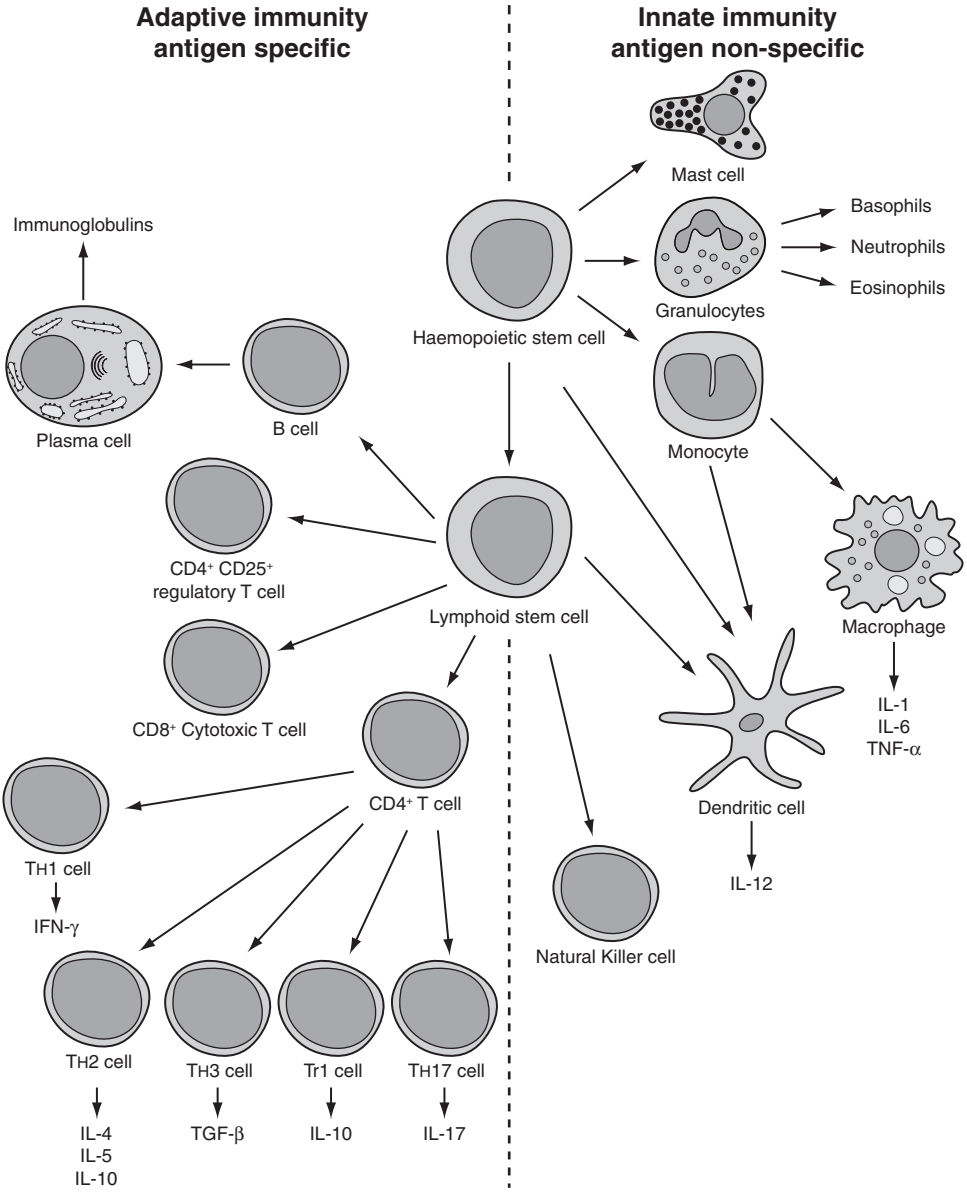

Figure 1.
Depicted in this figure are the important players of the innate and adaptive immune system. Their roles are described in the text.
IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; TGF, tumor growth factor; Th, T helper cell.

Table 1. The important cell types of the innate and adaptive immune system.

Cell type	Origin	Main location	Function	Important markers
Innate immune system				
Mast cell	Bone marrow	In mucosal and epithelial tissues near small blood vessels	Release granules containing histamine and active agents	Tryptase, Chymase
Granulocytes	Bone marrow	Blood	-Phagocytosis and activation of bactericidal mechanisms (neutrophils) -Killing of antibody-coated parasites (eosinophils) -Unknown (basophils)	CD15
Monocyte	Bone marrow	Blood	Precursor of macrophages	CD14
Macrophage	Bone marrow	Various tissues (e.g. spleen, lymphnodes, liver, lungs, skin)	Phagocytosis and activation of bactericidal mechanisms, antigen pre-sentation	CD68
Dendritic cell	Bone marrow	Various tissues (e.g. spleen, lymphnodes, liver, lungs, skin)	Transfer of antigen from periphery to lymphnodes, antigen presentation	CD1a, DC-SIGN, CD83
NK cell	Bone marrow	Blood	Kill some virus-infected cells by releasing lytic granules	CD16, CD56
Adaptive immune system				
T cell	Bone marrow, thymus	Thymus medulla, T cell areas of lymphnodes, spleen and tonsils	Cellular immunity	CD3
Helper T cells	Bone marrow, thymus	T cell areas of lymphnodes, spleen and tonsils	Recognize antigen if presented by antigen presenting cell; skewing immune response	CD3, CD4
Cytotoxic T cells	Bone marrow. thymus	T cell areas of lymphnodes, spleen and tonsils	Kill infected cells	CD3, CD8
Regulatory T cells	Bone marrow, thymus	T cell areas of lymphnodes, spleen and tonsils	Inducing tolerance	CD3, CD25, FOXP3
B cell	Bone marrow	B cell areas of lymphnodes	Production antibodies	CD19, CD20

NK, natural killer cell.

Innate immunity

The innate immune system is a first line of defence against pathogens and provides a fast response with a limited specificity. The key players are: 1) barriers, which can be chemical such as tears or saliva, or mechanical such as the skin; 2) cells, which compromise monocytes, the monocyte-derived dendritic cells and macrophages, neutrophils, natural killer cells and mast cells; and 3) soluble factors, of which complement factors and various cytokines and chemokines are examples. If the innate system fails to resolve the infection, the adaptive system will get triggered by cells of the innate immunity. This is seen as the interphase between innate and adaptive immunity.

Interphase

The cells responsible for the activation of the adaptive immune system and thus the key players of the interphase are the various antigen presenting cells (APC). Accessory macrophages are an example of these APC but the most specialized ones are the dendritic cells (DC). DC pick up antigen at the site of infection, travel through lymphatics to the lymph node and present the collected antigen to cells of the adaptive immune system. With the presence of MHC class II molecules and the co-stimulatory molecules CD80 and CD86, DC are well equipped to stimulate the naïve cells of the adaptive immune system. These cells will subsequently proliferate and play their role in the combat against the invading pathogens.

Adaptive immunity

The adaptive immune system is antigen specific, provides a memory and is typically activated a few days later than the innate system. The main contributors are T cells and B cells. B cells, which transform into plasma cells, are responsible for the production of antibodies, the so-called humoral immunity. Antibodies neutralize extracellular bacteria, viruses and toxins, activate complement and facilitate pathogen phagocytosis. T cells are responsible for the so-called cell-mediated immunity. T cells form a heterogeneous group of cells comprising effector cells, regulatory cells and memory cells and are typically divided into $CD4^+$ and $CD8^+$ T cells. The effector $CD8^+$ T cells are cytotoxic and important in killing other body cells infected with virus, intracellular bacteria, or which are otherwise damaged or dysfunctional. The effector $CD4^+$ T cells are important in providing help to other immune cells and are therefore called helper T cells (Th). A naïve T helper cell (a cell which has not encountered an antigen yet in the presence of an APC) can upon stimulation by an APC differentiate into three Th subtypes namely Th1, Th2 and Th17 cells. Th1 cells activate mononuclear cells (e.g. macrophages), and are in this way essential in the elimination of intracellular pathogens. Th2 cells play a central role in clearing extracellular pathogens as they are responsible for the transformation of B cells into plasma cells. The third member of Th cells the Th17 cell has just recently been identified.[37, 38] The function of Th17 cells is not yet completely understood but is suggested to be in host defence against mainly extracellular pathogens which have not been cleared efficiently by the other Th cells.

Next to these effector and helper T cells, there are also T cells with regulatory functions as the immune system needs to be tightly regulated since it is well equipped to lethally damage the host cells if it looses control. The main regulatory T cell subsets are the $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells (Tregs), the Tr1 cells (defined by a high production of the anti-inflammatory cytokine IL-10) and the Th3 cells (defined by a high production of the anti-inflammatory cytokine TGF-β).

Monocytes, macrophages and dendritic cells in more detail

Since monocytes are important targets for the studies described in this thesis, the cells are here described in a little more detail. Monocytes are closely related to macrophages and dendritic cells and are often referred to as the "mononuclear phagocyte system" or the "monocyte-macrophage-DC (MMD)" system. This group of hematopoietic cells has diverse characteristics and origins[39]. In general the cells originate in the bone marrow and migrate as monocytes through the blood to peripheral tissues. The main descendents of these circulating monocytes, the macrophages and DC, occur in virtually all organs. Moreover, each organ contains multiple different macrophage and DC subpopulations. However local myeloid precursors for macrophages and DC are also present in various organs, including the pancreas and the brain.

The best-known functions ascribed to macrophages -clearance by phagocytosis and digestion- and to DC -primary activation of the adaptive immune system- have long been the leading principle in distinguishing these cell types. However, the biologic reality is much more complex. The cells of the MMD system comprise a large family of functionally and/or developmentally related cells that form a continuum in which the cells recognized as prototypical 'macrophages' and 'DC' form the extremes of a spectrum. Both DC and macrophages are involved in the regulation of the immune response and both DC and macrophages are capable of up and down-regulating immune responses. In 'danger' situations the cells of the MMD system become pro-inflammatory by perceiving danger signals, and then guide an effective T cell response to eliminate the danger-evoking signal. In normal situations without any danger the cells stay in their "tolerogenic steady state", yet are active and do interact with T cells, but in such a way that tolerance to auto-antigens is upheld and induced (thus in particular to various populations of Treg cells). To execute these functions the cells of the MMD system must make contacts with T cells and they do so in a cluster formation with T cells. In these clusters antigen, MHC class II molecules, adhesion and co-stimulatory molecules as well as signalling intermediates are organized in supra-molecular activation clusters (SMACs or immune synapses).

To conclude, the monocyte-macrophage-dendritic cell system comprises a large family of functionally and/or developmentally related cells essential for both inducing tolerance towards autoantigens and for inducing an effective immune response towards dangerous intruders.

1.3 MOOD DISORDERS AND THE IMMUNE SYSTEM

Sickness behaviour

Various observations have led to the concept that the immune system plays a role in the pathogenesis of mood disorders. First, it was noted that acute infection in man was accompanied by a cluster of non-specific symptoms including decreased appetite, anorexia, weight loss, sleep disturbances, retardation of motor activity, reduced interest in social environment, loss of libido, impaired cognitive abilities, dysphoria, anhedonia and depressed mood. This behavioural symptom complex was called “sickness behaviour” and was seen as a natural response of the body to infection to force an infected individual to reorganise priorities.[40, 41] Many of these behavioural aspects were noted to resemble symptoms seen in unipolar depression. Second, it was observed that sickness behaviour was seen in cancer and hepatitis patients after treatment with pro-inflammatory cytokines such as IFN-α.[42-44] The behavioural symptoms almost immediately disappeared after termination of treatment indicating a causative role of cytokines in mediating this condition.[44]

The macrophage- T cell theory of depression

The possible role of the immune system in mood disorders was introduced by Smith as the macrophage-T cell theory of depression.[45] This theory was embraced in the scientific field. Different names were given such as IRS (inflammatory response system) model of depression[46] and the cytokine hypothesis of depression,[47] but the essential remained the same: unipolar depression is seen as a psychoneuroimmunological disorder, in which peripheral immune activation, through release of cytokines, is responsible for the variety of behavioural, neuroendocrine and neurochemichal alterations that are associated with this psychiatric condition. To further investigate this theory, the above mentioned observations were studied in experimental settings. In rodents endotoxin (which is accompanied by a rise in cytokine levels) and the pro-inflammatory cytokine IL-1 were administrated and indeed this induced the full spectrum of sickness behaviour in these animals hereby confirming the role of cytokines in this behavioural complex.[48-50] In addition, Reichenberg *et al.* injected *Salmonella abortus equi* as endotoxin in healthy volunteers.[51] This endotoxin submission increased cytokine levels, induced fever and led to anorexia, anxiety, depressed mood and memory impairments. The levels of the latter three symptoms were positively correlated with cytokine concentrations suggesting again a causative role of cytokines in inducing this behaviour.

Immune abnormalities in unipolar depression

That the immune system indeed is activated in patients with an unipolar depression has been shown in numerous reports.[52, 53] The following characteristics of immune activation in unipolar depression have been established: high levels of pro-inflammatory cytokines in serum, saliva and cerebral spinal fluid,[54-58] a high production of cytokines by

macrophages,[59] elevated serum levels of positive acute phase response proteins (such as CRP and haptoglobin),[60-62] reduced serum levels of negative acute phase response proteins (such as albumin and transferrin),[63, 64] elevated numbers of leucocytes, and higher serum levels of activation markers of cell-mediated immunity.[65]

Brain-to-immune communication and vice-versa (Figure 2)

The brain has modulatory effects on the immune system via the hypothalamic-pituitary-adrenal (HPA)-axis and the sympathetic arm of the autonomic nervous system (SNS). The HPA-axis is formed by the hypothalamic paraventricular nucleus (PVN), the anterior pituitary gland, and the adrenal cortex. Environmental stressors are received and monitored by the hypothalamus and will lead to the production of corticotrophin-releasing hormone (CRH). This hormone will stimulate the synthesis of adrenocorticotrophin hormone (ACTH) from the anterior pituitary gland. ACTH will subsequently induce the production of the gluco- and mineralocorticoids from the adrenal cortex. Depending on the level of glucocorticoids (GC), GC either have immune suppressive or immune stimulatory effects, low concentrations being stimulatory, while high concentrations have immune suppressive effects. The HPA-axis is subject to a classical negative feedback loop by the end product: GC inhibit the release of CRH and ACTH at both the hypothalamic and pituitary level.

The SNS system is also stimulated under stressful conditions. The SNS has both stimulatory and inhibitory effects on the immune system, and these effects are achieved via the production of catecholamines.

In Figure 2 macrophages are depicted as an example cell for this regulation and it can be seen from this picture that pro-inflammatory cytokines produced by macrophages, like IL-1, IL-6 and TNFα, are subject to the regulatory action of GC and catecholamines. These cytokines on their turn are able to regulate the HPA-axis and the SNS via negative feed back loops. But how can cytokines produced and circulating in the periphery communicate with the brain? Cytokines are known to be large and hydrophilic molecules and are therefore not likely to cross the blood-brain-barrier (BBB). So other mechanisms must be important (Figure 2). First, there are regions in the brain where the blood-brain-barrier is absent or less restrictive such as the circumventricular sites and the organum vasculosum of the lamina terminalis.[66] Passive transport is thought possible here. Second, it might also be that the BBB loses its integrity by certain pathological conditions (f.i. in MS) or by the cytokines themselves.[67, 68] Third, active transport is suggested to play a role in letting cytokines pass the BBB as transporters have been identified for IL-1, IL-6 and TNFα.[69] Fourth, there are reports on cytokines binding to receptors on cerebral vascular endothelial cells inducing in this way second messengers.[70] Via these endothelial second messengers cytokines can influence the brain without having to cross the BBB. Finally, a nervous communication between brain and the peripheral immune system needs mentioning involving the vagus nerve which innervates the immune organs and receives immunogenic stimuli from cytokines produced by macrophages, DC and other immune cells.[71]

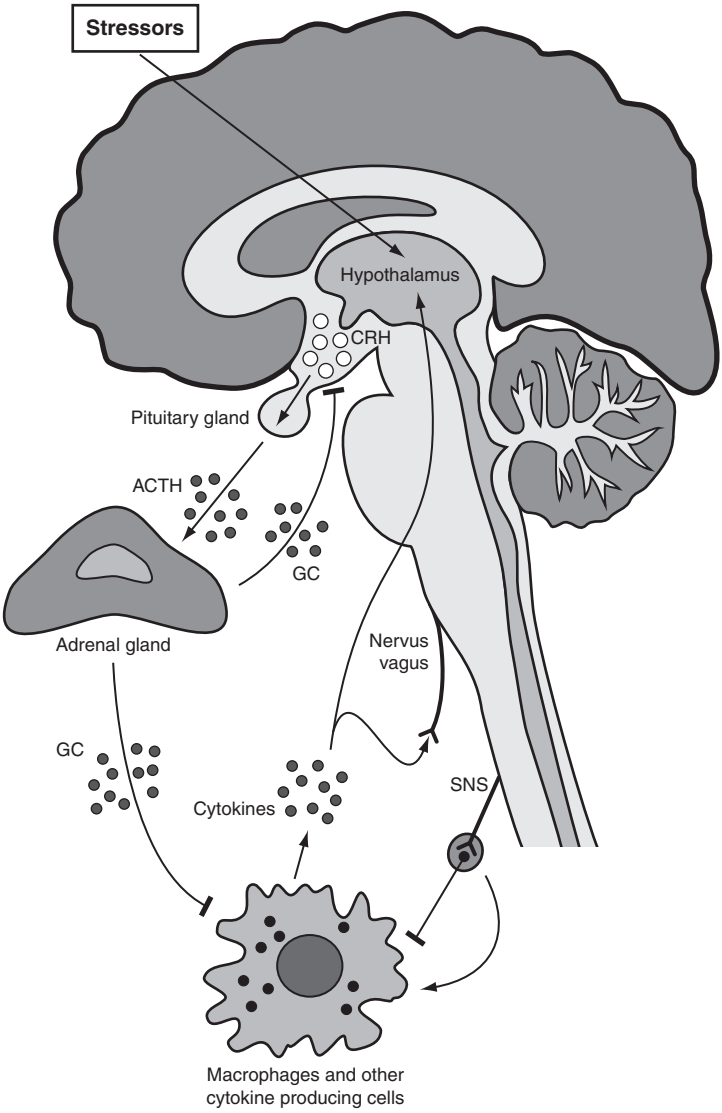

Figure 2. Interaction immune system and brain.
The immune system and brain communicate via neuronal and humoral pathways. The various pathways are depicted in this figure and described in the text.
CRH, corticotropin hormone; ACTH, adrenal corticotropin hormone; GC, glucocorticoids; SNS, sympathetic nervous system

Possible mechanisms underlying cytokine-induced depression

Cytokines and cytokine-stimuli reaching the brain may cause depression in various ways. It is well known that the neurotransmitters noradrenalin (NA) and serotonin (5-HT) play a pivotal role in the pathogenesis of major depression[72] and it has been suggested that cytokines can induce mood symptoms via their effect on these (and other) neurotransmitters. This effect can be reached via several routes. First, cytokines themselves can alter the turnover of the neurotransmitters in particular brain regions that are thought to be important in unipolar depression.[73] Second, cytokines can influence neurotransmitter levels via the enzyme indoleamine-2,3-dioxygenase (IDO). Indeed, cytokines such as IL-1, IL-6 and IFNs are known to induce this tryptophan metabolizing enzyme IDO. Tryptophan is the most important precursor of 5-HT. Over-stimulation of IDO by cytokines could thus reduce the tryptophan availability for the brain leading to reduced levels of 5-HT.[74, 75] Furthermore, certain metabolites of the IDO-mediated kynurenine pathway such as 3-hydroxy-kynurenine and quinolinic acid are thought to be neurotoxic.[76] A third possible mechanism by which cytokines can induce depression via an effect on neurotransmitter levels or function is via the HPA-axis.[52] As said before, the HPA-axis produces stress hormones such as cortisol as response to internal and external stressors. Chronically elevated cortisol levels have been described to decrease postsynaptic serotonin receptors and serotonin responsiveness and to reduce the β-adrenergic response to NA in the brain.[77-79] Furthermore, cortisol has effect on neuronal plasticity by reducing neurotrophic factors such as BDNF (brain derived neuronal factor).[80] The HPA-axis is known to be activated in unipolar depression and also bipolar disorder as demonstrated by high levels of cortisol in plasma, urine and cerebral spinal fluid of patients and an abnormal response to the dexamethason (dex) suppression test and dex/corticotrophin-releasing hormone test.[81-88] Normally, hyperactivation of the HPA-axis is prevented by negative feedback of the elevated cortisol levels. However, this feedback system seems deregulated in mood disorders.[89] There is evidence that cytokines are not only responsible for the activation of the HPA-axis but also for this deregulation of the feedback system. The induction of resistance of the glucocorticoid receptors in the hypothalamus and pituitary (and thus making these glucocorticoid receptors less sensitive for elevated cortisol levels) is thought to be a possible mechanism in this.[81, 90]

Immune abnormalities are also present in bipolar disorder

Most of the above is based on observations in unipolar depression. It can be assumed that immune aberrancies and the subsequent role for these aberrancies in the pathogenesis of the mood disorder could also apply for bipolar disorder since a) depression is part of the bipolar spectrum and b) it was observed that mania (the other element of the bipolar spectrum) could be induced by IFNα treatment in chronic hepatitis patients.[91] Reported evidence regarding immunological disturbances in bipolar disorder is presented in Table 2.

Table 2. Immunological disturbances in bipolar disorder.

Results	Patient characteristics	Reference
Serum/ plasma		
Cytokines and soluble receptors		
sIL-6R ↑ and sIL-2R ↑, no aberrancies IL-6	*N*=10, Untreated, Manic,	186
sIL2R ↑ and sIL6R ↑, normalization after Li treatment no changes in IL-2, IFNγ, IL-10 and IL-4	*N*=17, Untreated → Treated, 6 depressed, 2 hypomanic, 9 euthymic,	187
IL-1RA ↑ and IFNγ ↓, no aberrancies IL-2, IL-4 and IL-10, no effect of treatment	*N*=29, 13 Untreated, 16 Treated, Manic	188
sIL-2R ↑, after remission normalization	*N*=23, Untreated → Treated, Manic	189
sIL-2R ↑, no influence of mood status	*N*=172, 70 Untreated, 102 Treated, manic (?), depressed (?), euthymic(?)	190
No changes in IL-12	*N*=25, Untreated, Manic	191
IFNγ ↑, IL-4 ↑, TGF-β ↓ Normalization TGF-β after treatment	*N*=70, Untreated→Treated, Manic	192
IL-8 ↑ and TNFα ↑, no aberrancies for IL-6, IL-10 and sIL-6R	*N*=21, 12 Untreated, 9 Treated, Manic (12), Depressed (9)	193
Acute phase proteins		
Hp ↑, Fb ↑, alpha 1-AT ↑ and Hpx ↑, normalization after treatment	*N*=23, 15 Untreated, 8 Treated, Manic	60
manic BP: CRP ↑ (also compared to euthymic and depressed BP)	*N*=80, most (?) treated, Manic (30), Depressed (20), Euthymic (30)	194
Autoantibodies		
TPO-abs ↑, not associated with lithium treatment or mood status	*N*=226, 124 Untreated, 102 Treated, Manic (?) Depressed (?), Euthymic (?)	139
Prevalence ANA ↑	*N*=290, 144 Untreated, 146 Treated, Manic (27), Depressed (74), Cycling (17), Euthymic (165), Unknown (7)	195
Monocytes/ macrophages/ dendritic cells		
LPS stimulated monocytes: abnormal Il-1β/ IL-6 production ratio (↓IL-1β/↑ IL-6), normalization ratio after Li treatment, no association with mood status	*N*=80, 16 Untreated, 64 Treated, Manic (11), Depressed (15), Euthymic (50), cycling (4)	196
Defect in differentiation of monocytes into DC after GM-CSF/ IL-4 culture: low expression CD14 and low T cell stimulatory capacity, after Li treatment restoration of defects to even activation.	*N*=53, 12 Untreated, 41 Treated, Manic (8), Depressed (12), Cycling (2), Euthymic (31),	197

Table 2. Immunological disturbances in bipolar disorder (continued).

Results	Patient characteristics	Reference
Lymphocytes		
After mitogen stimulation: IL-2, IL-6, IL-10 and IFN-γ secreting cells↓, associated with Li treatment	*N*=50, 10 Untreated → Treated, 40 Treated, Euthymic	198
Mitogen stimulated PBMC: IFNγ production ↓, IL-10 production no aberrancies, same results in subsequent remission	*N*=20, 12 Untreated, 8 Treated , Manic	199
Higher levels of circulating activated T cells, unrelated to Li exposure or mood status.	*N*=172, 70 Untreated, 102 Treated, manic (?), depressed (?), euthymic(?)	190
No aberrancies in lymphocyte counts and subsets Mitogen induced lymphocyte proliferation ↑	*N*=11, Treated, Manic	200
Antibody-dependent cellular toxicity ↓	*N*=11, Treated, Manic	201
Mitogen-induced lymphocyte proliferation ↑, normalization after remission	*N*=23, Untreated → Treated, Manic	189
Activated lymphocytes ↑ in manic and depressed BP, not in euthymic BP	*N*=12, 3 Untreated, 9 Treated, Manic (3), Depressed (3), Euthymic (3)	202
Relative T cell resistance to dexamethason, no effect of Li treatment or mood status	*N*=54, 49 treated, 5 untreated, euthymic (29), depressed (15), manic (9), cycling (1)	203

Hp, haptoglobin; Fb, fibrinogen; Hpx, hemopexin; alpha 1-AT, alpha-1 antitrypsin; BP, bipolar patients; CRP, C-reactive protein; TPO, thyroidperoxidase; ANA, antinuclear antibodies, LPS, lipopolysaccharide, GM-CSF, granulocyte monocyte colony stimulating factor; Li, lithium.

1.4 FAMILY, TWIN AND ADOPTION STUDIES, IN PARTICULAR WITH REGARD TO BIPOLAR DISORDER

Family studies

Family, twin and adoption studies represent approaches to study familial and genetic influences on a specific disorder or trait. With the use of family studies it can be shown that a disorder aggregates in families. This however does not automatically mean that genes are responsible for this aggregation since next to sharing genes family members also share a part of their environment. However, a family study is still a useful tool to estimate the morbidity risk of a disorder in relatives. With regard to bipolar disorder a meta-analysis comprising eight family studies demonstrated a morbidity risk of 4-9% in first degree relatives.[92]

Twin studies

To be able to distinguish between genetic and environmental influences, twin studies are a very useful approach. For this purpose, monozygotic (MZ) and dizygotic (DZ) twins are studied.[93, 94] MZ twins are essentially genetically identical and DZ twins share on average only half of their genes. Since MZ as well as DZ twin pairs living together are assumed to match for a shared environment (meaning that the shared environmental influences on MZ twins will not be different from shared environmental influences on DZ twins), significantly higher rates in MZ twins must reflect the action of genes. Furthermore, a concordance rate of lower than 100% in MZ twin pairs indicates that environmental factors must play a role. Twin studies can be used to estimate the specific contribution of genetic and environmental factors to the variance in liability to the disorder. The sources of genetic and environmental variation that are usually considered are the additive genetic influences (A), shared environmental influences (C, such as socio-economic status, parenting style, childhood diet, shared infections, having a sick parent and peer influences of adolescent twins) and unique environmental influences that result in differences among family members (E, e.g. stressful live events). These factors together give the total phenotypic variance of the liability of the disorder and are described in a model called ACE.[93-95] The estimated proportion of phenotypic variance caused by the genetic influences is referred to as the heritability of the disorder. Heritability refers to the strength of genetic influences in a population (and not in a particular individual), and it may differ depending on which population is studied. More advanced statistical methods have become available to analyse twin data, for example methods that take into account the effect of certain covariates (such as gender) on a trait. Specific structural equation modelling (SEM) programs such as Mx[96] are available for this kind of analysis. We used these advanced twin mathematics in Chapter 4, in a study on the heritability of monocyte activation in bipolar disorder.

Twin studies in bipolar disorder

For bipolar disorder, the concordance rate in MZ twins ranges from 39-75% and in DZ twins from 0-8%.[97] This wide range of concordance rates can be explained by the use of different diagnostic methods in the various studies. As discussed above this higher observed concordance rate in MZ twins than in DZ twins means that there must be a genetic influence on the familial aggregation of bipolar disorder seen in family studies. Indeed the heritability (so the relative attribution of genes to the phenotype bipolar disorder) of bipolar disorder is estimated at 60-85%.[98, 99] However, given the observation that the heritability and the concordance rate of MZ twins is not 100% indicates that environmental factors must be important as well.

Adoption studies

Adoption studies are another approach to distinguish between genetic and environmental influences. These studies examine the shared environment of non-

genetically related family members and the shared genetics of family members who have different environments. If a high relative risk for a disorder or trait is found in both adoptee and adoptive parents, a shared environmental influence is suggested. In contrast, if the same risk is found in adoptee and biological parents, a genetic influence is expected.[97, 98, 100]

Adoption studies in bipolar disorder

For bipolar disorder there are not many adoptive studies available, since such studies are very difficult to conduct. Only two reports have been published. However, both studies demonstrated that biological parents of bipolar adoptee have higher rates of bipolar disorder than adoptive parents (5-7% vs. 1.8-2.8%).[101, 102] This again indicates that in bipolar disorder the genetic contribution is substantial.

1.5 ORGAN-SPECIFIC AUTOIMMUNITY

Autoimmune diseases are diseases in which the immune system attacks the tissues of its own host. Autoimmune diseases are divided in systemic and organ-specific autoimmune diseases. The organs that are most often under attack of its own immune system are the endocrine organs such as the pancreas (type 1 diabetes), the thyroid gland (autoimmune thyroid disease), the adrenal gland (Addison's disease), the parietal cells of the stomach (atrophic gastritis) and the gonads.

Type 1 diabetes

Type 1 diabetes (T1D) is an organ-specific endocrine autoimmune disease in which the insulin producing β-cells of the islets of Langerhans in the pancreas are destroyed by autoimmune processes. Because of this β-cell destruction, the production of insulin diminishes and finally comes to an end. This will lead to hyperglycaemia. Insulin shortage itself can lead to keto-acidoses which is, when untreated, a fatal outcome of T1D. Chronic hyperglycaemia has its own set of problems such as cardiovascular disease, chronic renal failure, and retinal and nerve damage.

Several autoantigens have been reported in T1D, including GAD65 (65 kDA glutamic acid decarboxylase), ICA69 (69 kDa islet cell antigen), IA2 (insulinoma-associated protein 2) and insulin.[103] Of these autoantigens insulin seems to be most important.[103] Firstly because GAD65 and ICA69 were shown to be dispensable for diabetes development in GAD65 and ICA69 knock-out mouse models.[104, 105] Second, because insulin deficiencies were found to abrogate the development of T1D.[106, 107] Third, insulin reactive $CD4^+$ T cells and $CD8^+$ T cells are present in islet infiltrates and these cells could adoptively transfer disease to syngeneic recipients.[108, 109] Fourth, in humans a relatively high frequency of insulin A-chain-reactive $CD4^+$ T cells was observed in the pancreas associated lymph nodes.[110] And finally since, a polymorphic variable number tandem repeats (VNTR) located

upstream of the insulin gene leads to susceptibility or resistance to T1D by influencing the level of expression of pro-insulin in the thymus.[111]

Antibodies against the above mentioned autoantigens are frequently present, not only in T1D patients but also in their first degree relatives. [112, 113] However, these autoantibodies probably do not have an active role in the pathogenesis of diabetes but are thought to be a secondary result of β-cell destruction caused mainly by $CD8^+$ T cells and macrophages. They are nevertheless helpful in prediction of progression of disease.

The pathogenesis of T1D is -like bipolar disorder- multifactorial which means that both genes and environment play a role. This can be concluded from the higher concordance rate of T1D in MZ twins (53%, 95% CI: 33-73%) as compared to DZ twins (13%, 95% CI: 0.05-0.21%)[114] and from the observation that the MZ concordance rate does not reach 100%. The various genes and environmental factors indicated in type 1 diabetes are summarized in Tables 3 and 4.

Latent autoimmune diabetes of the adults (LADA)

In this thesis the term autoimmune diabetes will be used. This term describes diabetes forms which are accompanied by diabetes-associated antibodies. Hence they include type

Table 3. Genes with multiple replication studies involved in T1D and AITD.

Gene	Description	Associated diseases	Reference
Insulin	Hormone produced by the pancreas	T1D	204
HLA class II	Antigen presentation		204
DR3; DR4		T1D	205, 206
DR3; DR5		GD, HT	207, 208
CTLA4	Inhibits T cell-activation by binding the co-stimulatory molecules CD80/CD86	T1D GD,HT	204 207
PTPN22	Involved in T cell signaling, binds to Csk, a kinase playing a role in down-regulating T cell activation	T1D GD, HT	204, 207
CD40	Involved in B cell differentiation	GD	209
TSHR	TSH receptor	GD	207
TG	Major protein product synthesized in the thyroid	AITD	207

HLA, human leukocyte antigen; CTLA4, Cytotoxic T-Lymphocyte Antigen 4; PTPN22, protein tyrosine phosphatise 22; Csk, C-terminal Src tyrosine kinase; TSHR, thyroid stimulating hormone receptor; TG, thyroglobin.

Table 4. Environmental factors involved in the pathogenesis of T1D and AITD.

Environmental factor	Associated diseases	References
Infections		
Enterovirus	T1D, TPO-abs	210, 211
Congenital rubella infection	T1D, HT	212, 213
Rotavirus	T1D	214
CMV	T1D	
Mumps	T1D	
Yersinia enterocolitica	GD	215
Diet		
Early exposure to cow's milk	T1D	210
Gluten	T1D, TPO-abs (in patients with celiac disease)	210, 216
Vitamin D	T1D, AITD	210, 217-221
Omega-3 fatty acids (protective)	T1D	222
Iodine excess	GD, HT	215
Selenium deficiency	HT	215
Growth		
Increased growth rate	T1D	210
Toxins		
N-nitroso compounds	T1D	210
Bafilomycin A1	T1D	210
Smoking	GD	215
Smoking (protective)	T1D, TPO-abs	223, 224
Ante and perinatal factors		
High maternal age (>35 yrs)	T1D	210
Maternal-child blood group incompatibility	T1D	210
Excessive weight gain in pregnancy	T1D	210
High birth weight	T1D	210
Low birth weight	TPO-abs	215
Stressful life events	T1D, GD	210, 215
Fetal microchimerism	GD, HT	215
Hormonal influences		
Oral contraceptives (protective)	TPO-abs	215
Longer reproductive span	HT	215

T1D, type 1 diabetes; HT, Hashimoto's thyroiditis; GD, Graves'Disease; TPO, thyroid peroxidase, CMV, cytomegalovirus; AITD, autoimmune thyroid disease.

1 diabetes but also latent autoimmune diabetes of the adults (LADA). LADA has an adult onset and patients are initially not insulin dependent. In this it resembles type 2 diabetes. However, genetically and immunologically it resembles T1D since it has a similar HLA genetic susceptibility and it is associated with islet autoantibodies. Furthermore, although not insulin dependent from the beginning, most patients develop an insulin dependency within three years.[115, 116]

Autoimmune thyroid disease

Another archetype of the organ-specific endocrine autoimmune diseases is autoimmune thyroid disease (AITD). AITD encompasses a diverse range of clinical entities including Hashimoto's thyroiditis (HT), Graves' disease (GD), atrophic autoimmune thyroiditis, postpartum thyroiditis, sporadic painless thyroiditis and thyroid associated opthalmopathy. The main clinical entities are Hashimoto's thyroiditis and Graves' disease. These two forms differ in phenotype but resemble each other in the histological features of thyroid lymphocytic infiltration and the presence of autoantibodies against thyroid peroxidase (TPO).[117] Furthermore, the natural course of hyperthyroid GD is without surgical or iodine ablation not uncommonly hypothyroidism.[118] Also, both HT and GD run in the same families, implying a shared genetic background of both diseases.[117]

Hashimoto's thyroiditis is the catabolic form of AITD and is characterised by a gradual destruction of the thyroid gland by a T cell-mediated autoimmune process leading to low levels of thyroid hormone (hypothyroidism). The main autoantigen next to TPO is thyroglobulin (Tg).

Graves' disease is the anabolic form of AITD. Here, antibodies directed against the thyroid stimulating hormone receptor (TSHR) of the thyroid stimulate the thyroid to grow and to produce more thyroid hormone leading to hyperthyroidism.[119]

As in T1D, AITD is the result of an inherited susceptibility and encountered environmental factors (GD: concordance rate MZ twins=22%, DZ twins=0% (range or CI not available),[120] HT: concordance rate MZ twins=55% (95% CI: 23-83%), DZ twins=0% (95% CI: 0-25%).[121] Possible genes and environmental factors that could play a role are listed in Tables 3 and 4.

1.6 CO-OCCURRENCE OF ORGAN-SPECIFIC AUTOIMMUNITY AND MOOD DISORDERS

T1D and AITD are frequently associated

T1D and AITD co-occur more frequently than normal within patients and families. The co-occurrence of these two organ-specific autoimmune diseases is described as subtype 3A of a syndrome called autoimmune polyendocrine syndrome (APS), reviewed in Lam-Tse *et al.*[122] This syndrome was initially defined as a multiple endocrine gland insufficiency associated to an autoimmune disease in a patient. These associations were

noted not to be at random but in particular combinations. Therefore, the syndrome was classified after careful clinical observations by Neufeld and Blizzard in four main types (Table 5).[123]

With regard to the pathogenesis of both AITD and T1D several similarities can be seen. In both diseases there is a destruction of target tissue by $CD8^+$ T cells and macrophages, a presence of autoantibodies and common genes and environmental factors have been suggested as possible actors in the disease (Tables 3 and 4).

Table 5. Classification of the autoimmune polyglandular syndromes (modified from Betterle *et al.*[225]).

APS-1	Chronic candidiasis, chronic hypoparathyroidism, Addison's disease (at least two present)	
APS-2	Addison's disease (always present) + autoimmune thyroid disease and/or type 1 diabetes	
APS-3a	Autoimmune thyroid disease + type 1 diabetes	Exclusion of Addison's disease and/or hypoparathyroidism
APS-3b	Autoimmune thyroid disease + chronic atrophic gastritis or pernicious anaemia	
APS-4	Combinations not included in the previous groups.	

APS, autoimmune polyglandular syndrome

A higher prevalence of mood disorders in diabetes patients

Diabetes is known to be frequently complicated by mood disorders. A meta-analysis conducted by Gavard *et al*. reported a prevalence of depression of 14% (range 9-27%) in diabetic patients.[124] A more recent one showed an aggregated mean (weighted by the number of patients included) of 9%.[125] In these reports, only studies were included that had used diagnostic interviews for diagnosis of depression. By reviewing studies that had used self-report depression scales the mean prevalence rose to 32% (range 22-60%)[124] and the aggregated mean value to 26%.[125] In these meta-analyses controlled studies were used but no distinction was made between type 1 and type 2 diabetes. In the systemic literature review of Barnard *et al.*, only studies of type 1 diabetes patients were included.[126] Here a prevalence of depression of 12% was found in controlled studies, and of 13.4% in uncontrolled studies. Furthermore, type 1 diabetes patients with a lifetime diagnosis of major depression show a worsened glycaemic control,[127] have higher risks for complications[128] and a higher mortality rate[129] than patients without a history of a psychiatric illness.

A higher prevalence of diabetes in patients with a mood disorder

In the same way, diabetes occurs more frequently in mood disorders. A meta-

analysis including nine studies illustrated that depressed adults have a 37% increased risk for developing type 2 diabetes.[130] With regard to bipolar disorder, bipolar patients were identified as a high risk population for developing diabetes, with a three times higher prevalence of diabetes in bipolar patients than in the general population.[131] Cassidy reported on an elevated frequency of diabetes (9.9%) in hospitalized bipolar patients.[132] However, it must be noted that the latter two studies did not differentiate between type 1 and type 2 diabetes. Furthermore, negative reports on the relation diabetes and mood disorders have been published as well.[133, 134]

Thyroid abnormalities and mood disorders are frequently associated

Several points of interest can be raised discussing the relationship between AITD and mood disorders. First, hypothyroidism is commonly accompanied by depressive symptoms. Second, subjects positive for TPO-abs were shown to have a higher risk to develop mood disorders.[135, 136] Third, various studies described abnormalities of thyroid function in patients with a mood disorder.[137, 138] And, finally, bipolar patients are more prone to develop thyroid autoimmunity as is shown by the presence of autoantibodies against the thyroid (TPO-abs, Tg-abs)[139, 140] in these patients.

Shared susceptibility for autoimmunity and mood disorders?

Also children of bipolar patients are at higher risk to develop endocrine autoimmunity as is demonstrated by a study conducted in our group.[141] In this research, especially in female bipolar offspring there was a higher prevalence of TPO-abs positivity than in healthy controls (16% vs. 4%). Furthermore, three adolescents had developed T1D (i.e. 2% versus 0.4% in the general population[142]). In addition, a twin study reported that healthy co-twins of bipolar index twins show a similar raise in their TPO-abs positivity.[143] In all studied groups thyroid autoimmunity was raised irrespective of mood symptoms. Thus family members of bipolar patients are more vulnerable to develop thyroid autoimmunity (and possibly also T1D), but these endocrine autoimmune diseases do develop independently from the vulnerability to develop mood disorders. These findings refute the concept that mood disorders and thyroid autoimmunity are cause or consequence of each other. This is further strengthened by the observation that depression is related to TPO-abs positivity rather than to thyroid dysfunction[135] and by the observation that the excess of depression seen in TPO-abs positive women was not corrected by thyroid hormone treatment.[144] Taken together this might imply the presence of a shared common vulnerability factor for mood disorders, thyroid autoimmunity and possibly also T1D. Various factors could be possible candidates to act as a common vulnerability factor, such as an increased activity of the HPA-axis, infections, a low intake or impaired metabolism of omega-3 polyunsaturated fatty acids, stress or immunological disturbances. We hypothesize that in particular the latter is important since activated monocytes/ macrophages/ dendritic cells (as part of an activated inflammatory response system) play a prominent role in the pathogenesis of T1D, AITD but also -as described in the beginning of this chapter- in mood disorders.

1.7 THE ROLE OF MONOCYTES AND MONOCYTE-DERIVED CELLS IN T1D AND AITD

There is extensive evidence supporting the involvement of monocytes, macrophages and dendritic cells in the pathogenesis of T1D and AITD. Part of that evidence is derived from animal models of spontaneous autoimmune thyroiditis and insulitis namely the NOD (non obese diabetic) mouse and BB-DP (bio breeding diabetes prone) rat. The development of these animal models had a major impact on our ability to understand the pathogenesis of T1D and AITD. Especially the study of early phases of disease became possible. However, one must keep in mind that although the models resemble the human situation in some aspects they certainly differ in others as well, especially with regard to the genetic background and environmental influences.

Functional aberrancies of peripheral monocytes, macrophages and dendritic cells in T1D and AITD

Monocytes and monocyte-derived cells are thought to be involved particularly in the early processes that lead to autoimmunity in T1D and AITD. In T1D and AITD both DC and antigen-presenting (accessory) macrophages are involved in the up- and down-regulation of autoantigen-specific T cells and the production of autoantibodies, including that of GAD65-abs and TPO-abs.[145] Only certain maturation/ differentiation stages of DC and macrophages (the so-called “mature immunogenic pro-inflammatory DC and macrophages” in “danger” conditions, see before) are capable of initiating such effector immune responses towards foreign and autoantigens.[146] In their normal “steady state” (thus under “non-inflammatory/non-danger” conditions, see before) DC and antigen presenting macrophages are important in maintaining tolerance and are referred to as “tolerogenic/ steady state” DC and “alternatively activated” macrophages.[146] Not only do such steady state cells play a role in negative selection of autoreactive T cells in the thymus, they are also essential in stimulating regulatory T cells in the periphery. Thus in the healthy state there is a continuous trafficking of DC and antigen presenting macrophages from the periphery to the draining lymph node carrying autoantigens along and inducing tolerance to these autoantigens.

Any aberrancy in monocytes or the monocyte-derived DC and accessory macrophages switching the cells into a “danger” mode and leading to malfunctioning of these tolerogenic processes (such as e.g. the introduction of a strong danger signal in the form of a mycobacterium or due to an aberrant intrinsic pro-inflammatory state of the cell) could in principle lead to a loss of tolerance by an escape of autoreactive T cells from control and/or an unbalance (either in number or in function) of T effector and T regulatory cells.[147, 148-155]

Interestingly, intrinsic aberrancies in monocytes, DC and antigen-presenting macrophages have extensively been described in the NOD mouse and the BB-DP rat, and - though less extensive - also in patients. The intrinsic aberrancies are summarized in two sub-categories underneath:

An aberrant pro-inflammatory status of monocytes, DC and antigen-presenting macrophages

Reports of an abnormal function of DC and accessory macrophages in both the NOD mouse and BB-DP rat before the onset of autoimmune disease include an aberrant generation of DC from bone-marrow precursors. In the NOD mouse the generated DC are pro-inflammatory and show high levels of NFκB.[156] In NOD studies from our own group we found a defective generation of classical DC from bone-marrow precursors, but a favoured generation of "pro-inflammatory" macrophage-like DC from such precursors.[157] In the BB-DP rat DC are also aberrant and produce low quantities of the anti-inflammatory cytokine IL-10.[158] Correction of the aberrant DC via DC transfers in the NOD mouse has been shown to lead to the prevention and/or delay of diabetes onset.[159, 160] Apart from the pro-inflammatory DC, there are also many reports on an aberrant pro-inflammatory status of macrophages and monocytes prior to the actual initiation of the autoimmune reaction. Our group has previously performed extensive functional studies on monocytes, DC and macrophages in the NOD mouse and the BB-DP rat and reported a.o. on the pro-inflammatory, mature status of circulating monocytes (overrepresentation of Ly-6C low monocytes) in the NOD mouse.[161] Also a high PTGS2 expression level in NOD monocytes and macrophages[162] has been reported as well as a high pro-inflammatory cytokine production.[163, 164] In the BB-DP rat, macrophages produce high quantities of TNF-α.[165] IL-1 β, an important pro-inflammatory cytokine from monocytes and monocyte-derived cells, has directly been implicated in the diabetes process, because IL-1 β plays an important role in the killing of the β cell.[166] Also, oxidative radicals are produced at higher level by both NOD and BB-DP macrophages.[167, 168]

In T1D and AITD patients a defective antigen presenting cell (APC) function of monocyte-descendent cells as well as pro-inflammatory abnormalities of monocytes (e.g. increased IL-1β, ROS and PTGS2 production) [169-171] have been reported, although rather scarce and inconsistent.[172, 173]

An aberrant trafficking and adhesion state of monocytes, DC and antigen-presenting macrophages

NOD DC and macrophages show an abnormal motility, trafficking and chemotactic responsiveness and an aberrant adhesion to fibronectin (FN).[174, 175] While NOD spleen DC, immature and mature bone marrow-derived DC show an increased adhesion to FN[174], NOD peritoneal macrophages and NOD bone-marrow-derived macrophages do not. On the contrary these cells have a decreased adhesion to FN. [175] This latter deficiency was due to a lower expression of the integrin receptor α4β5, an expression regulated by ERK2/3.[175] The aberrant regulation of integrins, determining the aberrant FN adherence of NOD monocytes DC and macrophages is relevant, since increased levels of FN are present in the NOD pancreas at the islet edges, particularly in the early postnatal period, where it is associated with an enhanced accumulation of monocytes and DC.[176] With regard to trafficking abnormalities, NOD monocytes have a reduced migration

capacity towards the chemokine CCL2.[177] Similarly, in the NOD mouse the recruitment of macrophages, DC and monocytes to inflammatory sites was reported as aberrant.[177]

An interesting parallel exists between the NOD mouse and T1D/AITD patients regarding the aberrant adhesion of monocytes to FN as monocytes of T1D patients displayed an increased adherence to FN (and to endothelial cells) and had a higher expression of the integrin CD11b (part of Mac-1) in contrast to monocytes from healthy controls and type 2 diabetic (T2D) patients.[174] Interestingly, adherence to FN of monocytes of T1D patients induced a significantly increased production of MRP8/14 (a pro-inflammatory compound of the S100 family) and of the pro-inflammatory chemokines CCL2 and CCL3, which are capable of attracting more monocytes and other inflammatory cells. [178] However, migration of T1D monocytes was found reduced towards these chemokines. These results suggest that an increased adhesion of diabetic monocytes to extracellular matrix components could contribute to inflammation by an increased production of pro-inflammatory compounds and chemokines. In general it is known that monocytes, after adherence to FN, are “activated” (to so-called P-monocytes) and have acquired the capability not only to produce more chemokines and cytokines, but also to change their shape (polarize).[179, 180]

To conclude, there is evidence that peripheral monocytes, macrophages and dendritic cells of T1D/AITD individuals/animals are not in “steady state”, but - most likely due to intrinsic abnormalities - become pro-inflammatory and abnormal in their adhesion/ migration. These abnormal set points of the cells most likely play a role in breaking tolerance and inducing an aberrant immune response towards β-cells and thyrocytes.

Current Concept on the Histopathological Events in T1D and AITD

In short, the immune histological events in the autoimmune insulitis/ thyroiditis of the NOD mouse and the BB-DP rat are the following:

In the initial phase DC and antigen-presenting macrophages start to excessively accumulate around the islets of Langerhans in the pancreas or around the thyroid follicles in the thyroid and are triggered (by a yet unknown event, perhaps their intrinsic disturbances?) not to act as steady state cells, but as immunogenic activated DC and macrophages. They pick up autoantigens (such as insulin and TPO), travel to the draining lymph nodes and there erroneously start to activate and expand autoreactive effector $CD4^+$ and $CD8^+$ T cells (instead of regulatory T cells).

The generated autoreactive effector $CD4^+$ T cells, after dissemination through the blood and infiltration to the target tissue, react in the second phase again with the in-the-target-tissue accumulated APC, and start to produce inflammatory cytokines such as IFN-gamma, attracting and activating scavenger macrophages, which together with the generated and infiltrated auto-reactive $CD8^+$ T cells are responsible for the destruction of the target tissue.[167]

It is thus not surprising that removal of DC and macrophages stops the autoimmune reaction and the destruction of the target tissue. Nikolic *et al.* demonstrated that depletion of monocytes from the circulation in the NOD mouse resulted in a temporarily disappearance

of macrophages and DC from the islets of Langerhans and postponed the onset of diabetes.[181] Similarly, in the BB-rat macrophage depletion prevented the development of lymphocytic insulitis and thyroiditis.[182] Also, transfer of peritoneal macrophages from diabetic NOD mice to naive NOD mice accelerated disease.[183] With regard to autoimmune thyroid disease, thyroglobulin-pulsed DC transferred into a control mouse strain induced thyroiditis.[184]

Above mentioned literature implies that next to DC macrophages play a prominent role in the pathogenesis of T1D/AITD, however a recent study[185] indicates that the depletion of prototypic macrophages alone had no impact on islet antigen presentation or on the induction of insulitis and diabetes. In contrast, depletion of prototypic DC did lead to a reduction in T cell activation and insulitis score and diabetes development. Thus, the aberrant pro-inflammatory DC are probably the most crucial cells in the first phases of endocrine autoimmunity (loss of tolerance, and activation autoreactive T cells), whereas the importance of aberrant macrophages for the initial (loss of tolerance, and activation autoreactive T cells) and final phase (destruction of the tissue) can be questioned. Destruction of the target tissue can apparently occur (at least in the NOD mouse) without macrophages being present, most likely because of the presence of autoreactive cytotoxic CD8 T^+ cells who are next to the macrophages the important killers of the immune system.

1.8 AIMS OF THIS THESIS/ RESEARCH QUESTIONS

Given:

1. The higher prevalence of thyroid autoimmunity in bipolar patients.
2. The higher prevalence of thyroid autoimmunity in family members (children and co-twins) of bipolar patients not related to the presence of mood disorders (suggesting a shared vulnerability factor).
3. The indications for an abnormal function of monocytes, macrophages and DC in bipolar disorder as part of an activated inflammatory response system.
4. The aberrant pro-inflammatory state of monocytes, macrophages and DC in the cluster of endocrine autoimmune diseases, of which AITD, T1D and autoimmune gastritis are the most prevalent and well-known examples.

The following research questions were put forward:

Is there a higher prevalence of islet and gastric autoantibodies in bipolar disorder?

Published data indicate a relationship of bipolar disorder with autoimmunity. We and others for instance demonstrated higher serum levels of TPO-abs in bipolar patients. Similarly, our group found a higher frequency of antinuclear antibodies in patients. To further establish this relationship, the presence of GAD65 autoantibodies (associated with autoimmune diabetes) and gastric H/K adenosine triphosphatase antibodies (associated with atrophic gastritis) were determined in the sera of bipolar patients (**Chapter 2**).

Which genes are aberrantly expressed in the circulating functionally aberrant monocytes of bipolar patients?

There is extensive data implicating a role of an aberrant immune system in the pathogenesis of unipolar depression. More recently our group a.o. has also suggested that for bipolar disorder. To further research this issue, abnormal gene expression was studied in the circulating monocytes of bipolar patients. Next to determining the presence of aberrant monocytes, the study was also conducted to search for biomarkers that could facilitate diagnosis, provide ways of prognosis and perhaps lead to new therapy targets for bipolar disorder. Affymetrix analysis was performed on the monocytes of bipolar patients and healthy controls. Genes with a more than four fold higher or lower expression in bipolar monocytes than in healthy monocytes and a known function in inflammation and inflammation-related processes were chosen for further verification via Q-PCR (**Chapter 3**).

Is the pro-Inflammatory signature present in the monocytes of bipolar patients the result of genes or environmental factors?

To determine whether the presence of a pro-inflammatory monocyte state in bipolar

disorder is due to genetic effects or due to environmental influences a twin study was conducted and data were analysed via a formal twin modelling method (**Chapter 4**).

Is the pro-inflammatory monocyte state the shared vulnerability factor for bipolar disorder and thyroid autoimmunity?

Not only bipolar patients but also their first degree relatives have higher serum levels of TPO-antibodies. In these family members the presence of antibodies was not related to any mood disturbances. It thus seems that they inherit the susceptibility for thyroid autoimmunity independent from their vulnerability to develop a mood disorder. Aberrant monocytes have been implicated in the pathogenesis of bipolar disorder but also in the pathogenesis of AITD. We deduced the following hypothesis: pro-inflammatory monocytes could be the shared vulnerability factor for bipolar disorder as well as for thyroid autoimmunity. Since in the research described in Chapter 3 (addressing question 2) a pro-inflammatory gene-expression signature was found in the monocytes of bipolar patients, we searched for this inflammatory signature in the monocytes of AITD patients as well to investigate whether this pro-inflammatory state could indeed be the shared vulnerability factor. In addition affymetrix analysis was performed on the monocytes of patients with AITD to search for extra inflammatory genes (**Chapter 5**).

Is the pro-inflammatory monocyte state as found in bipolar disorder also present in patients with autoimmune-mediated diabetes?

Since both AITD and bipolar disorder are known to be associated with diabetes and since monocytes are also important in the pathogenesis of autoimmune diabetes, the in bipolar patients and AITD patients detected pro-inflammatory gene-expression signature was also studied in the monocytes of diabetes patients. In addition affymetrix analysis was performed on the monocytes of patients with type 1 diabetes to search for extra genes (**Chapter 6**).

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II

A High Prevalence of Organ-Specific Autoimmunity in Patients with Bipolar Disorder

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ABSTRACT

Background In a previous study, we reported an increased prevalence of thyroid peroxidase antibodies (TPO-abs) in patients with bipolar disorder. Here, we report the prevalence of other organ-specific autoantibodies: H^+/K^+ adenosine triphosphatase antibodies (H^+/K^+ ATPase-abs), glutamic acid decarboxylase-65 antibodies (GAD65-abs) and GAD-67 antibodies (GAD67-abs).

Methods H^+/K^+ ATPase-abs, GAD65-abs and GAD67-abs were determined (via a commercially available enzyme linked immunosorbent assay for H^+/K^+ ATPase-abs, and standardized radio immuno-assays for GAD65- and GAD67-abs) in the sera of 239 patients with DSM-IV bipolar disorder, in 74 patients with DSM-IV schizophrenia and in 220 healthy control subjects.

Results The positivity prevalences for H^+/K^+ ATPase-abs and GAD65-abs (but not that of GAD67-abs) were raised in bipolar patients compared with those in healthy control subjects (11.7% vs. 6.1% and 11.3% vs. 2.6% respectively; $p<0.05$). Schizophrenia patients did not show such statistically higher prevalence. The elevated prevalence of H^+/K^+ ATPase-abs and GAD65-abs in bipolar disorder was associated with neither rapid cycling nor the use of lithium. Interestingly, the presence of GAD65-abs (and not that of TPO-abs and H^+/K^+ ATPase-abs) tended to be associated with the activity of bipolar disorder. The level of TPO-abs was negatively correlated with the serum level of sIL-2R, a measure of T-cell activation.

Conclusion Bipolar disorder is associated with organ-specific autoimmunity to the antigens TPO, H^+/K^+ ATPase and GAD65.

INTRODUCTION

Bipolar disorder is a severe, recurrent illness with a complex genetic, biological, and psychosocial etiology. There is increasing evidence that the immune system, in close interaction with the central nervous system and the endocrine system, plays a role in its pathophysiology. There is ample evidence to suggest that acute episodes of bipolar disorder (BD) are accompanied by an acute phase response and an activation of cell-mediated immune reactions, including an activation of the T cell system as evidenced by elevated levels of soluble IL-2 receptor (sIL-2R) in the serum of the patients.[1-7] An increased prevalence of thyroid autoantibodies, that is of thyroid peroxidase autoantibodies (TPO-abs), in BD has been shown as well, and this higher prevalence of thyroid antibodies was associated with a higher prevalence of clinically overt thyroid failure (hypothyroidism) due to autoimmune thyroiditis.[8]

Autoimmune thyroiditis represents an organ-specific or endocrine autoimmune disease. In organ-specific autoimmunity, more than one organ is often affected. The most frequent co-occurrences are between autoimmune thyroiditis and type 1 diabetes and between autoimmune thyroiditis and autoimmune atrophic gastritis.[9, 10] These syndromes are referred to as autoimmune polyendocrine syndrome (APS) type 3a and 3b, respectively.

Autoimmune atrophic gastritis is characterized by an increased prevalence of antibodies to gastric parietal cells.[11] These autoantibodies recognize gastric H^+/K^+ adenosine triphosphatase (H^+/K^+ ATPase), a highly specialized proton pump located in the unique intracellular membranes of gastric parietal cell membranes. The presence of these H^+/K^+ ATPase autoantibodies (H^+/K^+ ATPase-abs) provides a convenient diagnostic probe for chronic autoimmune (type A) atrophic gastritis.[12]

Type 1 diabetes has several serological markers. The most frequently used markers are autoantibodies against the 65-kDalton (65kD) isoform of glutamic acid decarboxylase (GAD65-abs), which are positive in approximately 80% of recent-onset cases.[13] Two forms of GAD occur in mammalian tissues: a 65kD form and a 67kD form. GAD65 and GAD67 are enzymes that catalyze the reaction of L-glutamate to γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nervous system. GAD65 and GAD67 are both highly expressed in the brain, but the two forms appear to be differentially expressed in pancreatic islets. In human islets, GAD65 is abundant, but GAD67 can barely be detected (or cannot be detected at all) using various detection methods.[14, 15-17]

Because of the high prevalence of autoimmune thyroiditis in bipolar patients and the known association of autoimmune thyroiditis with autoimmune atrophic gastritis, type 1 diabetes, or both, we investigated the presence of autoantibodies to H^+/K^+ ATPase and GAD65 in the sera of a group of well-characterized bipolar patients (*n*=239). The same blood samples had been used in previous studies conducted by our group on the prevalence of TPO-abs and the level of sIL-2R in bipolar patients.[1, 8]

As control subjects, we used a group of well-characterized schizophrenia patients (*n*=74) and a group of healthy individuals (*n*=220). Apart from autoantibodies to H^+/K^+

ATPase and GAD65, we also determined antibodies to gastric parietal cells using the classical indirect immunofluorescence (IIF) assay and antibodies to GAD67 as a possible sign of autoimmunity against “brain” GAD.

We report a higher prevalence of autoantibodies to H^+/K^+ ATPase and to GAD65 (but not to GAD67) in BD compared with healthy control subjects. The schizophrenia patients did not show statistically higher prevalences of these organ-specific autoantibodies.

METHODS AND MATERIALS

Patients and healthy control subjects

Patients: The subjects were 239 outpatients with bipolar I, II or not otherwise specified (NOS) disorder from the Stanley Foundation Bipolar Network (SFBN), a multi-center longitudinal treatment research program performed in the United States and The Netherlands described in detail elsewhere.[18, 19] A DSM-IV [20] diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I, Patient Edition (SCID-I/P) Research Version.[21] A detailed illness history, including age of onset, previous illness course, medical history, and past and present medication, was assessed at entry into the Network. Blood for the present study was drawn during one of the monthly follow-up visits, when a research clinician also determined the present mood by means of a detailed interview including the Young Mania Rating Scale,[22] the Inventory of Depressive Symptoms[23] and the Clinical Global Impressions Scale - Bipolar Version.[24] Based on the retrospective and prospective Life Chart Methodology[25] patients were defined as rapid cyclers (DSM-IV: four or more episodes of mania/hypomania, depression, or both in the previous year) or nonrapid cyclers.

Subjects were 122 (51%) women and 117 (49%) men, mean age 45 (range 23-83) years. At the time of blood collection, 124 (52%) were euthymic, 84 (35%) depressed, and 31 (13%) manic or hypomanic, all meeting syndromal DSM-IV criteria; 103 (43%) had a rapid-cycling course. Medication use was as follows: 84% used or had used lithium, 43% used or had used carbamazepine, 70% used or had used valproate, 42% used or had used antidepressants, and 19% used or had used antipsychotics.

Healthy Control Subjects: Healthy control subjects were recruited from three groups of healthy individuals (the staff of the Altrecht Institute for Mental Health Care, Utrecht; Department of Immunology, Erasmus MC, Rotterdam, The Netherlands; Department of Medicine, University of Washington Medical Centre, Seattle, USA), representing 129 Dutch and 91 U.S. control subjects. All 220 healthy subjects gave written information about medication use and medical history. All were free of any psychiatric or medical illness and none used any psychotropic or other medication (apart from anticonceptive hormonal therapy). Control subjects were 145 (66%) women and 75 (34%) men, mean age 32 (range 18-59) years. The racial composition of both the patient group and the control group was comparable, being mainly of Caucasian origin.

A second control group consisted of 74 inpatients and outpatients with a DSM-IV diagnosis of schizophrenia,[20] made by means of the Structured Clinical Interview for DSM-IV Axis I, Patient Edition (SCID-I/ P) Research Vision.[21] A research clinician assessed current and past medication use at the time of blood sampling. Subjects were 16 (22%) women and 58 (78%) men, mean age 41 (range 19-61) years.

Blood was drawn and serum collected, which was stored immediately at -20 °C. All samples were sent frozen to the Department of Immunology of the Erasmus MC and stored there, enabling us to test patient and control material in the same series of experiments at appropriate times. The average duration of storage before testing was the same for the patient and control groups (i.e., about 3 to 4 years). It is our experience that titers of autoantibodies do not change during such storage time when sera are not thawed and frozen several times during storage, which was not the case in this study. Blood samples had been used before in previous studies by our group.[1, 8]

The Institutional Review Boards of the participating centers approved the study protocol. All subjects gave informed consent.

Laboratory methods

The H^+/K^+ ATPase antibodies in serum were determined by a specific enzyme-linked immunosorbent assay (ELISA; QUANTA Lite^{tm} GPA ELISA, INOVA Diagnostics, San Diego, USA). According to the manufacturer recommendations, a prediluted negative, a low positive, and a high positive control (supplied by manufacturer) were aliquoted in duplicate in the wells of a preincubated 96-well ELISA plate. Patient sera were diluted to a measurable solution with horseradish peroxidase sample diluent (1:101) and afterwards added to the plate as well. The remaining part of the assay was performed according to the manufacturer's manual. Absorbance at 450-620 nm was measured with a microplate reader Multiskan RC (Thermo Life Sciences, Breda, The Netherlands). The H^+/K^+ ATPase-abs level in the unknown samples was calculated according to the manual: ([Sample OD]/[OD of the H^+/K^+ ATPase-abs low positive control]) x 25 units. Using the control values, the concentrations of the samples were calculated based on their optical density values. The inter-assay variation was less than 10%. Our internal control was comparable with the manufacturer's controls and stable over all the plates we used.

Antibodies to gastric parietal cells were determined via indirect immunofluorescence (IIF) according to standard routine diagnostic procedures in use at the Department of Immunology at the Erasmus MC. Briefly, serum was incubated at 1:10 dilution on cryostat sections from rat stomach (IMMCO diagnostics, New York, USA) followed by incubation with a second antibody (i.e., fluorescein isothiocyanate-conjugated sheep anti-human immunoglobulin; De Beer Medicals, Diessen, The Netherlands). In each assay, positive and negative serum controls were included. These positive and negative sera in the IIF assay were also used in the previously described ELISA for H^+/K^+ ATPase-abs. All sections were evaluated by at least two technicians. In case of positive fluorescence, serum was screened for mitochondrial antibodies using rat kidney sections (IMMCO diagnostics,

New York, USA). When positive for mitochondrial antibodies, sera were considered dubious and were not included in the analysis.

The glutamate decarboxylase-65 antibodies (GAD65-abs) and glutamate decarboxylase-67 antibodies (GAD67-abs) were detected by radioimmunoassay (RIA) as described by Grubin *et al.*[26] Recombinant 35S-GAD65 and GAD67 were made in an *in vitro* transcription and translation reaction (Promega, Madison, WI, USA) described elsewhere.[27, 28] Samples were analyzed in triplicate. Antibody levels were expressed as a relative index to correct for inter-assay variation using the World Health Organization standard for GAD65-abs and GAD67-abs as positive control subjects.[29] To determine the relative index, positive and negative controls were included in all assays.[26] The threshold for positivity for both GAD antibodies was taken at the 97th percentile of the GAD index levels of the 220 healthy control subjects.

The TPO-abs levels had been measured previously[8] with ELISA (Milenia assay, DPC, Breda, The Netherlands) according to the manufacturer's recommended protocol. A level of 10 units/ml or higher was considered as positive for the presence of TPO-abs.

The sIL-2R levels had been measured previously[1] by automatic Immulite chemiluminescent enzyme immunometric assay (DPC) according to manufacturer instructions.

Statistical analysis

Data were analyzed using the SPSS statistical package for Windows. Logistic regression and analysis of covariance (ANCOVA) were used to determine differences between groups, corrected for age and gender.

To investigate the difference in GAD65-abs and GAD67-abs index between control subjects and bipolar patients, a log transformation was performed on the data to obtain Gaussian distribution. Correlations and associations between the various autoantibodies were examined using Spearman's Rho test. All tests were tested for two-tailed significance and a *p* value below 0.05 was considered to be statistically significant.

RESULTS

Antibodies to H^+/K^+ ATPase

The bipolar patients had a higher prevalence of antibodies to H^+/K^+ ATPase compared with the healthy control subjects (11.7% versus 6.1%, p=0.049, corrected for age and gender, Table 1). The percentage of rapid cyclers as well as medication use was similar in H^+/K^+ ATPase-abs positive and negative bipolar patients (Table 2). There were also no relationships between H^+/K^+ ATPase-abs positivity and duration of illness, present mood, or subtype of BD (Table 2).

The schizophrenia patients, our comparison group, had a prevalence of H^+/K^+ ATPase-abs of only 6.8%, a value that was similar to that of the healthy control subjects (p=0.45

Table 1. Prevalence of H^+/K^+ ATPase-, GAD65- and GAD67-antibodies in healthy control, bipolar and schizophrenia subjects and the mean GAD65-abs and GAD67-abs index.

	HC	BD	*p*	SCH	*p*
	(*n*=220)	(*n*=239)	vs. HC	(*n*=74)	vs. HC
H^+/K^+ ATPase-abs positivity[1] (%)	6.10%	**11.70%**	*.049*	6.80%	*.45*
GAD65-abs positivity[1] (%)	2.60%	**11.30%**	*.002*	4.10%	*.49*
GAD65-abs index[2]	.018 ± .035	**.025 ± .019**	*.038*	.014 ± .071	*.70*
GAD65-abs index[3]	.70 ± .016	**.71 ± .009**	*.016*	.70 ± .031	*.57*
GAD67-abs positivity[4] (%)	3.00%	6.80%	*.16*	9.50%	*.17*
GAD67-abs index[5]	-.032 ± .167	-.006 ± .400	*.16*	-.005 ± .39	*.30*
GAD67-abs index[6]	.67 ± .078	.68 ± .137	*.25*	.68 ± .15	*.47*

[1] Logistic regression, adjusted for age, gender, and GAD67-abs index.
[2] Mean ± SD; ANCOVA on index value, adjusted for age, gender, and GAD67-abs index.
[3] Mean log-transformed ± SD; ANCOVA on log index, adjusted for age, gender, and GAD67-abs log index.
[4] Logistic regression, adjusted for age, gender, and GAD65-abs index.
[5] Mean ± SD; ANCOVA on index value, adjusted for age, gender, and GAD65-abs index.
[6] Mean log-transformed ± SD; ANCOVA on log-index, adjusted for age, gender, and GAD65-abs log-index.

ANCOVA, analysis of covariance; H^+/K^+ ATPase-abs, H^+/K^+ adenosine triphosphatase antibodies; GAD65-abs, glutamic acid decarboxylase-65-antibodies; GAD67-abs, glutamic acid decarboxylase-67-antibodies; vs., versus; HC, healthy control subject; BD, bipolar disorder; SCH, schizophrenia.

versus healthy control subjects, Table 1).

Apart from the H^+/K^+ ATPase-abs measured in ELISA, we also tested for antibodies to gastric parietal cells using the classical and original IIF assay. Using this assay, differences between bipolar patients and healthy control subjects could not be found (we therefore did not test the schizophrenia patients with the IIF assay). We consider this discrepancy between the two assays to be the result of the poorer sensitivity and specificity of the IIF assay compared with the newer H^+/K^+ ATPase-abs ELISA. Reading of the IIF requires subjective interpretation and is subject to variability in the quality and reproducibility of the tissue sections used to manufacture the slides (indeed, the negative standard used for IIF gave weak positive results in the H^+/K^+ ATPase-abs ELISA).

Antibodies to GAD65 and GAD67

The prevalence of GAD65-abs positivity (defined as a level higher than the 97th percentile of levels found in the healthy control subjects) was significantly higher in the

Table 2. Characteristics of bipolar patients positive and negative for, respectively, H^+/K^+ ATPase antibodies (H^+/K^+ ATPase-abs) and GAD65 antibodies (GAD65-abs).

Characteristics	H^+/K^+ ATPase-abs positive		H^+/K^+ ATPase-abs negative			GAD65-abs positive		GAD65-abs negative		
	n[1]	%	n[2]	%	p[3]	n[1]	%	n[2]	%	p[3]
Rapid cycling	13/25	52%	78/193	40%	*.58*	12/27	44%	80/191	42%	*.52*
Medication use										
lithium	22/28	79%	181/212	85%	*.99*	22/27	82%	181/212	85%	*.83*
carbamazepine	10/27	37%	95/211	45%	*.86*	11/27	41%	93/210	44%	*.66*
valproate	23/28	82%	144/212	68%	*.50*	18/27	67%	148/212	70%	*.92*
antidepressives	13/28	46%	83/204	41%	*.36*	14/26	54%	82/206	40%	*.75*
antipsychotics	6/28	21%	39/204	19%	*.85*	4/26	15%	41/206	20%	*.23*
	Mean ± SD		Mean ± SD			Mean ± SD		Mean ± SD		
Years since first medication	12.04 ± 9.37 (*n*=25)		14.80 ± 9.93 (*n*=193)		*.70*	13.65 ± 8.37 (*n*=23)		14.51 ± 10.06 (*n*=194)		*.88*
Years since first symptoms	24.35 ± 14.08 (*n*=26)		22.98 ± 12.06 (*n*=201)		*.48*	25.54 ± 13.00 (*n*=24)		22.96 ± 12.19 (*n*=194)		*.54*
Present mood										
euthymic	12/24	50%	98/188	52%	*.23*	11/26	42%	100/186	54%	*.02*
depressive + manic	12/24	50%	90/188	48%	*.74*	15/26	58%	86/186	46%	*.07*
depressive	9/24	37%	65/188	35%	*.11*	10/26	39%	63/186	34%	*.15*
manic	3/24	13%	25/188	13%	*.11*	5/26	19%	23/186	12%	*.06*
Bipolar subtypes										
Bipolar I	19/28	68%	169/210	81%	*.58*	20/26	77%	167/211	79%	*.94*
Bipolar II	9/28	32%	34/210	16%	*.95*	6/26	23%	37/211	18%	*.92*
Bipolar NOS	0/28	0%	7/210	3%	*.94*	0/26	0%	7/211	3%	*.92*
BMI	28.55 ± 6.31 (*n*=26)		27.56 ± 6.25 (*n*=197)		*.46*	27.91 ± 6.66 (*n*=24)		27.64 ± 6.23 (*n*=198)		*.15*
sIL-2R expression[4]	366.14 ± 117.27 (*n*=14)		429.09 ± 203.04 (*n*=131)		*.16*	422.42 ± 128.79 (*n*=12)		421.81 ± 203.17 (*n*=132)		*.85*

[1] Number of bipolar patients with indicated characteristic present per number H^+/K^+ ATPase-abs positive bipolar patients (or respectively per number GAD65-abs-positive bipolar patients)

[2] Number of bipolar patients with indicated characteristic present per number H^+/K^+ ATPase-abs negative bipolar patients (or respectively per number GAD65-abs-negative bipolar patients)

[3] Logistic regression adjusted for age and gender

[4] Determined in sera, IU/ml

n, numbers; H^+/K^+ ATPase-abs, H^+/K^+ adenosine triphosphatase antibodies; GAD65-abs, glutamic acid decarboxylase-65-antibodies; BMI, body mass index weight/ (length)^2; sIL-2R, soluble interleukin-2 receptor

group of bipolar patients compared with the control subjects. A prevalence of 11.3% GAD65-abs positivity was found in bipolar patients compared with the 2.6% positivity in the control subjects (p=0.002, corrected for age and gender, Table 1). This was also reflected in the calculated mean GAD65-abs index that was significantly higher in bipolar patients compared with the control subjects. Both the normal mean value and the log transformed mean value (to obtain a Gaussian distribution) were significantly different, also after adjustments for age and gender (p=0.038 and p=0.016 respectively; Table 1).

In contrast to the prevalence of GAD65-abs positivity, that of GAD67-abs positivity was not significantly higher in the group of bipolar patients and a prevalence value of 6.8% versus 3% (p=0.16) was found (Table 1).

The data in Table 2 show that there was no correlation between GAD65-abs positivity and rapid cycling or medication use in the bipolar group or between GAD65-abs positivity and years since first symptoms or duration of treatment. In the GAD65-abs positive and negative groups, body mass index (BMI) did also not differ. In line with this observation there was also no correlation between BMI and GAD65-abs positivity (r=0.01, p=0.88). Interestingly, bipolar patients with GAD65-abs tended to have more active forms of the illness (either mania/hypomania or depression) compared with the GAD65-abs negative bipolar patients. Table 2 shows symptomatic BD in 58% of GAD65-abs positive patients and 46% in GAD65-abs negative patients, although this difference did not reach statistical significance (adjusted for age and gender, p=0.065). When we compared GAD65-abs positivity in patients with symptomatic BD with values found in euthymic patients, this trend was also visible: values of 15% versus 9.9% (data not shown) were found respectively, but again this difference was not statistically significant (adjusted for age and gender p=0.085).

With regard to our comparison group, the schizophrenia patients showed a normal prevalence and index value of GAD65-abs, which was comparable to that of the control group (Table 1). The prevalence of GAD67-abs positivity was relatively high in the schizophrenia group, that is 9.5%, yet the difference with the control group was not statistically significant (Table 1).

Comparisons of antibodies to H^+/K^+ ATPase and GAD65/GAD67 to antibodies to TPO and to serum levels of soluble IL-2 receptor (sIL-2R)

In a previous study, we reported on a higher prevalence of antibodies to TPO and autoimmune hypothyroidism in the same cohort of bipolar patients as studied here.[8] We also reported on a higher level of sIL-2R in the patients of this bipolar cohort and took this as a sign of T-cell activation[1]. In this study, we correlated the positivity for antibodies to H^+/K^+ ATPase and to GAD65 with each other and to positivity for antibodies to TPO in our test groups. We were unable to find any significant correlations.

We also studied the relationship between the serum levels of sIL-2R and the three organ-specific autoantibodies. Tables 2 and 3 show that the levels of sIL-2R did not

correlate with the level of antibodies to GAD65 or with the level of antibodies to H^+/K^+ ATPase. Table 3, however, also shows that the levels of sIL-2R did significantly and negatively correlate to the titre of TPO-abs (interestingly, for all three autoantibodies, correlation coefficients were negative). Also, TPO-abs positive patients had a lower sIL-2R level compared with TPO-abs negative patients (i.e. 336 versus 435 pg/ml [mean]), but this did not reach statistical significance.

Table 3. Correlations of autoantibodies with sIL-2R.

	sIL-2R[1]
H^+/K^+ ATPase-abs	*r*=-.10
	p=.13
GAD65-abs	*r*=-.10
	p=.13
GAD67-abs	*r*=-.09
	p=.20
TPO-abs	*r*=-.29
	p<.001

[1] Spearman's rho test
H^+/K^+ ATPase-abs, H^+/K^+ adenosine triphosphatase antibodies; GAD65-abs, glutamic acid decarboxylase-65-antibodies; GAD67-abs, glutamic acid decarboxylase-67-antibodies; TPO-abs, thyroperoxidase antibodies; sIL-2R, soluble IL-2 receptor

DISCUSSION

In this cross-sectional study, we found that bipolar patients not only have a higher prevalence of antibodies to TPO as reported previously[8], but also a higher prevalence of antibodies to H^+/K^+ ATPase and GAD65 compared with healthy control subjects. These higher prevalences of H^+/K^+ ATPase-abs and GAD65-abs were not found in our comparison group of schizophrenia patients (this also applied for a normal 5% prevalence of TPO-abs in the schizophrenia patients; unpublished results). The prevalence of GAD67-abs was not increased in bipolar patients.

The tendency to develop organ-specific autoimmunity did not correlate with a rapid-cycling course of BD or the use of medications. Although it is known that lithium in particular has immune-modulating effects,[30] we did not find a correlation between past or present lithium exposure and TPO- abs,[8] H^+/K^+ ATPase-abs, or GAD65-abs. Likewise, Vestegaard and Schou [31] found that lithium did not induce diabetes mellitus. To the best of our knowledge, there are no data on the prevalence of autoimmune atrophic gastritis in lithium-treated subjects.

Although our sample of bipolar patients is one of the largest studied, the sample size still limits the power of statistical analysis. Another limitation of the study is that the healthy control group and the comparison schizophrenia group are not entirely comparable with the bipolar patient group because they differed slightly in the female:male ratio and in age. Gender and age do affect the prevalence of TPO-abs and H^+/K^+ ATPase-abs (higher in women at older age,[11, 32] yet it is questionable whether this also applies for GAD65-abs).[33] To rule out a possible confounding effect of age and gender, we adjusted for these variables in our statistical models. Hence, we are confident that the higher prevalence of TPO-abs, H^+/K^+ ATPase-abs and GAD65-abs in BD reported here, is not the result of such confounding effects. In addition, the values found in this study for positivity of the organ-specific autoantibodies in the healthy control group and the comparison schizophrenia group are in the range of values found in population-based control groups in the literature (i.e. around 4-7%).[34, 35]

There are, however, putative confounding factors that we could not correct for because exact and reliable information was not available for a substantial proportion of our controls. One of these is the smoking behavior (we assume that almost all of our control subjects did not smoke, whereas most of the patients did). Smoking is known to activate the cell-mediated immune system, yet effects on the prevalence of organ-specific autoantibodies range from unknown (H^+/K^+ ATPase-abs, GAD65-abs) to nonexistent, to protective (TPO-abs).[32]

It is also of interest that the GAD65-abs index has been reported to relate to the BMI; both positive[36] and negative correlations[37-40] have been found. In our patients, a correlation did not exist between the BMI and GAD65-abs (see Table 2). A correlation of BMI to GAD65-abs was not possible in our control group because we lacked reliable information on the weight of our healthy subjects. It must be noted in this regard that our patient group was slightly overweight (i.e. mean BMI of 28, normal 20-25).

The impact of mood state on the functioning of the immune system of bipolar patients is controversial. Hornig *et al.*[2] Maes *et al.*[4] and Rapaport *et al.*[6] showed that the activation of the cell-mediated immune system and of the acute phase response did not normalize with patients' remission. These authors concluded that the immune arousal of patients with a mood disorder was a trait phenomenon; however, Tsai *et al.*[7] showed a normalization of cell-mediated immune system activation and acute phase response in patients in remission and concluded that there is a state dependency of the immune activation.

We previously reported on the activation of the T cell system in BD and used as one of the parameters the sIL-2R levels. We showed higher levels of sIL-2R in the sera of depressed, manic/hypomanic and euthymic patients compared with healthy control subjects, yet the levels of sIL-2R in manic/hypomanic patients were the highest. We concluded that the cellular immune activation is both a trait and a state phenomenon of the disorder.[1]

We show here some influence of the illness on positivity for GAD65-abs, albeit a borderline statistically significant effect (p=0.065). The trend detected was that there

were more euthymic subjects among bipolar GAD65-abs negative than bipolar GAD65-abs positive patients. The latter had a higher prevalence of both depression and mania compared with euthymic patients. In our previous study on the prevalence of TPO-abs in BD, we found no association with current mood.[8]

Interestingly, we found negative correlations between the serum level of sIL-2R and the titer of the three organ-specific autoantibodies, and this reached statistical significance for TPO-abs. A possible explanation for the discrepancy might be found in the actual function of the raised sIL-2R in the serum of the bipolar patients. The function of this shed receptor, although indicating T cell activation, is to capture circulating IL-2. Raised levels of the shed receptor thus result in the actual down regulation of T cell activation, representing a negative feedback system.[41] This down regulation might also affect the activity of autoreactive B cells and the production of autoantibodies.

GAD65-abs is an important marker of type 1 diabetes. The risk to develop type 1 diabetes is, however, not only dependent on the positivity for GAD65-abs, but is also, and in particular, influenced by the titer of the GAD65-abs and the co-occurrence of other islet-related autoantibodies such as antibodies to insulin and insulinoma-associated protein 2 (IA-2) or islet cell antibody (ICA).[12, 42] The prevalence of diabetes is elevated in bipolar patients,[43, 44] however, most reports on the subject did not distinguish between type 1 and 2 diabetes. Regenold *et al.*[45] limiting themselves to type 2 diabetes, showed a higher prevalence of diabetes mellitus in bipolar patients. We are not aware of any study that systematically investigated the prevalence of type 1 diabetes in bipolar patients or the co-occurrence of GAD65-abs, ICA, IA-2, or insulin autoantibodies. In the entire Stanley Foundation Bipolar Network cohort, 27 out of 967 patients (i.e. 2.8%) self-reported that they had diabetes mellitus, and 6 of those 27 were insulin dependent (R.W. Kupka, personal communication). Hence, at least 6 out 967 bipolar patients (i.e. 0.62%) had insulin-dependent diabetes, a figure that is slightly higher than the prevalence figures for type 1 diabetes in the literature for community-based population groups in the United Kingdom of the same age as our SFBN cohort (i.e. 0.4%).[46] Values range in such community-based population groups from 0.2% at 20 years of age to 0.8% at 80 years of age, however.[46] Moreover, prevalence also depends on geography (e.g. low in European Mediterranean countries, high in Nordic countries). We therefore cannot conclude that there is a putative-raised prevalence of type 1 diabetes in BD because we lack solid prevalence figures for type 1 diabetes in both the Stanley Foundation group and the matched healthy control group.

The same arguments can be used in a discussion of whether the raised prevalence of H^+/K^+ ATPase-abs in BD is a sign of a higher risk for autoimmune atrophic gastritis because gastric autoantibodies are characteristic of (type A) autoimmune gastritis.[11] Controlled epidemiological studies need to be performed to answer this question.

Because our findings suggest that BD is associated with organ-specific autoimmunity, the question of whether bipolar mood disorder is itself an autoimmune disease can be raised. GAD65 expression is not restricted to the pancreas, but is also present in nervous

tissue. The specific GAD65-abs in bipolar patients may thus be viewed as not directed against pancreatic tissue alone, but against nervous tissue as well. Would our observation of a raised prevalence of GAD65-abs in bipolar patients mean that BD belongs to the spectrum of brain-specific autoimmune diseases, with GAD65-abs in fact directed against GAD65-positive nervous tissue in the brain? It has been shown that the expression of GAD65 and GAD67 is reduced in the brains of bipolar patients,[47-49] whereas autoantibodies to brain structures have also been detected in mood disorders and schizophrenia[50-53] GAD67-abs were not elevated in our bipolar patients (this report), however, whereas GAD67 is expressed in nervous tissue. A high titre of GAD67-abs (together with a high titer of GAD65-abs) is a hallmark of an uncommon neurological autoimmune disease Stiff Man Syndrome, which is clinically associated with insulin-dependent diabetes mellitus.[54]

Collectively, although the low prevalence of GAD67-abs suggests that an autoimmune reaction to nervous tissue is not likely in BD, we cannot rule out with certainty that the disorder itself is an organ-specific autoimmune disease. More types of antibodies to other autoantigens of the brain need to be determined.

In conclusion, our data are compatible with the view that patients with bipolar disorder show various signs of an enhanced organ-specific autoimmune reaction to TPO, H^+/K^+ ATPase and GAD65. The positivity of TPO-abs indeed led to a greater prevalence of autoimmune thyroid failure in our bipolar patients.[8] It remains unclear whether the higher prevalence of antibodies to H^+/K^+ ATPase and GAD65 are linked to a higher risk for developing autoimmune atrophic gastritis and type 1 diabetes.

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III

A Discriminating mRNA Signature for Bipolar Disorder formed by an Aberrant Expression of Inflammatory Genes in Monocytes

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ABSTRACT

Context Mood disturbances are associated with an activated inflammatory response system.

Objective To identify a discriminating and coherent expression pattern of pro-inflammatory genes in monocytes of patients with bipolar disorder.

Design A quantitative-PCR (Q-PCR) case-control gene expression study on purified monocytes of bipolar patients, offspring of bipolar patients and of healthy controls (HC) after having selected 22 discriminating inflammatory genes using whole genome analyses.

Setting Academic research setting, The Netherlands.

Patients Forty-two bipolar patients together with 25 HC and 54 offspring of a bipolar parent (13 had and 3 developed a mood disorder during follow up) together with 70 healthy children were studied in Q-PCR.

Main outcome measure Inflammatory gene expression levels in monocytes.

Results We detected in the monocytes of bipolar patients a coherent mutually correlating set ("signature") of 19 aberrantly expressed (p<0.01) mRNAs of inflammatory (PDE4B, IL1B, IL6, TNF, TNFAIP3, PTGS2, PTX3), trafficking (CCL2, CCL7, CCL20, CXCL2, CCR2, CDC42), survival (BCL2A1, EMP1) and MAPkinase (MAPK6, DUSP2, NAB2, ATF3) genes. Fifty-five% (23/42) of bipolar patients had a positive signature test versus 18% of HC (positive test: positive for PDE4B, i.e. a mRNA expression over one standard deviation of the mean of the level found in HC, plus 25% of the other genes similarly positive). Positive signature test were also present in 11/13 (85%) of offspring with a mood disorder, 3/3 (100%) of offspring developing a mood disorder and 17/45 (45%) of euthymic offspring versus 13/70 (19%) of healthy children. Lithium and antipsychotic treatment down regulated the gene expression of most inflammatory genes.

Conclusion

The monocytes of a large proportion of bipolar patients and bipolar offspring show an inflammatory gene expression signature. This coherent set of genes opens new avenues for biomarker development with possibilities for disease prediction in individuals genetically at risk and for the sub-classification of bipolar patients who could possibly benefit from anti-inflammatory treatment.

INTRODUCTION

The "macrophage-T cell theory of depression"[1, 2] postulates an activated inflammatory response system (IRS) in mood disorders and considers this activated IRS as a driving force behind the illness, since pro-inflammatory cytokines are capable of destabilizing brain function making the brain vulnerable to stress and unknown endogenous factors such that major mood disturbances are the consequence. Indeed in animal models the behavioral changes induced by pro-inflammatory cytokines ("sickness behavior") are comparable to the symptoms of depression as seen in humans.[3] Also in humans low intravenous dosages of endotoxin increase the level of these cytokines and induce depressive symptoms.[4, 5] Furthermore, depressive symptoms and mania can be precipitated by interferon-α treatment[6] while anti-TNF therapy given for psoriasis resulted in a markedly improved mood.[7] Last but not least, in patients with mood disorders increased serum, saliva and cerebral spinal fluid levels of several pro-inflammatory compounds have been found such as of IL-6, IL-1β, TNF-α, PGE2 and CCL2,[5, 8-11] although there are also negative reports.[12] In addition, there have been reports on high serum levels of positive acute phase proteins (f.i haptoglobin, alpha-1 antitrypsin, ceruloplasmin, C-reactive protein) and low levels of negative acute phase proteins (f.i albumin) in patients with mood disorders, indicating that mood disorders are accompanied by an acute phase response.[13-15]

We here report outcomes of a Q-PCR study on monocytes of bipolar patients in which we found 19 aberrantly expressed genes involved in inflammation and inflammation-related processes after having identified and selected 21 such pro-inflammatory genes in Affymetrix whole genome gene expression profiling on purified $CD14^+$ monocytes of a limited set of bipolar patients. The expression levels of the inflammatory and inflammation-related genes were mutually strongly correlating in four functional pathways forming a monocyte "gene expression signature". To investigate whether this set of aberrantly expressed genes exists prior to disease we additionally tested the monocytes of 54 children of a bipolar parent, of whom 13 had and 3 developed a mood disorder within 2-3 years after blood collection.[16]

MATERIAL AND METHODS

Patients and healthy controls

Bipolar patients

Outpatients with DSM-IV bipolar I or II disorder were recruited from two studies, i.e. the Dutch site of the Stanley Foundation Bipolar Network (SFBN), an international multi-center research program described elsewhere in detail[17, 18] (*n*=19 for Affymetrix analysis studies and *n*=19 for Q-PCR verification) and from an ongoing Dutch twin study on bipolar disorder described in detail in Vonk *et al.*[19] (*n*=23 index cases for Q-PCR studies). Clinical characteristics of bipolar patients used for Q-PCR are shown in Table 1.

Table 1. Characteristics of bipolar patients and healthy controls used for Q-PCR, and IL-1β, IL-6, CCL2 and CCL7 ELISAs.

		Bipolar patients		Healthy controls	
Group size		42		25	
Age (years)[1]		42	(26-57)	40	(23-56)
Gender	Male	16	(38%)	11	(44%)
	Female	26	(62%)	14	(56%)
Diagnosis	Bipolar I	35	(83%)		
	Bipolar II	7	(17%)		
Duration illness (years)[1]		16	(3.5-40)		
Age of onset illness (years)[1]		25	(6-49)		
Mood	Euthymic	26	(62%)		
	Depressed	7	(17%)		
	Manic	7	(17%)		
	Unknown	2	(5%)		
Psychotropic medication	Lithium	24	(57%)		
	Carbamazepine	13	(31%)		
	Valproate	15	(36%)		
	Lamotrigine	2	(5%)		
	Thyroxine	7	(17%)		
	Antipsychotics	8	(19%)		
	Antidepressives	16	(38%)		
	Benzodiazepines	15	(36%)		

[1] Mean (range)

Offspring of a bipolar patient

The children reported here belong to an ongoing prospective study among the adolescent offspring of a bipolar patient in The Netherlands. The children were not related to the bipolar patients mentioned above. The study design, recruitment procedure and study population have been described in detail.[16, 20, 21] In brief, 86 bipolar patients and their spouses and 140 offspring aged 12 to 21 years were examined between November 1997 and March 1999 (Time 1). Fourteen months after the first assessment, 132 children, aged 13-23 years, were available for reassessment (Time 2). At Time 2 we were able to collect immune cells for our study of 54 children (26 boys, 28 girls). At the third assessment (Time 3), 41 months after Time 2, 129 children (aged 16-26 years) belonging to 80 families were still participating.[16] For further characteristics of the bipolar offspring see Table 2.

Table 2. Characteristics of bipolar offspring and healthy controls used for Q-PCR.

		Bipolar offspring		Healthy young adults	
Group size		54		70	
Age (years)[1]		18	(12-26)	16	(11-29)
Gender	Male	26	(52%)	33	(47%)
	Female	28	(48%)	37	(53%)
		Time 2[2]	Time 3[2]		
Diagnosis	Euthymic	41	38		
	Any mood disorder	13	16		
	Bipolar disorder				
	Bipolar I disorder	1	3		
	Bipolar II disorder	2	1		
	Depressive disorder				
	Major depressive disorder	2	4		
	Dysthymic disorder	4	3		
	Depressive disorder NOS	4	5		

The bipolar offspring are not related to the bipolar patients mentioned in Table 1.

[1] Mean (range), at Time 2
[2] Time 1: First psychiatric evaluation (between November 1997 and March 1999)
Time 2: Second psychiatric evaluation, 14 months after Time 1
Moment of blood drawing and monocyte collection
Time 3: Third psychiatric evaluation, 41 months after Time 2
NOS, not otherwise specified

Psychiatric assessments

A DSM-IV diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I (SCID; in the bipolar patients of the SFBN, in the bipolar cases of the twin study and in the bipolar offspring study at Time 3) or the Schedule for Affective Disorders and Schizophrenia, children's version (K-SADS, in the bipolar offspring study at Time 1 and 2). The bipolar patients of the SFBN were assessed at monthly follow-up visits by means of the Young Mania Rating Scale (YMRS), the Inventory of Depressive Symptomatology (IDS), and the Clinical Global Impressions Scale-Bipolar Version (CGI-BP).

Healthy controls

Adult healthy controls (HC) were recruited via enrolling laboratory, medical staff and medical students. The offspring study was controlled by healthy Dutch high school students. The inclusion criteria for the HC were an absence of psychiatric disorders such as psychosis, mood disorders and anxiety disorders but also of chronic fatigue syndrome and fibromyalgia, and an absent history of these disorders in first-degree family members. HC had to be in self-proclaimed good health and free of any obvious medical illness for at least two weeks prior to the blood withdrawal, including acute infections and allergic reactions. HC did not use any psychotropic or other medication.

The Medical Ethical Review Committee of the University Medical Center Utrecht approved the studies. Written informed consent was obtained from all participants after a complete description of the study was given.

Laboratory methods

Blood collection and preparation

Blood was collected in a clotting tube for serum preparation (frozen and stored at -80°C) and in tubes containing sodium-heparin for immune cell preparation. From the heparinized blood peripheral blood mononuclear cell (PBMC) suspensions were prepared via low-density gradient centrifugation as described in detail before[22] within 5 hours for all samples to avoid activation of the monocytes. PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments at appropriate times.

Isolation of monocytes

On the day of testing, stored PBMC suspensions were quickly thawed and directly diluted in RPMI 1640 medium with 25 mM Hepes and Ultraglutamin 1 containing 10% fetal calf serum to wash out the dimethylsulfoxide. The viability of the cells was invariably over 90% (trypan-blue exclusion staining). Monocytes were prepared from the PBMC suspensions by MACS (magnetic cell sorting system) CD14 separation according to manufacturer's (Miltenyi Biotec) recommendations. Purity of monocytes was routinely determined for each sample by morphological screening of the wet preparation after trypan blue staining. In addition, purity was determined by routine flow cytometry analysis (FACSCalibur, Beckton Dickinson, Amsterdam, The Netherlands, antibody CD14 APC, BD Pharmingen) on randomly chosen samples.

Affymetrix whole genome gene expression profiling

RNA was isolated from the purified monocytes using RNAeasy columns as described by the manufacturer and previously.[23] RNA was first converted into cDNA and subsequently into cRNA. Fragmented cRNA was hybridized to U95Av2 microarrays (Affymetrix,

manufacturers protocol). For all experiments reported here, the 5' / 3' ratios of GAPDH were 2 or less (usually 0.9 - 1.1).

All raw data obtained via Affymetrix are available as MIAMExpress submission E-MEXP-1275 (http://www.ebi.ac.uk/miamexpress/).

Quantitative-PCR (Q-PCR)

RNA was isolated from monocytes using RNeasy mini-prep columns (Qiagen, Hilden, Germany, manufacturer's protocol). After extraction, the RNA concentration was determined and RNA was stored at -80°C until use.

To obtain cDNA for Q-PCR, we used the optimized extensively described BIOMED-1 protocol.[24] One μg RNA was reversed transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham Biosciences, Roosendaal, The Netherlands) for 50 min at 42°C.

Q-PCR was performed with Taqman Universal PCR mastermix (Applied Biosystems, Foster City, CA). All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand) on the basis of the detected transcript clusters of the bipolar discriminating genes detected in Affymetrix analysis (see Appendix 1 and results section). PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, and finally 1 min at 60°C. PCR amplification of the housekeeping gene *ABL* was performed for each sample to allow normalization between the samples. *ABL* was chosen as the housekeeping gene because it was previously shown that *ABL* was the most consistently expressed housekeeping gene in hematopoietic cells.[25] The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values (CT gene- CT housekeeping gene, for these ΔCT values see Supplementary Tables 1 and 2), by the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, Applied Biosystems, Foster City, California, see Tables 3 and 6). To correct for inter-assay variance we set the mean of the studied genes found in the healthy control groups in the same assay for each gene to 1 ($\sum\Delta CT\ HC= 0$, $2^{-0}=1$). The fold change values of the genes in patient monocytes were expressed relative to this set mean of 1 (Tables 3 and 6).

Using the aberrantly expressed genes in the monocytes of the patients, various signatures could be defined. The definitions were given depending on the percentage positive genes, positive being defined as a mRNA expression over one standard deviation of the level found in healthy controls.

Bioinformatics and statistics

Scanned micro array images were analyzed using Affymetrix Microarray Suite 4.2 software. Further analysis was performed using Rosetta Resolver (www.rosettabio.com) software. Classification of the genes was done with Ingenuity Systems software (www.ingenuity.com). Statistical analysis of the data was performed using the SPSS 11.0 package for Windows. Data was tested for normal distribution using the Kolmogorov-

Smirnov test. Depending on distribution pattern and total number of subjects, either parametric (normal distribution, >50 subjects) or non-parametric tests (skewed distribution, <50 subjects) were used. Differences in means between groups were determined using the Mann-Whitney test or the Kruskal-Wallis test (>2 groups). Correlations were determined via Pearson/Spearman correlation coefficients, linear regression and univariate analysis of (co)variance (AN(C)OVA). Frequencies were compared using the Chi-Squared test. The specific tests used are indicated in the legends of figures and tables.

RESULTS

Affymetrix analysis of monocytes of bipolar patients to search for potential inflammatory biomarker genes

Affymetrix analysis was performed on MACS purified monocytes of 5 non-lithium-treated bipolar patients (BP, ages 12-36 years, mean 22 years, 3 females, 2 males) and of 6 healthy controls (HC, ages 12-39 years, mean 20 years, 3 females, 3 males) with the aim to search in particular for inflammation-related genes. The in total 11 cell files are available as MIAMExpress submission E-MEXP-1275 (http://www.ebi.ac.uk/miamexpress/). We analyzed the data using the Rosetta Resolver program and only considered for further study genes which were more than 2 fold statistically significantly differentially expressed (p<0.01) between BP and HC. This resulted in 71 discriminating genes for non-lithium-treated BP (64 up and 7 down-regulated). Major functional networks found to be involved in Ingenuity analysis were indeed inflammation and cell movement, but also cellular growth and differentiation. Pathways involved were the MAPK pathway, the IL-6 signaling pathway and the apoptosis pathway. To select for genes, which could serve as potential monocyte biomarkers for the bipolar “inflammatory” condition we took the top 7 genes from the list (i.e. *PDE4B, ATF3, MAPK6, DUSP2, TNFAIP3, CXCL2* and *BCL2A1*) and genes >2 fold statistically significantly differentially expressed with a well-known involvement in inflammation, the MAPK pathway, the IL-6 pathway and in cell movement, i.e. *IL1B, IL6, TNF, PTGS2, PTX3* and *CCL20*.

In addition we analyzed MACS purified pooled monocytes of lithium-treated BP and of HC. Cell pools were mainly used to limit costs and avoid inter-individual differences. One pool consisted of monocytes of 7 lithium-treated BP (1 male, 6 females, mean age 39 years, range 27-57 years) compared to a pool of healthy control monocytes (1 male and 6 females, mean age 40 years, range 24-56 years); the other pool consisted of 7 lithium-treated BP (4 males, 3 females, mean age 44 years, range 37-57 years) compared to a pool of healthy control monocytes (4 males, 3 females, mean age 45 years, range 39-53 years). The raw data of the in total 4 pools are also available as MIAMExpress submission E-MEXP-1275 (http://www.ebi.ac.uk/miamexpress/). After Rosetta Resolver analysis we found 187 discriminating genes (114 up and 73 down-regulated) between lithium-treated BP and HC (2 fold difference). Ingenuity analysis showed that lithium

treatment down-regulated genes involved in inflammation, but induced the de novo expression of genes involved in cell growth, differentiation, survival and apoptosis. Since we were searching for potential inflammatory biomarkers we selected in this set of genes those well known to be involved in inflammation, the MAPK and IL-6 pathway and in cell movement, these were *HSPA1A, NAB2, CDC42, ADAM17, CCL2, CCR2, CCL7, CX3CR1* and *EMP1.* The via-Affymetrix-selected in total 22 genes were further validated via Q-PCR.

Q-PCR analysis of monocytes of bipolar patients

Table 3 (column A) shows that of the 22 mRNAs tested 19 could be verified in Q-PCR to be significantly differentially expressed ($p \leq 0.01$) in the monocytes of 42 newly selected bipolar patients (BP) as compared to 25 healthy controls (HC) (see Supplementary Table 1 for ΔCT values). These were the inflammatory genes *PDE4B, IL1B, IL6, TNF, TNFAIP3, PTGS2* and *PTX3*, the chemokinesis/motility genes *CCL2, CCL7, CCL20, CXCL2, CCR2* and *CDC42*, the MAPK pathway genes *MAPK6, DUSP2, NAB2* and *ATF3* and the cell survival/apoptosis genes *BCL2A1* and *EMP1*. All were over expressed in BP monocytes, except for CCR2.

Table 4 shows that the actual mood status of the patients is related to the inflammatory gene expression. During a manic episode the mRNA expression of MAPK6 and CCL2 was significantly increased in monocytes of manic versus euthymic BP; during depressive episodes these mRNAs were also raised but those of *IL6, PTX3, EMP1* and *BCL2A1* too. Although active disease thus is related to the mRNA expression of these molecules, almost all 19 mRNAs were still significantly higher in euthymic BP as compared to HC, except for *CCL2* and *EMP1*.

Table 4 also shows that lithium and antipsychotic treatment reduced the expression of *PDE4B* and *TNF*. We therefore analyzed the Q-PCR monocyte data for BP without lithium or antipsychotic treatment separate from those on lithium and/or antipsychotic treatment (Table 3, columns B and C). From this analysis it is clear that in BP off lithium and antipsychotics the gene expressions of *PDE4B, IL1B, Il6, TNF, TNFAIP3, PTGS2, PTX3, CCL20, CXCL2, BCL2A1* and *DUSP2* were in particular high, while being reduced by lithium and antipsychotic treatment. *CCL2, CCL7, CDC42, CCR2, ATF3, NAB2* and *MAPK6* expressions were not affected by lithium and/or antipsychotic treatment. Interestingly *EMP1* expression was not raised in BP off lithium and/or antipsychotics, while lithium treatment had a significantly stimulating effect on *EMP1* expression (Tables 3 and 4). The Q-PCR data thus verify the limited Affymetrix data, showing a decrease in inflammatory and a rise in apoptotic genes in monocytes of lithium-treated BP. *EMP1* was also influenced by other medications: a higher expression was found with carbamazepine, while a lower expression was found with valproate (Table 4).

The other variables tested for (age, gender, Body Mass Index (BMI), age at onset disease, duration of illness and duration of lithium use) (via linear regression) lacked any significant effect on the aberrant mRNA expressions.

Table 3. Q-PCR analysis of monocytes of bipolar patients (*n*=42) and healthy controls (*n*=25). Fold change values.

	Fold change[1]							
	A		B		C			
	All BP vs HC[2]		BP without Lithium and Antipsychotic treatment vs HC[2]		BP with Lithium and/or Antipsychotic treatment vs HC[2]		B/C[3]	
Gene symbol	*n*=42	*p*[4]	*n*=11	*p*[4]	*n*=31	*p*[4]		*p*[4]
1) Inflammation								
PDE4B	4.92	*<.001*	13.73	*<.001*	3.42	*<.001*	4.01	*.001*
IL6	14.40	*.001*	37.92	*.005*	9.56	*.006*	3.97	*.29*
Il1B	5.82	*.002*	14.65	*.003*	4.05	*.010*	3.62	*.05*
PTX3	4.56	*<.001*	8.47	*.001*	3.55	*.003*	2.39	*.14*
PTGS2	3.14	*.007*	5.75	*.008*	2.46	*.030*	2.38	*.17*
TNF	2.56	*.01*	8.26	*.006*	1.56	*.06*	5.29	*.01*
TNFAIP3	3.17	*.001*	11.68	*<.001*	1.99	*.01*	5.87	*.001*
ADAM17	1.32	*.09*	1.07	*.83*	1.41	*.05*	.75	*.51*
HSPA1A	.89	*.69*	1.78	*.15*	.73	*.27*	2.44	*.05*
2) Chemokinesis/ motility								
CCL7	13.78	*.001*	12.44	*.02*	14.29	*.003*	.87	*.70*
CCL20	11.66	*.018*	55.49	*.006*	6.02	*.10*	9.22	*.07*
CXCL2	6.36	*.001*	17.62	*.001*	4.43	*.009*	3.98	*.04*
CCL2	4.93	*.004*	3.06	*.15*	5.84	*.003*	.52	*.42*
CDC42	2.56	*<.001*	3.67	*.001*	2.25	*<.001*	1.63	*.23*
CCR2	.43	*.010*	.59	*.18*	.40	*.019*	1.48	*.46*
CX3CR1	.36	*.02*	.66	*.34*	.30	*.016*	2.20	*.41*
3) Cell survival/ apoptosis								
BCL2A1	2.48	*.001*	5.39	*.001*	1.88	*.006*	2.87	*.04*
EMP1	2.81	*.005*	1.62	*.48*	3.42	*.001*	.47	*.15*
4) Mapk pathway								
DUSP2	4.85	*<.001*	10.18	*<.001*	3.73	*.001*	2.73	*.09*
ATF3	4.32	*<.001*	5.57	*<.001*	3.95	*<.001*	1.41	*.58*
NAB2	3.27	*.005*	3.73	*.08*	3.12	*.006*	1.20	*.81*
MAPK6	2.47	*<.001*	3.09	*.03*	2.29	*.001*	1.35	*.38*

[1] The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene- CT housekeeping gene), via the ΔΔCT method (User Bulletin, Applied Biosystems). The fold change of the HC is set to 1.
The ΔCT values are available in supplementary Table 1.

[2] Values >1: patients have a higher expression than control group.
Values <1: patients have a lower expression than control group.
Healthy controls: *n*=25

[3] BP without Lithium and Antipsychotic treatment (for at least one year) versus BP with Lithium and/or Antipsychotic treatment.
Values >1: patients without treatment have a higher expression than patients with treatment.
Values <1: patients without treatment have a lower expression than patients with treatment.

[4] Groups were compared using the Mann-Whitney test.
Purity of monocytes was over 90% (as determined by morphology on each sample) and over 92% as determined by FACS analysis. Yield of monocytes was 28% (±10%) in the BD group and 21% (±8%) for the HC group (n.s) of the Ficoll-isolated peripheral blood mononuclear cells.

BP, bipolar patients; HC, healthy controls

Table 4. Correlations of mood status and medication use of bipolar patients (*n*=42) with the aberrant mRNA expressions of the genes.

Mood	Genes[1]	Depressed (*n*=7) vs. Euthymic (*n*=26)[2]			Manic (*n*=7) vs. Euthymic (*n*=26)[2]			
		B[3]	95% CI	*p*	B[3]	95% CI	*p*	*p*[4]
	MAPK6	4.24	1.74-10.40	*.002*	3.68	1.46-9.24	*.007*	*.004*
	IL6	208	9.21-4708	*.002*	12.58	.29-36.71	*.177*	*.006*
	CCL2	38.85	5.18-291.83	*.001*	11.96	1.50-95.14	*.021*	*.003*
	PTX3	9.48	2.20-40.84	*.004*	3.36	.65-17.32	*.142*	*.013*
	BCL2A1	5.29	1.58-17.65	*.008*	2.97	.86-10.26	*.083*	*.025*
	EMP1	5.05	1.47-17.34	*.012*	2.09	.59-7.45	*.245*	*.040*
Psychotropic Medication	Genes[1]	Effect of indicated medication[5]						
		B[3]	95% CI	*p*				
- Lithium	*PDE4B*	.36	.17-.73	*.007*				
	TNF	.25	.087-.71	*.011*				
	EMP1	2.82	1.19-6.69	*.020*				
- Carbamazepine	*EMP1*	4.71	1.80-12.3	*.002*				
- Valproate	*EMP1*	.38	.16-.93	*.035*				
- Antipsychotics	*PDE4B*	.17	.070-.42	*<.001*				
	TNF	.21	.058-.74	*.017*				
- Antidepressives	All molecules			*>.05*				
- Benzodiazepines	All molecules			*>.05*				
- Thyroxine	All molecules			*>.05*				

[1] Genes tested were the 19 genes that were statistically significantly differentially expressed in the patient groups as compared to healthy controls (see Table 3).
[2] Determination of the influence of mood on mRNA expression of molecules via ANCOVA analysis. The values of patients with a euthymic mood are set to 1.
[3] Regression coefficient. A value above 1 corresponds with a positive correlation. The higher the value the stronger the correlation. Values below 1 correspond with a negative correlation.
[4] Overall p value of mood in model.
[5] Linear regression with lithium, carbamazepine, valproate, antipsychotics, antidepressives and benzodiazepines included in model; thyroxine was analyzed in a separate model. The values of patients not on the indicated medication are set to 1 in both models.
Data represent relative mRNA expression.

An inflammatory gene expression signature in bipolar patients

The expression levels of the 19 mRNAs were virtually all mutually strongly correlating in expression (see Supplementary Figure 1). The strongest correlations were found between *PDE4B* and the other mRNAs and *PDE4B* was the gene most consistently over-expressed. There are reasons to consider *PDE4B* as a key molecule in the pro-inflammatory state

of the bipolar monocytes (see discussion). We therefore tried out various definitions for the presence or absence of a "*PDE4B*-associated pro-inflammatory mRNA signature" (Table 5). The definitions given depend on the percentage positives of the 19 genes, positive being defined as a mRNA expression over one standard deviation of the level found in healthy controls (see also legend Table 5). In all definitions *PDE4B* was positive. Using these definitions it is evident that a *PDE4B* "positivity" in monocytes combined with a positivity for the other signature genes of up to 50% discriminates bipolar patients from healthy controls and that a proportion of up to 60% of bipolar patients is characterized by a "*PDE4B* associated pro-inflammatory, chemokinesis, cell survival mRNA signature" (depending on the signature definition). It is also clear in this analysis that lithium and/or antipsychotic treatment down-regulated the signature expression (Table 5).

Table 5A. The presence of a *PDE4B*-associated mRNA signature in healthy controls and bipolar patients.

Signature definitions[1]	HC[2]	All BP		BP without Lithium and Antipsychotic treatment[3]		BP with Lithium and/ or Antipsychotic treatment	
	Total *n*=38	Total *n*=42		Total *n*=11		Total *n*=31	
	n (%)	n (%)	*p*[4]	n (%)	*p*[5]	n (%)	*p*[6]
PDE4B positive	8 (21)	25 (60)	*<.001*	10 (91)	*<.001*	15 (48)	*.017*
+ at least 25% of genes positive	7 (18)	23 (55)	*.001*	9 (82)	*<.001*	14 (45)	*.016*
+ at least 50% of genes positive	6 (16)	18 (43)	*.008*	7 (64)	*.002*	11 (35)	*.059*
+ at least 75% of genes positive	4 (11)	7 (17)	*.43*	3 (27)	*.16*	4 (13)	*.76*
+ at least 90% of genes positive	0 (0)	2 (5)	*.17*	2 (18)	*.007*	0 (0)	
+ at least 100% of genes positive	0 (0)	1 (2)	*.34*	1 (9)	*.06*	0 (0)	

[1] Various definitions are given depending on the percentage positives of the 19 genes that were statistically differentially expressed ($p<0.01$) in BP as compared to HC (see Table 3). Positive is defined as a mRNA expression over one standard deviation of the mean of the level found in healthy controls. In all definitions *PDE4B* is positive.

[2] The same 25 HC were used as described in Table 1, but also 13 extra HC were included in this analysis to increase power (mean age 32 yrs, 77% females).

[3] BP without Lithium and Antipsychotic treatment for at least one year.

[4] Chi-Squared test: All BP versus HC.

[5] Chi-Squared test: BP without Lithium and Antipsychotic treatment versus HC.

[6] Chi-Squared test: BP with Lithium and/or Antipsychotic treatment versus HC.

HC, healthy controls; BP, bipolar patients

Table 5B. A ROC analyses on the *PDE4B*-associated mRNA signatures defined in Table 5A on bipolar patients without lithium and antipsychotic treatment (*n*=11) and healthy controls (*n*=38).

Signature definitions	Area	SE[1]	*p*	Asymptotic 95% Confidence Interval		Sensitivity	Specificity
				Lower Bound	Upper Bound		
PDE4B positive	.85	.065	*<.001*	.72	.98	.91	.79
+ at least 25% of genes positive	.82	.077	*.001*	.67	.97	.82	.82
+ at least 50% of genes positive	.74	.094	*.017*	.56	.92	.64	.84
+ at least 75% of genes positive	.58	.10	*.40*	.38	.79	.27	.89
+ at least 90% of genes positive	.59	.11	*.36*	.38	.80	.18	1.00
+ at least 100% of genes positive	.55	.10	*.65*	.34	.75	.09	1.00

[1] Under the nonparametric assumption

The inflammatory gene expression signature in offspring of bipolar patients

Table 6 shows that the 13 bipolar offspring with a lifetime diagnosis of a mood disorder showed a raised expression of the mRNAs for all 18 signature genes tested (CCR2 was not tested for) at Time 2 (the time of monocyte testing) (for ΔCT values see Supplementary Table 2). When expressed as "signature positivity" (Table 7): 11 of the 13 children with a lifetime diagnosis of a mood disorder at Time 2 were positive for the pro-inflammatory signature if it was defined as "*PDE4B* plus 25% of the 18 genes positive". Four of those 13 children had a lifetime diagnosis of bipolar disorder, all 4 were signature positive. The other 9 children had a lifetime diagnosis of depression, 7 of them had a pro-inflammatory signature. There was no relationship of positivity and negativity for this signature and the precise psychiatric diagnosis of the unipolar mood disorder (major depressed, dysthymia or depression NOS).

There were 3 children who were healthy at the time of blood collection at Time 2, but who had developed a depression 41 months later at Time 3. All 3 subjects had monocytes with the pro-inflammatory signature (defined as described above) at Time 2 (Tables 6 and 7).

There was also one offspring who had a lifetime diagnosis of depression at Time 2 and who had converted to a lifetime diagnosis of bipolar disorder at Time 3; this subject had a full-blown pro-inflammatory signature (defined as "*PDE4B* positive plus 100% of the 18 genes positive") at Time 2 (data not shown).

Table 6 in addition shows that healthy offspring of a bipolar parent (i.e. no lifetime mood disorder at any time point tested) did show aberrancies in the expression of the

Table 6. Q-PCR analysis of monocytes of bipolar offspring (*n*=54) and healthy young adults (*n*=70). Fold change values.

	Fold change[1]							
	Offspring with mood disorder[2] vs. Healthy young adults[3]		Euthymic offspring becoming depressed vs. Healthy young adults[3]		Euthymic offspring vs. Healthy young adults[3]		Offspring with mood disorder vs. Euthymic offspring[4]	
Gene symbol	*n*=13	*p*[5]	*n*=3	*p*[5]	*n*=38	*p*[5]		*p*[5]
1) Inflammation								
PDE4B	5.79	*<.001*	6.77	*.010*	2.79	*.001*	2.14	*.21*
IL6	935.70	*<.001*	2147.77	*.004*	23.43	*<.001*	46.67	*.001*
Il1B	32.81	*<.001*	49.65	*.007*	8.78	*.001*	4.04	*.48*
PTX3	18.19	*<.001*	14.40	*.026*	3.04	*.001*	5.73	*.003*
PTGS2	16.67	*<.001*	19.74	*.006*	3.49	*.001*	4.69	*.025*
TNF	20.71	*<.001*	23.59	*.005*	3.49	*.002*	6.08	*.009*
TNFAIP3	8.14	*<.001*	9.03	*.018*	1.88	*.028*	4.41	*<.001*
2) Chemokinesis/ motility								
CCL7	450.53	*<.001*	894.27	*.004*	50.19	*<.001*	9.84	*.032*
CCL20	400.12	*<.001*	421.96	*.009*	20.46	*<.001*	19.75	*.017*
CXCL2	40.50	*<.001*	41.29	*.008*	7.56	*<.001*	5.38	*.008*
CCL2	7.02	*<.001*	16.65	*.008*	2.92	*.002*	2.83	*.15*
CDC42	5.46	*<.001*	4.74	*.005*	4.50	*<.001*	1.18	*.56*
3) Cell survival/ apoptosis								
BCL2A1	8.59	*<.001*	9.69	*.007*	4.28	*<.001*	2.05	*.11*
EMP1	5.69	*<.001*	3.46	*.052*	3.38	*<.001*	1.54	*.063*
4) Mapk pathway								
DUSP2	11.11	*<.001*	10.57	*.008*	3.29	*.001*	3.35	*.005*
ATF3	6.28	*<.001*	3.63	*.13*	1.30	*.78*	4.35	*<.001*
NAB2	2.87	*<.001*	5.81	*.017*	2.38	*<.001*	1.38	*.65*
MAPK6	4.13	*<.001*	2.76	*.22*	2.44	*<.001*	1.57	*.098*

[1] The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene- CT housekeeping gene), via the ΔΔCT method (User Bulletin, Applied Biosystems). The fold change of the HC is set to 1.
The ΔCT values are available in supplementary Table 2.

[2] Mood disorder: 4 children had a lifetime diagnosis of bipolar disorder, 9 children had a lifetime diagnosis of depression.

[3] Values >1: bipolar offspring have a higher expression than healthy young adults.
Values<1: bipolar offspring have a lower expression than healthy young adults.

[3] Healthy young adults *n*=70.

[4] Values >1: bipolar offspring with a mood disorder have a higher expression than euthymic offspring.
Values<1: bipolar offspring with a mood disorder have a lower expression than euthymic offspring.

[5] Groups were compared using the Mann-Whitney test.

Monocytes of bipolar offspring were collected at Time 2. Psychiatric evaluations were done at Time 1 (between November 1997 and March 1999), Time 2 (14 months after Time 1) and Time 3 (41 months after Time 2).
The bipolar offspring are divided in: offspring with a mood disorder at Time 2 and Time 3 (*n*=13), offspring euthymic at Time 2 but developing depression at Time 3 (*n*=3), offspring euthymic at Time 2 and Time 3 (*n*=38).

Purity of monocytes was over 90% (as determined by morphology on each sample) and over 93% as determined by FACS analysis. Yield of monocytes was 19% (±13%) in the bipolar offspring group and 29% (±12%) for the healthy young adult group (n.s.) of the Ficoll-isolated peripheral blood mononuclear cells.

Table 7A . The presence of a *PDE4B*-associated mRNA signature in bipolar offspring and in healthy young adults.

Signature definitions[1]	Healthy young adults	Offspring with mood disorder[2]		Euthymic offspring becoming depressed		Euthymic offspring	
	Total *n*=70	Total *n*=13		Total *n*=3		Total *n*=38	
	n (%)	n (%)	*p*[3]	n (%)	*p*[4]	n (%)	*p*[5]
PDE4B positive	15 (21)	11 (85)	*<.001*	3 (100)	*.002*	17 (45)	*.011*
+ at least 25% of genes positive	13 (19)	11 (85)	*<.001*	3 (100)	*.001*	17 (45)	*.004*
+ at least 50% of genes positive	13 (19)	11 (85)	*<.001*	3 (100)	*.001*	17 (45)	*.004*
+ at least 75% of genes positive	5 (7)	10 (77)	*<.001*	3 (100)	*<.001*	12 (32)	*.001*
+ at least 90% of genes positive	2 (3)	5 (38)	*<.001*	1 (33)	*.009*	1 (3)	*.95*
+ at least 100% of genes positive	0 (0)	4 (31)	*<.001*	1 (33)	*<.001*	0 (0)	

[1] Various definitions are given depending on the percentage positives of the 18 genes that were statistically differently expressed (p<0.01) in BP as compared to HC (see Table 3)(CCR2 was not tested for in the bipolar offspring). Positive is defined as a mRNA expression over one standard deviation of the mean of the level found in healthy controls. In all definitions *PDE4B* is positive.

[2] Mood disorder: 4 children had a lifetime diagnosis of bipolar disorder, 9 children had a lifetime diagnosis of depression.

[3] Chi Squared test: Offspring with mood disorder versus Healthy young adults.

[4] Chi Squared test: Euthymic offspring becoming depressed versus Healthy young adults.

[5] Chi Squared test: Euthymic offspring versus Healthy young adults.

Monocytes of bipolar offspring were collected at Time 2. Psychiatric evaluations were done at Time 1 (between November 1997 and March 1999), Time 2 (14 months after Time 1) and Time 3 (41 months after Time 2). The bipolar offspring are divided in: offspring with a mood disorder at Time 2 and Time 3 (*n*=13), offspring euthymic at Time 2 but developing depression at Time 3 (*n*=3), offspring euthymic at Time 2 and Time 3 (*n*=38).

18 pro-inflammatory, chemokinesis and cell survival genes in their monocytes, and the inflammatory gene signature defined as "*PDE4B* plus 25% of the genes positive" was found in 45% of them versus 19% in healthy young adults (Table 7). Yet, expression levels of particularly the pro-inflammatory compounds *IL6, TNF, TNFAIP3, PTX3, PTGS2, CCL7, CCL20, CXCL2* and the Mapk pathway *DUSP2* and *ATF3* were significantly higher in offspring with a mood disorder than in healthy euthymic offspring (Table 6).

Table 7B. A ROC analyses on the *PDE4B* associated mRNA signatures defined in Table 7A on bipolar offspring with a mood disorder (*n*=13) and the healthy young adults (*n*=70).

Signature definitions	Area	SE[1]	*p*	Asymptotic 95% Confidence Interval		Sensitivity	Specificity
				Lower Bound	Upper Bound		
PDE4B positive	.82	.065	<.001	.69	.94	.85	.79
+ at least 25% of genes positive	.83	.064	<.001	.70	.96	.85	.81
+ at least 50% of genes positive	.83	.064	<.001	.70	.96	.85	.81
+ at least 75% of genes positive	.85	.071	<.001	.71	.99	.77	.93
+ at least 90% of genes positive	.68	.094	.042	.49	.86	.38	.97
+ at least 100% of genes positive	.65	.096	.079	.47	.84	.30	1.00

[1]Under the nonparametric assumption.

Expression at the protein level of the aberrantly expressed monocyte signature mRNAs *IL1B, IL6, CCL2, CCL7 and CCR2*

We measured (via standard commercially available ELISAs) the protein expression levels of the signature genes *IL1B, IL6, CCL2* and *CCL7* in bipolar patients (BP) and healthy controls (HC) described in Table 1. Although the serum levels of all 4 cytokines were higher in BP, only that of IL-1β was statistically significantly raised (2 times higher in BP vs. HC, Figure 1A). The serum level of IL-1β was neither influenced by mood (*p*=0.58, Kruskal-Wallis test) nor by lithium, antipsychotic or any other treatment. There was no effect of age, gender and BMI on the levels of the cytokines.

We also measured the expression of CCR2, the receptor for CCL2 on monocytes via standard FACS analysis and found that CCR2 was mainly expressed on the mature $CD14^{+}CD16^{+}$ set of monocytes (Figure 1B). CCR2 expression was not significantly different between monocytes of BP and HC. CCR2 expression levels were independent from mood status and medication usage.

DISCUSSION

We here show that monocytes of the majority of bipolar patients and bipolar offspring (particularly in those who later develop a mood disorder) have an altered mRNA expression of genes involved in inflammation and inflammation related processes.[26-47]

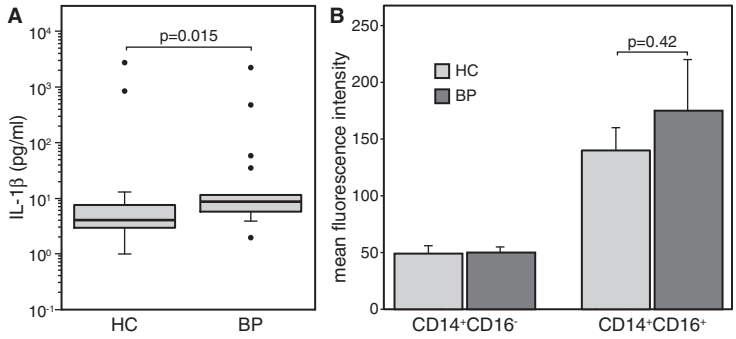

Figure 1. Serum IL-1β levels (A) and the CCR2 expression on monocytes (B) of bipolar patients (BP) and healthy controls (HC).
A). Boxplot of log transformed IL-1β is given. The serum IL1-β level was determined on the serum of 18 Dutch patients who were lithium naive and 25 healthy controls used for Q-PCR via a commercially available ELISA (R&D Systems, Minneapolis MN, USA) used according to the manufacturers protocol. The box indicates the lower and upper quartiles. The line within the box represents the median. The whiskers extend to the 2.5 and 97.5 percentiles. The outliers are characterized by the filled dots. Data were log transformed to obtain a normal distribution of the cytokines/chemokines. Since this was not possible for IL-1β (and IL-6 and CCL7), the non-parametric Mann-Whitney test was used for statistical evaluation of IL-1β levels.
B). The expression of CCR2 on monocytes in Mean Fluorescence Intensity levels as determined by FACS analysis. Means and standard deviations are given of $CD14^+CD16^-$ monocytes and $CD14^+CD16^+$ monocytes. The Mann- Whitney test was used to compare groups. For the test we used the monocytes of 12 bipolar patients (6 males, 6 females, mean age 47 (range 38-56) years, euthymic: 7, depressed: 5, manic: 1, all on lithium) and 9 HC. In the test PBMC (5.10^5 cells/ml) were stained (10 min, room temperature) with 25µl of mouse anti-human FITC- (fluorescein) or PE-conjugated (phycoerythrin) monoclonal antibodies (mAbs), and afterwards washed. The following mAbs were used: anti-IgG1 FITC (Becton Dickinson (BD), San Jose, USA), anti-IgG1 PE (BD), anti-CD14 PE (Beckman Coulter (BC), Hialeah, USA), anti-CD16-FITC (1:10, CLB, Amsterdam, The Netherlands), anti-CD16-PE (1:10, BD) and anti-CCR2-PE (1:5, R&D systems, Abingdom, UK). Immediately after staining, cells were measured using a FACScalibur flowcytometer (BD) gating out debris and dead cells via light scatter properties. Data were analysed by CellQuestPro software (BD Pharmingen, Alphen a/d Rijn, The Netherlands), monocytes were gated out by means of cell size (forward scatter) and irregular shape (side scatter).

Due to the coherent and mutually strongly correlating aberrant expression, the mRNAs form a “mRNA signature” representing a set of pro-inflammatory genes that discriminate bipolar patients from healthy controls.

Based on the literature an interaction model of the aberrantly expressed genes can be constructed (Figure 2). This model illustrates the mutual interdependency of the molecules. The expression of both positive and negative signals in the network is an indication that in the aberrant monocytes of bipolar patients regulation is operative. For inflammatory cytokines, mRNA and protein levels often do not correlate. Indeed the about 6-fold raised expression of *IL1B* at the monocyte mRNA level was only reflected in a 2-fold raised protein level in the serum of bipolar patients, while CCL-2, CCL7, IL-6 and CCR2 protein expression were not significantly different. Clearly a regulation at the protein transcription

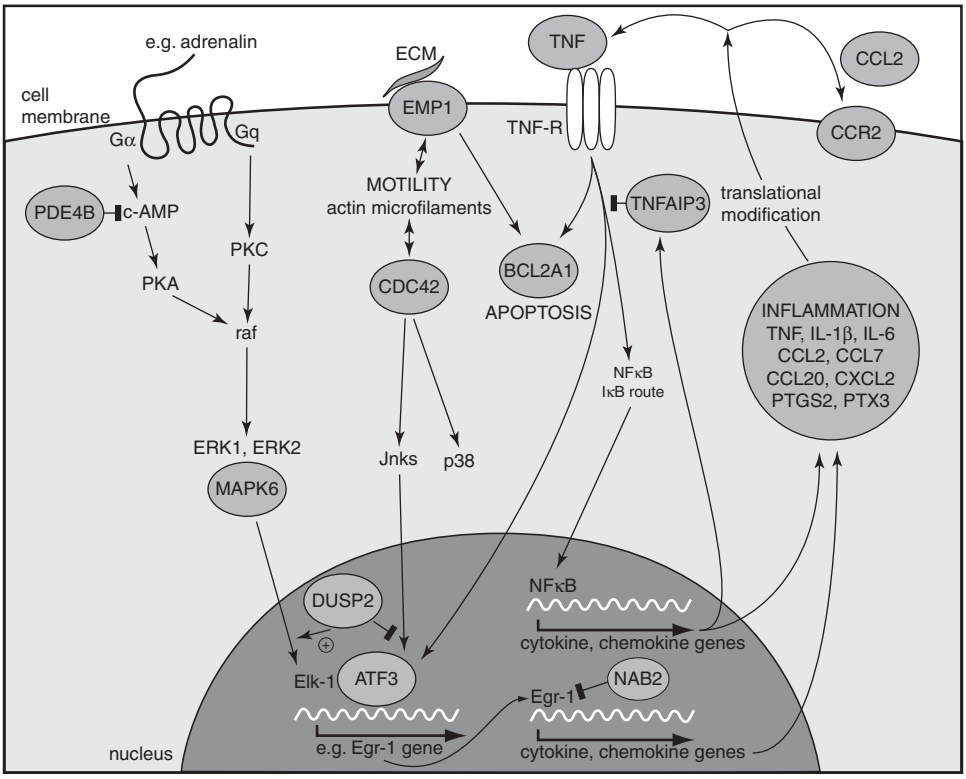

Figure 2. The hypothetical relationship of the 19 pro-inflammatory signature genes.
The hypothetical relationship of the 19 molecules (encircled) of the pro-inflammatory, chemokinesis signature. The MAP kinases (MAPKs) are fundamental regulators of immune cell function.[26] There are three main classes: the ERKs, the p38 proteins and the Jun N terminal kinases (Jnks), which shuttle after an activating phosphorylation into the nucleus and initiate the transcription of immediate early genes of pre-existing transcription factors.[26] A pre-existing transcription factor is Elk-1, but also ATF3.[26] ATF3 is bi-directionally regulated by the Jnk (positive) and ERK (negative) pathways[27] and is induced during cellular stress. Dual-specificity phosphatases (DUSPs) regulate MAPK activity through de-phosphorylation and also anchor or shuttle MAPKs.[28, 29] DUSP2 (encoding PAC1) localizes to the nucleus and is one of the most highly induced transcripts in activated immune cells. A recent report shows that DUSP2 has a positive function in macrophage mediated inflammatory responses via a lowering of the Jnks and a compensatory rise in ERKs and p38[28]. Of the actual MAPKs MAPK6/ERK3 is over expressed. CDC42 acts as a Rho GTPase signaling molecule upstream from the MAPKs[31] but also as a molecule related to the cytoskeletal organization of the cell, its motility and chemotactic potential.[30] Also TNF-α feeds into the MAPK system and activates the Jnk cascade and ATF3 expression.[27]
Not only activators of the MAPK signaling pathway, but also repressors are part of the signature. NAB2 is a co-repressor molecule that directly binds Egr-1 and inhibits its trans-activating potential.[32] Egr-1 is induced by Elk-1 and the Egr family plays a key role in coordinating subsequent waves of gene expression after the immediate early gene response induced by MAPKs.[26, 32] Another molecule with repressor activity, but part of the signaling cascade down stream from the TNF-α receptor is TNFAIP3: TNFAIP3 blocks the activation of the Jnk cascade by TNF-α[33] and in this way acts anti-apoptotic and anti-inflammatory.[34] Yet another layer of negative regulation is seen at the level of the pro-inflammatory chemokine CCL2 and its receptor CCR2: an up regulation of the mRNA for CCL2 was opposed by a down regulation of the mRNA of CCR2.
EMP1 is a tetraspan transmembrane protein playing a role in cell-cell adhesion and interactions with the ECM.[35] Although the function of the molecule is not entirely known, it appears to be involved in cell survival and growth.[36] BCL2A1 is a well-known apoptotic molecule.[37] The pro-inflammatory cytokines and components are discussed in the text.
MAPK, mitogen-activated protein kinase; ERK, Extracellular regulated kinase; ATF3, activating transcription factor 3; DUSP, dual specificity phosphatase; TNF, tumor necrosis factor; Jnk, Jun N terminal kinases; NAB2, NGFI-A binding protein; Egr-1, early growth response 1; TNFAIP3, tumor necrosis factor, alpha-induced protein 3; CCL2, chemokine (CC-motif) ligand 2; CCR2, chemokine (CC-motif) ligand 2 receptor; EMP1, epithelial membrane protein 1; ECM, extra-cellular matrix; BCL2A1, BCL2-related protein A1

level is operative in the monocytes of bipolar patients to ensure a close-to-normal function, and the question thus arises which environmental or endogenous condition will create a failure of the monocyte to keep control over its aberrant gene expression avoiding a higher protein production of the pro-inflammatory compounds. Psychological stress (both acute and chronic) might be such condition (via adrenaline and glucocorticoid signaling)[48] and indeed stressors have an up-regulating effect of IL-1β and IL-6.[49] In addition, stressful life events triggered the onset of mood disorder episodes in the here-reported children of a bipolar patient,[50] showing a possible interaction between the existence of an aberrant monocyte pro-inflammatory gene expression signature and environmental stress factors. A co-factor playing a role in this stress induced immune activation might be a relative resistance of immune cells to glucocorticoids, which has been reported in patients with bipolar disorder.[22] Such decreased glucocorticoid sensitivity has also been thought to be the mechanism behind the well-known hypothalamic-pituitary-adrenal axis disturbances in patients with mood disorders[51-53] and these disturbances have led to the concept that impaired glucocorticoid receptor signaling is a crucial factor in the pathogenesis of mood disorders.[54]

It is important to note that the over expression of pro-inflammatory cytokines, mediators and chemokines in the monocytes of bipolar patients was in particular correlated to the over-expression of PDE4B, a c-AMP degrading enzyme. It is tempting to speculate that this molecule acts as a key molecule in the pro-inflammatory state of bipolar monocytes apart from the fact that it was the most consistently over-expressed gene, because 1) cAMP is known to be inhibitory for inflammatory cells,[55] 2) of the c-AMP specific isoenzymes PDE4 is expressed in all the inflammatory and immune modulating cells,[40] 3) a high activity of PDE4B leads to a pro-inflammatory state,[40, 41] 4) the PDE4B gene is crucial for the pro-inflammatory action of monocytes in gene knockout studies,[42] 5) the *in vitro* differentiation of monocytes to inflammatory macrophages leads to an up-regulation of PDE4B.[43] and 6) PDE4 inhibitors broadly inhibiting functions of inflammatory cells[40, 41] are in a far stage of development. Using such medication not only results in anti-inflammatory effects like reducing TNF-α production,[42, 45] but also in antidepressive effects when tested in animal models[47] and clinical trials.[56]

Our findings also suggest that an assessment of the monocyte gene signature might be useful for prognostic purposes. In a previous report on offspring of bipolar patients we confirmed that bipolar disorder is more prevalent in such offspring and is preceded by one or more episodes of (until then unipolar) depressive episodes.[20] Here we show that bipolar offspring with a lifetime diagnosis of bipolar or (still) unipolar depressive disorder were positive for the mRNA signature (similar to our adult bipolar patients), but more importantly that all three children who were psychiatrically healthy at the time of first monocyte testing, but had developed a depression 3 years later, had a positive monocyte gene signature. This suggests that a positive gene signature precedes the onset of the first mood episode in individuals at risk and is not the consequence of the psychiatric condition. Also a large proportion of the still healthy euthymic offspring showed an aberrant gene

signature. We hypothesize that especially these “signature positive” offspring are at risk to develop a depression and possibly eventually a bipolar mood disorder. We are currently planning a 10-years follow-up of our offspring study and like to validate the signature test as a potential inflammatory biomarker set linked to mood disorders.

Our data on a raised expression of the genes for pro-inflammatory cytokines and related compounds support the “macrophage-T cell theory of mood disorders”.[57-59] However, there are also reports refuting a high cytokine production from immune cells in mood disorders[60, 61] and our finding of a discrepancy between mRNA and protein levels of cytokines may in part explain this controversy. It also underscores the superiority of gene expression over protein detection in diagnosing the activated IRS of bipolar patients.

We also found *PTGS2* (COX-2, involved in the production of PGE2[62]) and *PTX3* to be part of the pro-inflammatory gene expression signature. Increased PGE2 levels have been described in the saliva,[11] serum[63] and cerebrospinal fluid[10] of depressed patients and PGE2 seems to be directly involved in the sickness behavior of animals.[64] An *in vitro* study reported an increased PGE2 secretion from blood cells of depressed patients[65] and a COX-2 inhibitor has been reported to enhance the therapeutic effects of reboxetine in major depressed patients.[66] PTX3 is expressed and released by cells of the monocyte-macrophage lineage exposed to a variety of inflammatory signals, including IL-6[38] and has a non-redundant role in resistance to selected microbial agents.[39] PTX3 is also expressed in the brain and involved in neuronal plasticity and degeneration.[67] PTX3 has been found over-expressed in the fibroblasts of melancholic patients.[68]

With regard to a genetic background for the aberrant pro-inflammatory gene signature in mood disorder it is relevant to note that gene polymorphisms in important signature genes (i.e. *IL-1B, TNF* and *CCL2*) have been described as linked to the presence or treatment response of the disorders.[69-71] However the inflammatory program is also activated in response to infection. As bipolar disorder has been linked to infection by viral and intracellular pathogens[72-74] a simple explanation for the inflammatory signature could be that the monocytes are reacting to infection.

The majority of our bipolar patients used various psychotropic medications and the medication influenced the expression level of the signature genes. The strongest effects were for lithium and antipsychotics. There is a large amount of literature on the immune modulating effects of antipsychotics and lithium.[75-77] With regard to pro-inflammatory cytokine production reports are inconsistent.[60, 78-80] In this *in vivo* study the mRNA levels for important inflammatory cytokines/compounds were reduced in monocytes by lithium and/or antipsychotic treatment but did not completely normalize to the level of healthy controls.

Lithium also has profound effects on the MAPK system.[81, 82] In rat brain it stimulates phosphorylation of signaling molecules in the ERK branch of the MAPK route,[81] while it also influences the expression of the ERK-pathway regulated apoptosis molecule BCL2A1.[82] In our bipolar patients with already raised mRNAs of the MAPK pathway genes and of *BCL2A1*, we could not find a stimulating effect of lithium on MAPK pathway genes nor on *BCL2A1* in the monocytes.

In summary, we here describe an inflammatory monocyte gene expression signature in patients with bipolar disorder reflecting their activated IRS. This signature is also present in bipolar offspring, particularly in those who later develop a mood disorder.

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Author contributions: Prof. H.A. Drexhage had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the analysis. The statistical analysis were conducted by R.C. Padmos under the guidance of ir. C. Looman.

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Supplementary Table 1. Q-PCR analysis of monocytes of bipolar patients and healthy controls. ΔCT values.

	ΔCT (mean ± sd)[1]			
	HC	All BP	BP without Lithium and Antipsychotic treatment[1]	BP with Lithium and/or Antipsychotic treatment
Gene symbol	*n*=25	*n*=42	*n*=11	*n*=31
1) Inflammation				
PDE4B	-.40 ± 2.51	-2.64 ± 2.05	-3.01 ± 2.08	-2.51 ± 2.20
IL6	5.78 ± 3.17	2.97 ± 4.85	-.19 ± 5.34	4.32 ± 5.34
IL1B	-4.56 ± 2.89	-7.11 ± 3.08	-8.36 ± 3.48	-6.67 ± 3.48
PTX3	4.73 ± 1.71	-.31 ± 2.37	-1.96 ± 2.61	.39 ± 2.61
PTGS2	-1.50 ± 2.22	-3.50 ± 2.38	-4.39 ± 2.78	-3.12 ± 2.78
TNF	-2.57 ± 2.33	-4.03 ± 2.00	-5.62 ± 2.40	-3.36 ± 2.40
TNFAIP3	-1.03 ± 3.08	-2.61 ± 2.80	-3.22 ± 2.37	-2.39 ± 3.07
ADAM17	-1.32 ± .95	-1.81 ± 1.04	-.93 ± 1.26	-2.06 ± 1.26
HSPA1A	-2.82 ± 1.28	-2.67 ± 1.56	-3.70 ± 0.97	-2.41 ± .97
2) Chemokinesis/ motility				
CCL7	4.45 ± 4.37	.76 ± 5.32	1.52 ± 4.49	.48 ± 4.91
CCL20	4.73 ± 4.41	2.41 ± 5.43	-1.44 ± 5.63	4.06 ± 5.63
CXCL2	-2.89 ± 3.23	-5.51 ± 3.06	-6.52 ± 3.20	-5.15 ± 3.20
CCL2	.47 ± 1.88	-1.93 ± 3.23	-1.26 ± 2.85	-2.18 ± 2.85
CDC42	-1.10 ± 1.43	-2.42 ± 1.39	-2.53 ± 1.40	-2.38 ± 1.40
CCR2	3.23 ± 1.69	4.48 ± 1.56	3.75 ± .92	4.69 ± .92
CX3CR1	-4.50 ± 1.49	-3.04 ± 2.56	-3.64 ± .95	-2.87 ± .95
3) Cell survival/ apoptosis				
BCL2A1	-6.15 ± 1.63	-7.42 ± 1.75	-8.30 ± 1.93	-7.11 ± 1.69
EMP1	.02 ± 1.93	-1.50 ± 2.75	.08 ± 2.59	-2.06 ± 2.50
4) Mapk pathway				
DUSP2	.25 ± 2.93	-1.92 ± 2.23	-1.97 ± 2.36	-1.91 ± 2.34
ATF3	-1.03 ± 2.16	-3.05 ± 1.67	-2.95 ± 1.61	-3.08 ± 1.77
NAB2	2.43 ± 2.24	.81 ± 2.90	1.26 ± 3.39	.65 ± 2.65
MAPK6	-1.96 ± 1.22	-3.22 ± 1.33	-3.73 ± 1.42	-3.04 ± 1.30

[1] ΔCT=CT gene X- CT housekeeping gene *ABL*.
[2] Bipolar patients without lithium and/ or antipsychotic treatment for at least one year.

Purity of monocytes was over 90% (as determined by morphology on each sample) and over 92% as determined by FACS analysis. Yield of monocytes was 28% (± 10%) in the BD group and 21% (± 8%) for the HC group (n.s) of the Ficoll-isolated peripheral blood mononuclear cells.

Abbreviations: BP, bipolar patients; HC, healthy controls.

Supplementary Table 2. Q-PCR analysis of monocytes of bipolar offspring and healthy young adults. ΔCT values.

	ΔCT (mean ± sd)			
	Healthy young adults	Offspring with mood disorder[2]	Euthymic offspring becoming depressed	Euthymic offspring
Gene symbol	*n*=70	*n*=13	*n*=3	*n*=38
1) Inflammation				
PDE4B	-2.45 ± 2.09	-4.98 ± 1.61	-5.21 ± .07	-3.93 ± 2.12
IL6	3.13 ± 4.97	-6.74 ± 3.62	-7.94 ± 1.19	-1.42 ± 5.45
Il1B	-3.77 ± 3.34	-8.80 ± 2.26	-9.40 ± .66	-6.90 ± 4.63
PTX3	-.68 ± 2.25	-4.86 ± 2.27	-4.52 ± 2.66	-2.28 ± 2.24
PTGS2	-3.19 ± 2.30	-7.16 ± 2.30	-7.50 ± .55	-5.14 ± 2.83
TNF	-2.96 ± 2.26	-7.33 ± 1.63	-7.52 ± .53	-4.76 ± 3.16
TNFAIP3	-3.26 ± 2.24	-6.28 ± 1.17	-6.43 ± 1.02	-4.16 ± 1.70
2) Chemokinessis/ motility				
CCL7	7.08 ± 3.07	-1.67 ± 2.66	-2.72 ± 1.72	1.44 ± 4.64
CCL20	2.83 ± 5.14	-5.81 ± 3.02	-5.89 ± .56	-1.52 ± 5.67
CXCL2	-2.65 ± 3.17	-7.99 ± 1.66	-8.02 ± .89	-5.57 ± 3.02
CCL2	.82 ± 1.95	-1.99 ± 1.73	-3.24 ± 1.10	-.72 ± 2.93
CDC42	-.22 ± 1.39	-2.67 ± .90	-2.47 ± 0.33	-2.39 ± 1.74
3) Cell survival/ apoptosis				
BCL2A1	-5.04 ± 1.79	-8.14 ± 1.70	-8.31 ± .13	-7.14 ± 2.08
EMP1	.74 ± 1.66	-1.77 ± 1.00	-1.05 ± 1.75	-1.02 ± 1.56
4) Mapk pathway				
DUSP2	-2.45 ± 2.52	-5.14 ± 1.02	-5.06 ± .25	-3.38 ± 2.04
ATF3	-1.68 ± 2.14	-4.33 ± 1.53	-3.54 ± 1.90	-2.06 ± 1.02
NAB2	2.48 ± 1.42	.96 ± 1.30	-.06 ± 1.32	1.23 ± 1.46
MAPK6	-1.41 ± 1.42	-3.46 ± 1.49	-2.88 ± 2.18	-2.70 ± 1.39

[1] ΔCT = CT gene X-CT housekeeping gene *ABL*.
[2] Mood disorder: 4 children had a lifetime diagnosis of bipolar disorder, 9 children had a lifetime diagnosis of depression.

Monocytes of bipolar offspring were collected at Time 2. Psychiatric evaluations were done at Time 1 (between November 1997 and March 1999), Time 2 (14 months after Time 1) and Time 3 (41 months after Time 2).
The bipolar offspring are divided in: offspring with a mood disorder at Time 2 and Time 3 (*n*=13), offspring euthymic at Time 2 but developing depression at Time 3 (*n*=3), offspring euthymic at Time 2 and Time 3 (*n*=38).

Purity of monocytes was over 90% (as determined by morphology on each sample) and over 93% as determined by FACS analysis. Yield of monocytes was 19%(±13%) in the bipolar offspring group and 29% (±12%) for the healthy young adult group (n.s) of the Ficoll-isolated peripheral blood mononuclear cells.

	PDE4B																
DUSP2	0.80	*DUSP2*															
ATF3	0.79	0.85	*ATF3*														
TNF	0.74	0.76	0.62	*TNF*													
IL1B	0.78	0.83	0.74	0.79	*IL1B*												
IL6	0.74	0.66	0.64	0.62	0.87	*IL6*											
CXCL2	0.72	0.76	0.64	0.64	0.91	0.89	*CXCL2*										
CCL20	0.73	0.66	0.57	0.69	0.90	0.93	0.92	*CCL20*									
PTX3	0.79	0.70	0.73	0.67	0.84	0.91	0.84	0.86	*PTX3*								
PTGS2	0.82	0.81	0.76	0.76	0.81	0.73	0.68	0.74	0.80	*PTGS2*							
TNFAIP3	0.82	0.78	0.62	0.77	0.77	0.68	0.72	0.70	0.67	0.72	*TNFAIP3*						
BCL2A1	0.71	0.81	0.64	0.73	0.85	0.76	0.82	0.78	0.71	0.74	0.87	*BCL2A1*					
CCL7	0.43	0.57	0.44	0.33	0.69	0.74	0.84	0.73	0.71	0.42	0.39	0.62	*CCL7*				
MAPK6	0.55	0.66	0.61	0.50	0.69	0.72	0.73	0.66	0.70	0.50	0.54	0.73	0.74	*MAPK6*			
CDC42	0.65	0.74	0.67	0.50	0.76	0.75	0.83	0.73	0.70	0.54	0.64	0.80	0.77	0.83	*CDC42*		
NAB2	0.26	0.50	0.35	0.21	0.32	0.26	0.46	0.23	0.42	0.20	0.31	0.39	0.62	0.57	0.53	*NAB2*	
CCL2	0.19	0.34	0.31	0.13	0.51	0.56	0.61	0.53	0.57	0.21	0.12	0.39	0.86	0.66	0.59	0.57	*CCL2*
EMP1	0.31	0.32	0.41	0.00	0.39	0.51	0.55	0.41	0.62	0.24	0.14	0.24	0.72	0.50	0.45	0.57	0.72	*EMP1*
CCR2	-0.26	-0.29	-0.31	-0.35	-0.44	-0.31	0.05	-0.23	-0.38	-0.60	-0.65	-0.51	-0.43	-0.08	0.09	-0.61	-0.29	-0.22

Supplementary Figure 1.
Correlation of mRNAs, data represent Pearson's correlation coefficients, tested on the relative mRNA expression of the genes in 67 individuals: 42 bipolar patients and 25 healthy controls (see Table 1). Black represents: r>0.5, grey represents: 0.5>r. Significance ($p<0.05$) was reached in all colored fields, white fields are not significant.

IV

A Twin Study: Pro-inflammatory Monocytes in Bipolar Disorder Mainly the Result of a Common Environmental Factor

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ABSTRACT

Context Previously a pro-inflammatory gene expression signature was found in the monocytes of bipolar disorder (BD) patients.

Objective To determine the contribution of genetic and environmental influences on the association of the monocyte gene expression signature with BD.

Design A Q-PCR case-control study using monocytes in a bipolar twin study. Determination of the influence of additive genetic, common and unique environmental factors by structural equation modeling (ACE).

Setting Dutch academic research center.

Participants Eighteen monozygotic (MZ) BD twin pairs, 23 dizygotic (DZ) BD twin pairs, 18 MZ healthy twin pairs and 16 DZ healthy twin pairs.

Main outcome measure The expression levels in monocytes of the 19 previously identified signature genes.

Results The familial occurrence of the association of the monocyte gene expression signature with BD found in the within trait/cross twin correlations ("twin-correlations") was due to shared environmental factors (both MZ and DZ ratios in twin-correlations approximate one, ACE modeling data: 94% (95% CI: 53-99%) explained by common (shared) environmental factors). Although the majority of individual signature genes followed this pattern, there was a small sub-cluster of chemotaxis/motility genes where genetic influences could dominate.

Conclusions The association of the pro-inflammatory monocyte signature with bipolar disorder primarily is the result of a common shared environmental factor.

INTRODUCTION

Bipolar disorder is a complex illness in which multiple genes and environmental factors determine the pathogenesis. The reported heritability ranges from 60% to 85%,[1, 2] which indicates that genes play a major role in the pathogenesis of bipolar disorder. However, the environment is also important, which can be concluded from the fact that the concordance rate of bipolar disorder is only 40-70% in monozygotic twins.[3]

Recently, we reported the presence of a pro-inflammatory state of circulating monocytes in a large proportion of bipolar patients and we detected the expression of a coherent set of 19 inflammation- or inflammation-related genes ("a mRNA signature") in the monocytes of up to 60% of bipolar patients. The inflammatory signature could also be found in the monocytes of a large proportion of offspring of bipolar patients and especially in those children developing a mood disorder, showing that the pro-inflammatory state of monocytes precedes the actual mood symptoms.[4] Our findings thus lend support for an activated inflammatory response system being a causal factor for mood symptoms in bipolar disorder. A role for an activated inflammatory response system in the etiology of mood disorders has been implicated previously and was postulated as the "macrophage-T cell theory of depression" or the "inflammatory cytokine theory of depression".[5-7]

Our offspring study mentioned above also showed that the presence of the inflammatory state of monocytes (as evidenced by the positive pro-inflammatory signature) is familial, but the study could not differentiate between genetic and shared environmental factors determining the inflammatory signature expression. Twin studies are needed for that particular distinction. We embarked on such a study and conducted a twin study to 1) determine the contributing part of genes and environmental factors to individual differences in signature expression and 2) to determine to which extend the observed association of bipolar disorder with the inflammatory signature is due to the same genetic effects and environmental factors.

For that purpose we performed Q-PCRs of the 19 genes in the circulating monocytes of 18 monozygotic bipolar twin pairs, 23 dizygotic bipolar twin pairs, 18 monozygotic healthy twin pairs and 16 dizygotic healthy twin pairs. We adopted a formal genetic model-fitting approach, the so-called structural equation modeling (SEM). This method is capable of quantifying genetic and environmental influences on the correlation between bipolar disorder and signature expression.

SEM has extensively been described and used in schizophrenia and bipolar disorder before.[8-10]

METHODS

Participants

Outpatients with DSM-IV bipolar I or II disorder were recruited from an ongoing Dutch twin study on bipolar disorder described elsewhere in detail.[11] In short, participants were

twin pairs in which at least one twin suffered from bipolar disorder. A DSM-IV diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I (SCID). Present mood states were evaluated via the Young Mania Rating Scale (YMRS) and the Inventory for Depressive Symptomatology (IDS). The twins did not have a history of drug or alcohol dependency for at least six months, nor did they have a severe medical illness, verified with a medical history assessment. The healthy control twins did not have an Axis I psychiatric disorder, or an Axis II personality disorder. Furthermore, they had no first-degree relatives with a severe psychiatric disorder such as schizophrenia, psychotic disorders, mood disorders, anxiety disorders and substance use disorders.

In this study 75 twin pairs were included: 41 bipolar twin pairs and 34 control twin pairs. Of the 41 bipolar twin pairs, 6 were monozygotic (MZ) concordant (both index twin and co-twin had bipolar disorder), 12 pairs were MZ discordant (co-twin did not have bipolar disorder), 4 pairs were dizygotic (DZ) concordant and 19 pairs were DZ discordant. Nine discordant (i.e. non-bipolar) co-twins had another DSM-IV diagnosis: 7 had a major depressive disorder and 2 had schizophrenia. At time of blood drawing, almost all patients were euthymic except for 4 patients who met the criteria for a depressive episode (Inventory for Depressive Symptomatology >12).

Of the control twin pairs, 18 were MZ and 16 were DZ. The demographics of all twin pairs are summarized in Tables 1 and 2.

The Medical Ethical Review Committee of the University Medical Center Utrecht approved the studies. Written informed consent was obtained from all participants after a complete description of the study was given.

Table 1. Demographics twin pairs.

	Bipolar twin pairs					**Control twin pairs**		
	Total	MZ CC	MZ DC	DZ CC	DZ DC	Total	MZ	DZ
Number of pairs	41	6	12	4	19	34	18	16
Age (range), yrs	42 (21-61)	36 (21-44)	41 (22-55)	43 (34-51)	43 (29-61)	42 (23-58)	40 (23-58)	44 (27-53)
Female, n (%)	56 (68)	8 (67)	18 (63)	6 (75)	24 (63)	53 (78)	30 (83)	23 (72)

MZ, Monozygotic; DZ, Dizygotic; CC, Concordant; DC, Discordant

Table 2. Characteristics bipolar patients.

		Bipolar patients				
		Total	MZ	DZ	Index Twins	Co Twins
Group size		51	24	27	41	10
Age[1]		41 (21-61)	39 (21-55)	43 (29-61)	42 (21-61)	39 (21-51)
Female, n (%)		35 (69)	17 (71)	18 (67)	29 (71)	6 (60)
Diagnosis, n (%)	Bipolar I	38 (74)	20 (83)	18 (67)	31 (76)	7 (70)
	Bipolar II	11 (22)	4 (17)	7 (26)	10 (24)	1 (10)
	Bipolar NOS	2 (4)	0	2 (7)	0	2 (20)
Age onset disease[1]		29 (14-50)	28 (14-50)	30 (18-45)	29 (14-50)	28 (18-44)
Duration disease[1]		12 (1-35)	11 (1-30)	14 (3-35)	13 (2-35)	11 (1-27)
Mood	Euthymic	47 (92)	22 (92)	25 (93)	39 (95)	8 (80)
	Depressed	4 (8)	2 (8)	2 (7)	2 (5)	2 (20)
Psychotropic medication, n (%)	Lithium	39 (76)	20 (83)	19 (70)	31 (76)	8 (80)
	Carbamazepine	3 (6)	3 (13)	0	1 (2)	2 (20)
	Valproate	6 (12)	3 (13)	3 (11)	6 (15)	0
	Lamotrigine	1 (2)	1 (4)	0	1 (2)	0
	Thyroxine	5 (10)	2 (8)	3 (11)	5 (12)	0
	Antipsychotics	10 (20)	2 (8)	8 (30)	9 (22)	1 (10)
	Antidepressives	16 (31)	6 (25)	10 (37)	14 (34)	2 (20)
	Benzodiazepines	13 (25)	9 (38)	4 (15)	11 (27)	2 (20)

[1] mean (range), years
MZ, Monozygotic; DZ, Dizygotic

Laboratory methods

Zygosity determination

Zygosity was determined by DNA fingerprinting, using 9-11 high polymorphic microsatellite markers in the laboratory of the Division Genetics, UMC Utrecht.

Blood collection and preparation

Blood was collected in a clotting tube for serum preparation (frozen and stored at -80°C) and in sodium-heparin tubes for immune cell preparation. From the heparinized blood peripheral blood mononuclear cell (PBMC) suspensions were prepared by low-density gradient centrifugation as described in detail before[12] within 8 hours to avoid *ex vivo* activation of the monocytes. PBMCs were frozen in RPMI 1640 with 25 mM Hepes and Ultraglutamin containing 10% fetal calf serum and 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to store the samples in the center of blood collection, to ship the samples deep-frozen to Rotterdam and to test patient and control immune cells in the same series of experiments.

Isolation of monocytes

CD14 positive monocytes were isolated from frozen PBMCs from selected subjects (Table 1) by magnetic cell sorting system (Miltenyi Biotec). The viability and purity of monocytes was >95% (determined by morphological screening after trypan blue staining and flow cytometry analysis).

Quantitative-PCR (Q-PCR)

RNA was isolated from monocytes using RNeasy columns (Qiagen, Hilden, Germany). After extraction, the RNA concentration was determined and RNA was stored at -80°C until use.

To obtain cDNA for the Q-PCR, we used the optimized extensively described BIOMED-1 protocol.[13] One μg RNA was reversed transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham Biosciences, Roosendaal, The Netherlands) for 50 min at 42°C.

Q-PCR was performed with Taqman Universal PCR mastermix (Applied Biosystems, Foster City, CA). All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand, see Appendix 1). PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR amplification of the reference gene *ABL* was performed for each sample to allow normalization between samples. *ABL* was chosen as the reference gene because it was previously shown that *ABL* was the most consistently expressed endogenous control in hematopoietic cells.[14] The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene-CT reference gene), by the ΔΔCT method ($2^{-\Delta\Delta CT}$,

User Bulletin 2, Applied Biosystems, Foster City, California). To correct for inter-assay variance we set the mean of the studied genes found in the healthy control index twins in the same assay for each gene to 1 ($\sum\Delta CT$ HC index twins= 0, 2^{-0}=1). The fold change values of the genes in patient monocytes were expressed relative to this set mean of 1 of the healthy controls. These fold change values were used in the statistical methods described below.

Statistical analysis

For the structural equation modeling (SEM) approach a statistical package Mx was used for analysis[15] and bivariate twin models were set up to describe the data. In these models, the variance of a certain trait (such as signature positivity) or the covariance between two traits (such as signature positivity and bipolar disorder) can be due to additive genetic (A), common environmental (C, such as socio-economic status and childhood diet) and/or unique environmental influences (E , e.g. accidents) (See Figure 1).

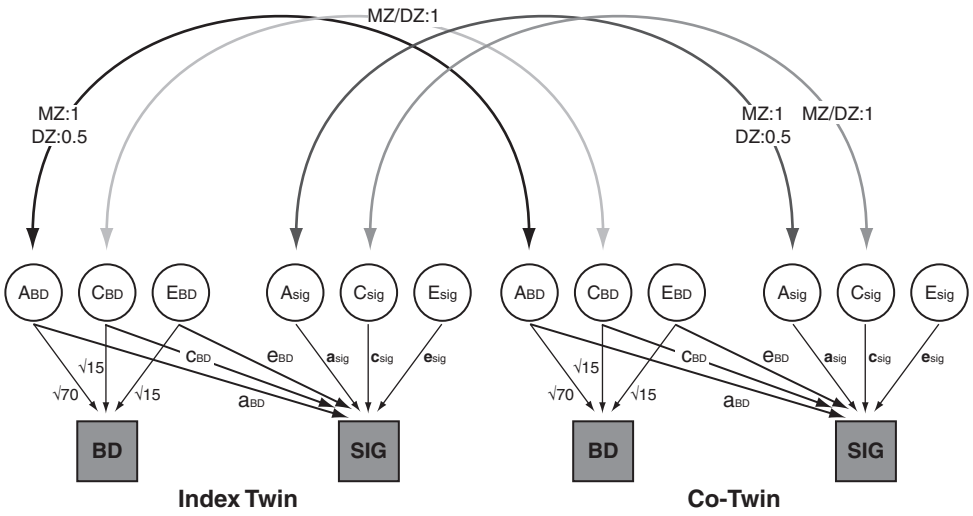

Figure 1. Path diagram of a bivariate twin model (Cholesky decomposition).
Bipolar Disorder (BD, yes or no) and monocyte signature expression (sig, 5 point scale) of index twin and co-twin are influenced by additive genetic (A), common environmental (C) and unique environmental (E) non-observed latent factors. One set of factors loads on both observed traits, the other set loads on signature expression only. The magnitude of the effects of A, C and E on BD (reflected in the factor loadings) are fixed at population values so that h^2, c^2 and e^2 are 70%, 15% and 15% respectively. Factor loadings of the two sets of latent factors on signature expression are estimated (a_{BD}, c_{BD} and e_{BD} for the factors that load on both traits, and a_{SIG}, c_{SIG} and e_{SIG} for the factors that load on monocyte signature expression only). A strong genetic correlation is reflected in a large a_{BD} compared to a_{SIG} and vice versa (same for common environmental and unique environmental influences). The 6 different parameters can be estimated because the latent factors show a distinctive pattern of correlations among each other: additive genetic factors between twins are correlated 1 for MZ twins and 0.5 for DZ twins, while common environmental factors correlate 1 for both types of twin pairs. Unique environmental factors are by definition non correlated.

Identical (MZ) twins share all of their genes, therefore they correlate 1 for additive genetic effects, whereas non-identical twins (DZ) share on average only half of their genes and thus correlate 0.5 for the additive genetic effects. However, with regard to the shared environment both MZ and DZ twin pairs correlate 1 as for example parenting, exposure to infections or diet is in most cases experienced the same for both MZ and DZ twins living together. In contrast, the correlations between unique environmental influences are modeled as zero since they are by definition not shared between twins. This means that if a higher correlation for a certain trait is found in MZ twins than in DZ twins it must be due to genetic effects (since the correlation for environmental influences is the same in DZ and MZ twins) but if in MZ and DZ twin pairs the same correlation (but different from zero) is found than it must be due to shared environment.

Bivariate twin modeling was used to estimate the magnitude of the effects of A, C and E on signature positivity and its interdependence with bipolar disorder, which is expressed in parameter estimates a, c and e respectively. The relative contribution of A to the variance of signature positivity (univariate heritability) and to the covariance between bipolar disorder liability and signature positivity (bivariate heritability) was expressed as a percentage genetic variance over total (co) variance. The univariate (narrow sense) heritability is thus given by $h^2_{univariate}$ = Var (A)/ (Var (A) + Var (C) + Var (E) and the bivariate heritability by $h^2_{bivariate}$ = ICov (A)I / (ICov (A)I + ICov (C)I + ICov (E)I). In a similar way univariate and bivariate c^2 and e^2 are defined.

Prior to structural equation modeling, various correlations were determined in MZ and DZ twins: 1) cross-trait/ within-twin correlations, the so-called trait-correlations (so in our case bipolar disorder with signature positivity within the same subject), 2) within-trait/ cross-twin correlations, the so-called twin-correlations (signature positivity index twin with that of their co-twin, for MZ and DZ twin pairs), and 3) cross-trait/ cross-twin correlations, the so-called cross-correlations (bipolar disorder of index twin with signature positivity of their co-twin and vice versa, for MZ and DZ twin pairs). A significant trait-correlation suggests a common etiological influence on both traits. Significant twin- or cross-correlations imply that this common influence is familial. And MZ/DZ ratios of 2:1 of the twin-or cross-correlations suggest this influence to be due to genetic effects (A) while ratios of 1:1 imply the effect of shared environment (C). A non-significant cross-correlation combined with a significant trait-correlation means that the common etiological influence on both traits is probably due to a unique environmental influence (E). Using the information from these correlations the phenotypic correlation (r_{ph}) between two traits (bipolar disorder and signature positivity) can be determined and decomposed into genetic (r_g), common environmental (r_c) and unique environmental (r_e) components. The r_g indicates to which degree the same genes influence both bipolar disorder and signature positivity, the r_c indicates to which degree the same shared environmental factors influence both bipolar disorder and signature positivity, and the r_e provides information about the possible effect of unique environmental factors on both traits. By combining information from the r_g, r_c and r_e correlations with the a^2, c^2 and e^2, the influence of genetic,

common and unique environmental factors to the total correlation between bipolar disorder and signature positivity could be established.

Ninety-five percent confidence intervals (95% CI) of the parameter estimates were calculated and used to draw conclusions.[16]: the parameter estimate can be fixed at all the values in the confidence interval without resulting in a significantly worse fit of the model (at α= 0.05).

For these statistical analysis liability threshold models were used meaning that both affected and unaffected individuals have a liability to develop bipolar disorder and that if a certain threshold was crossed a person has bipolar disorder and if not the person is considered healthy. However, since twin pairs in our sample were specifically selected on bipolar disorder, we could not estimate h^2, c^2 and prevalence on this sample. Hence, parameters were fixed to values which agree with the literature (h^2= 70%, c^2=15%, prevalence= 1%).[2, 17, 18] We allowed for common environmental influences on bipolar disorder to be able to detect a common environmentally mediated association between signature positivity and bipolar disorder. Fixing h^2, c^2 and prevalence to different values (h^2= 80% or 60%, c^2= 25 or 5%, prevalence= 0.5%) did not alter conclusions, although parameter estimates were slightly different (results on request).

The Mx program cannot process dichotomous and continuous data together; therefore the monocyte gene expression levels were categorized into a 5 category ordinal scale (approximately 20% per category).

RESULTS

Association of bipolar disorder with signature positivity

Column A of Table 3 shows that the cross-trait/within-twin correlations (the "trait-correlations") of the monocyte pro-inflammatory signature are significant, thus bipolar disorder is associated with the presence of the monocyte pro-inflammatory signature in this twin sample confirming our previous results in singletons.[4] The signature is present -depending on definition- in 20-41% of the bipolar index cases (Table 4).

The contribution of genes and environmental factors on signature positivity

A familial effect on the presence of pro-inflammatory monocytes is indicated by the significance of the within trait/ cross twin correlations ("twin-correlations", Table 3, column B). This familial effect is most likely mainly due to shared environmental factors, which is suggested by the MZ and DZ ratio of the twin-correlations, which approximates one (Table 3, column B). The ACE modeling data presented in column A of Table 5 also demonstrate that the variance of signature positivity is probably not caused by genetic effects (as h^2 =0%, 95% CI: 0-44%), but is most likely due to shared environmental influences (c^2=95%, 95% CI: 53-99%).

Table 3. Cross trait/ within twin correlations ("trait-correlations"), within-trait/cross-twin correlations ("twin-correlations"', irrespective of disease) and cross-trait/cross-twin correlations ("cross-correlations", associated with bipolar disease) on signature positivity and mRNA expression.

	Column A	Column B		Column C	
	Cross-trait correlation (95% CI)	Within-trait/ cross-twin correlation (95% CI)		Cross-trait/ cross-twin correlation (95% CI)	
		MZ (36 twin pairs)	DZ (39 twin pairs)	MZ (36 twin pairs)	DZ (39 twin pairs)
Signature definition					
PDE4B positive + at least 50% of genes positive	.45 (.37 to .69)	.95 (.63 to .99)	.95 (.71 to .97)	.44 (.16 to .70)	.52 (.26 to .76)
mRNA expression					
1) Inflammation					
PDE4B	.16 (.04 to .27)	.85 (.69 to .92)	.88 (.76 to .94)	.16 (.04 to .28)	.14 (.01 to .28)
IL6	.27 (.15 to .37)	.85 (.69 to .93)	.90 (.77 to .95)	.20 (.08 to .33)	.21 (.08 to .33)
IL1B	.31 (.20 to .41)	.83 (.67 to .91)	.90 (.79 to .95)	.25 (.13 to .37)	.22 (.09 to .34)
PTX3	.22 (.11 to .33)	.77 (.56 to .88)	.89 (.78 to .94)	.24 (.11 to .36)	.14 (.01 to .27)
PTGS2	.28 (.16 to .38)	.62 (.36 to .78)	.66 (.40 to .80)	.22 (.08 to .34)	.26 (.11 to .39)
TNF	.22 (.10 to .33)	.82 (.65 to .91)	.84 (.68 to .92)	.20 (.07 to .32)	.19 (.06 to .32)
TNFAIP3	.28 (.17 to .39)	.81 (.63 to .90)	.80 (.63 to .89)	.21 (.08 to .33)	.15 (.02 to .28)
2) Chemokinesis/ motility					
CCL2	.25 (.13 to .36)	.69 (.46 to .82)	.58 (.29 to .76)	.23 (.09 to .35)	.10 (.00 to .24)
CCL7	.21 (.10 to .32)	.84 (.68 to .92)	.72 (.52 to .84)	.24 (.11 to .35)	.15 (.01 to .29)
CCL20	.29 (.17 to .39)	.82 (.65 to .91)	.88 (.76 to .94)	.25 (.12 to .37)	.20 (.08 to .33)
CXCL2	.25 (.14 to .36)	.84 (.69 to .91)	.76 (.57 to .87)	.27 (.14 to .39)	.22 (.08 to .35)
CCR2	-.14 (-.27 to -.02)	.48 (.01 to .74)	.54 (.24 to .73)	-.26 (-.42 to -.07)	-.19 (-.33 to -.04)
CDC42	.16 (.04 to .27)	.62 (.41 to .78)	.58 (.30 to .75)	.12 (-.01 to .26) ns	.05 (-.09 to .20) ns
3) Cell survival/ apoptosis					
BCL2A1	.24 (.12 to .34)	.58 (.29 to .76)	.71 (.49 to .84)	.19 (.05 to .32)	.23 (.09 to .37)
EMP1	.14 (.03 to .25)	.71 (.48 to .84)	.64 (.37 to .79)	.10 (-.04 to .23) ns	.04 (-.10 to .18) ns
4) Mapk pathway					
DUSP2	.19 (.08 to .30)	.53 (.26 to .72)	.51 (.13 to .72)	.14 (.00 to .28)	.17 (.02 to .32)
ATF3	.22 (.11 to .33)	.54 (.24 to .72)	.53 (.22 to .72)	.14 (.00 to .28)	.19 (.04 to .34)
NAB2	.00 (-.12 to .11) ns	.62 (.35 to .78)	.74 (.54 to .86)	-.08 (-.21 to .05) ns	-.09 (-.22 to .05) ns
MAPK6	.33 (.22 to .44)	.90 (.79 to .95)	.85 (.72 to .92)	.33 (.21 to .44)	.25 (.12 to .37)

If 95% confidence interval does not include zero, the correlation is significant.
ns= non significant

Table 4. The presence of a *PDE4B*-associated mRNA signature in bipolar index twins and healthy twin pairs.

Signature definitions[1]	HC[2]	MZ and DZ BD Index twins	
	Total *n*=68	Total *n*=41	
	n (%)	*n* (%)	*p*[4]
PDE4B positive[3]	13 (19)	17 (41)	*.011*
+ at least 25% of genes positive	12 (18)	17 (41)	*.006*
+ at least 50% of genes positive	2 (3)	13 (32)	*<.001*
+ at least 75% of genes positive	2 (3)	8 (20)	*.004*

[1] Various definitions are given depending on the percentage positives of the 19 genes that had been found statistically differentially expressed in BP as compared to HC (see Padmos *et al*.[4], and Table 3).
[2] Healthy index twins as well as their co-twins were included in this analysis.
[3] Positivity of the genes is defined as an mRNA expression +1 SD higher than the mean level found in the healthy controls. PDE4B is positive in all definitions.
[4] Chi-Squared test: Bipolar index twins versus HC.
MZ, Monozygotic; DZ, Dizygotic; BD, Bipolar Disorder, HC, Healthy Controls

The contribution of genes and environmental factors on the association of bipolar disorder with signature positivity

Also for the presence of pro-inflammatory monocytes in association with bipolar disorder a familial effect is indicated by the significance of the cross-trait/ cross-twin correlations ("cross-correlations", Table 3, column C). This familial effect is again probably the result of mainly shared environmental factors, which is suggested by the MZ and DZ ratio of the cross-correlations, which again approximates one (Table 3, column C). The ACE modeling data confirms this large contribution of common environment to the covariance between bipolar disorder and signature positivity as it is shown in Table 5 (column C) that C must be at least for 45% responsible for the association of signature positivity with bipolar disorder. This means that similar shared/common environmental factors influence the co-occurrence of bipolar disorder and signature expression.

Association of bipolar disorder with the mRNA expression of the separate signature genes

The analysis of the separate signature genes confirmed the above-described findings. Column A of Table 3 shows that bipolar disorder is associated with aberrant mRNA expression levels of almost all genes tested (again because of the significance of the trait-correlations).

Table 5. Estimated influences of additive genetic (h^2), common (c^2) and unique (e^2) environmental factors on monocyte gene expression signature (column A) and the association monocyte gene expression signature-bipolar disorder (Column B).

	Column A			Column B	Column C		
	Sources of variance on signature positivity/ mRNA expression irrespective of disease			Phenotypic correlation	Sources of covariance between bipolar disease and signature positivity/ mRNA expression		
	h^2 (%) (95% CI)	c^2 (%) (95% CI)	e^2 (%) (95% CI)	rtotal=(r_{ph}) (95% CI)	h^2_{biv} (%) (95% CI)	c^2_{biv} (%) (95% CI)	e^2_{biv} (%) (95% CI)
Signature definition							
PDE4B positive + at least 50% of genes positive	0 (0 to 44)	95 (53 to 99)*	5 (1 to 21)*	.36 (.15 to .56)*	1 (0 to 50)	94 (45 to 100)*	5 (0 to 24)
mRNA expression							
1) Inflammation							
PDE4B	0 (0 to 24)	87 (64 to 92)*	13 (7 to 23)*	.16 (.00 to .31)	15 (0 to 100)	84 (0 to 100)	2 (0 to 48)
IL1B	1 (0 to 19)	86 (67 to 92)*	13 (7 to 23)*	.31 (.15 to .45)*	21 (0 to 98)	61 (0 to 98)	17 (0 to 48)
IL6	0 (0 to 24)	87 (64 to 93)*	13 (6 to 23)*	.27 (.11 to .41)*	0 (0 to 36)	78 (0 to 100)	22 (0 to 61)
PTX3	0 (0 to 34)	81 (64 to 90)*	16 (9 to 27)*	.22 (.06 to .37)*	56 (0 to 100)	39 (0 to 100)	5 (0 to 28)
PTGS2	0 (0 to 46)	64 (23 to 78)*	35 (21 to 53)*	.27 (.12 to .41)*	10 (0 to 37)	74 (45 to 100)*	15 (0 to 50)
TNF	2 (0 to 20)	83 (51 to 91)*	17 (8 to 29)*	.22 (.06 to .37)*	4 (0 to 100)	85 (0 to 100)	11 (0 to 56)
TNFAIP3	3 (0 to 42)	79 (43 to 88)*	19 (9 to 31)*	.28 (.12 to .43)*	41(0 to 100)	33 (0 to 74)	26 (0 to 73)
2) Chemokinesis/ motility							
CCL2	22 (0 to 80)	47 (0 to 74)	31 (16 to 52)*	.25 (.09 to .40)*	83 (0 to 100)	11 (0 to 41)	7 (0 to 58)
CCL7	23 (0 to 68)	61 (18 to 81)*	16 (8 to 33)*	.21 (.05 to .36)*	62 (0 to 100)	28 (0 to 100)	10 (0 to 32)
CCL20	1 (0 to 23)	84 (63 to 91)*	15 (8 to 25)*	.28 (.13 to .43)*	28 (0 to 100)	59 (0 to 100)	13 (0 to 35)
CXCL2	15 (0 to 57)	69 (28 to 87)*	16 (8 to 31)*	.25 (.09 to .40)*	37 (0 to 100)	56 (0 to 100)	8 (0 to 28)
CCR2	2 (0 to 67)	50 (0 to 70)	47 (25 to 73)*	-.14 (-.32 to .04)	35 (0 to 94)	34 (0 to 100)	30 (0 to 60)
CDC42	7 (0 to 73)	55 (0 to 73)	38 (21 to 59)*	.16 (.00 to .31)	74 (0 to 100)	9 (0 to 50)	17 (0 to 100)
3) Cell survival/ apoptosis							
BCL2A1	0 (0 to 34)	65 (34 to 78)*	34 (21 to 52)*	.24 (.08 to .37)*	17 (0 to 41)	72 (43 to 100)*	11 (0 to 51)
EMP1	16 (0 to 75)	56 (1 to 79)*	29 (15 to 49)*	.14 (-.02 to .30)	67 (0 to 100)	9 (0 to 61)	25 (0 to 100)
4) Mapk pathway							
DUSP2	5 (0 to 66)	48 (0 to 69)	47 (28 to 68)*	.19 (.04 to .33)*	19 (0 to 45)	66 (0 to 100)	16 (0 to 95)
ATF3	1 (0 to 62)	53 (2 to 70)*	46 (26 to 66)*	.22 (.07 to .36)*	22 (0 to 43)	59 (0 to 100)	19 (0 to 83)
NAB2	0 (0 to 35)	68 (35 to 80)*	31 (19 to 48)*	-.01 (-.16 to .15)	14 (0 to 100)	51 (0 to 100)	35 (0 to 100)
MAPK6	11 (0 to 39)	79 (52 to 91)*	10 (4 to 21)*	.34 (.18 to .48)*	46 (0 to 100)	52 (0 to 65)	2 (0 to 23)

*Parameter estimate is significantly different from zero.

The contribution of genes and environmental factors on the expression of the separate signature genes

For most genes a familial effect was found on the expression of the separate signature genes (the twin-correlations were significant, Table 3, Column C) and -as for the entire signature- this effect was primarily due to common environment (MZ and DZ twin correlations approximate one, Table 3, Column B).

Also the separate signature gene expressions were fitted into an ACE model. The estimated heritabilities (h^2) and environmental variances (c^2 and e^2) of the gene expression levels indicate that the variance of gene expression is for almost all genes mostly explained by common environmental influences (Column A). The additive genetic effect does not seem to explain any of the variance of the gene expression levels.

The contribution of genes and environmental factors on the association of BD with the mRNA expression of the separate signature genes

The familial effect seen on the association of bipolar disorder and most inflammatory signature genes is suggested to be shared environment as MZ and DZ cross-correlation ratios approximate one (Table 3).

However, it must be noted that for some of the motility/ chemotaxis/ cell survival genes *(i.e CCL2*, *CCL7*, *EMP1* and *CDC42*) the MZ cross-correlations are higher than the DZ cross-correlations (approximating a 2:1 ratio), suggesting that genetic effects influence the association of those particular genes with bipolar disorder.

The influence of h^2,c^2 and e^2 on the covariance of gene expression with bipolar disorder is given in Table 5 (column C). For most genes it is demonstrated that h^2 and/or c^2 must explain some of the covariance since the 95% CI of e^2 never reaches 100%. However, it is not possible to distinguish between h^2 effects and c^2 effects since for most genes the 95% CI of both effects reaches from 0 till 100. Therefore, more information can be obtained from the analysis of signature positivity described above, in which separate gene expression levels are combined into a signature.

DISCUSSION

This study points to common environmental factors as the main contributing factors to the inflammatory state of monocytes. By applying structural equation modelling we demonstrated (by univariate analysis) that of the total variance for signature positivity 95% (95% CI: 53-99%) could be explained by shared environmental factors.

In addition this study demonstrates that common environmental factors are also the main contributing factors to the association of the pro-inflammatory monocytes with bipolar disorder. By using a bivariate model it was shown that of the total covariance between bipolar disorder and signature positivity 94% (95% CI=45-100%) was due to shared common environment and not to genetic effects (h^2=1%, 95% CI=0-50%) or unique environmental factors (e^2=5%, 95% CI=0-24%).

Theoretically, the correlation between bipolar disorder and pro-inflammatory monocytes can be explained in three ways. First, bipolar disorder itself induces the pro-inflammatory state of monocytes (e.g. the stressful state of the illness might induce monocyte activation). Second, the pro-inflammatory monocytes cause mood disorders in patients (the "macrophage theory of depression"). And third, there could be a separate underlying factor that influences bipolar disorder as well as monocytes independently from each other (e.g. patients and their family members are present in an infectious/ stressful environment which affects their monocyte system as well as their brain). Because our data demonstrate that the pro-inflammatory state of the monocytes is mainly explained by a shared environmental factor, while it is known from literature that bipolar disorder itself is heritable for 60-85%, we can use the patterns of cross-twin/ cross-trait correlations ("cross-correlations") to falsify some of these theoretical explanations[19]: if bipolar disorder itself would be responsible for the monocyte activation the observed MZ/DZ cross-correlation ratios should approximate 0.5 since genes are very important for bipolar disorder itself (and not for signature expression) and therefore, since DZ share only half of their genes, their observed correlation should be less than in MZ twins. However, in our study the MZ/DZ cross-correlation ratios approximate 1. It is therefore reasonable to conclude that bipolar disorder is not a causative factor for monocyte activation. Regarding the other two explanations (whether pro-inflammatory monocytes cause bipolar disorder or whether a separate underlying factor influences both bipolar disorder as well as the monocyte) our data do not provide a solution.

It must be noted that this strong effect of common environment did not apply for all genes in the signature. Some of the motility/ chemotaxis/ cell survival genes (i.e. *CCL2, CCL7*, *EMP1* and *CDC42*) were clearly influenced by genetic factors and interestingly these genes have been found by us in a previous study[20] to belong to a separate sub-cluster of motility/chemotaxis and adhesion genes, which are -although weaker- also correlated to the core inflammatory genes (such as PDE4B, the cytokines and inflammatory compounds). It is tempting to speculate that the expression of this sub-cluster of genes in monocytes is more under the control of genetic factors and that common environmental factors play a minor role.

Our study has limitations that needs considering. First, since a shared common environment is such an important factor in explaining variance of signature expression, the question arises whether our experimental set up could be the shared environmental factor, and in this way creates biased results. However, although for most twin pairs blood drawing, blood processing and experiments were done on the same time ("the putative common shared environmental factor"), we also had twin pairs that were handled separately in each step. Also in these twin pairs the same high concordance rates were observed in both the MZ and DZ twin pairs. Also, by examining data from only one experiment (so excluding experiment as a confounding factor) the same results were seen. We are therefore confident that the observed data are indeed not caused by our experimental set up but by other shared environmental factors.

Second, although our bipolar disorder twin sample is, as far as we know, the largest twin sample studied to date, the sample is still not that large, that definite conclusions can be drawn.

So which shared environmental factors have been reported to be associated with both bipolar disorder and pro-inflammatory monocyte activation? To begin with, infections are good candidates. Studies have been reported that being born or raised in a city is a risk factor for developing bipolar disorder. This is thought to be due to household crowding and the consequent high exposure to infectious agents.[21] The viruses mentioned in the literature as being associated with bipolar disorder are Herpes Simplex virus (HSV), Cytomegalovirus (CMV) and Borna virus.[22-24] However, data are very inconsistent with regard to the presence of virus specific antibodies in serum as well as with regard to detecting virus RNA in brain or in peripheral blood mononuclear cells of bipolar patients.[22-24] As for other infections, Toxoplasma gondii, an intracellular protozoan parasite, is capable of latency and brain infiltration[25] and is therefore an interesting candidate for further study. In schizophrenia various investigations show a correlation with Toxoplasmosis infection,[26] especially with prenatal exposure. Indeed the period of time in which twins have the best chance to be exposed to the same pathogens is before the age of 20 years and especially in uteri. Prenatal infections mentioned to correlate with psychiatric diseases include besides toxoplasmosis, influenza,[27] rubella, and HSV.[28]

One of the next possible environmental factors that is experienced in uteri and can influence both the immune system as well as the brain, is prenatally experienced stress. Increasing amounts of evidence suggest that exposure to prenatal stress is a risk factor for psychopathology. Prenatally stressed rats show higher emotional reactivity, higher levels of anxiety and depressive-like behaviour.[29] In humans, low birth weight is considered an index of prenatal stress, and indeed low birth weight has been shown a risk factor for later development of mood symptoms[30] and even bipolar disorder.[31] Also, the amount of stress experienced by the mother during pregnancy was positively correlated to emotional, cognitive and behavioural problems of the offspring.[32] It is suggested that stress exposure at critical time points during fetal development may 1) influence the hypothalamic-pituitary-adrenal axis, leading to glucocorticoid resistance (which might play a role in immune activation) and hypercortisolism, 2) alter brain development, and 3) change neurotransmitter systems, all events as a result of high maternal cortisol levels.[29, 32-34]

Apart from infections and stress, diet and in particular the lack of consumption of sufficient omega-3 fatty acids is a third possible shared environmental factor linking bipolar disorder with the immune system.[35]

In conclusion our twin study shows that common environmental factors are most likely responsible for the pro-inflammatory monocyte activation seen in bipolar disorder. Chronic infection, stress and an insufficient diet are good candidates to act as such common environmental factors. When acting on a susceptible genetic background the environmentally induced pro-inflammatory monocyte activation can be seen as a factor precipitating disease.

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V

Inflammatory Monocytes, the Shared Vulnerability Factor for Bipolar Disorder and Autoimmune Thyroid Disease?

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ABSTRACT

Context In support of the "Macrophage-T cell theory of mood disorders" we recently described a coherent aberrant expression of 19 inflammatory genes (an "inflammation signature") in the monocytes of bipolar patients. Apart from having various signs of low-grade inflammation, patients with bipolar disorder (BD), their children and their monozygotic co-twins are also frequently affected by autoimmune thyroid disease (AITD), in children and twins often in the absence of mood symptoms, suggesting the existence of a shared vulnerability factor for BD and AITD.

Objective To study whether the monocyte inflammatory state as found in BD (and reflected in the inflammation signature) is the shared vulnerability factor, since inflammatory monocytes and their descendent cells have also been implicated in the pathogenesis of AITD.

Design Q-PCR analysis for 22 inflammatory genes in monocytes of 42 BD, 35 AITD patients and 38 healthy controls (HC). The tested 22 inflammatory genes were the 19 genes previously found in BD plus 3 "extra" genes found in whole genome analysis of monocytes of AITD patients (this paper).

Results The specific pro-inflammatory state of monocytes of BD (as evidenced by the presence of a the coherent set of inflammatory genes) was not only found in up to 60% (18/42) of bipolar patients, but also in 43% (15/35) of AITD patients. However signature positivity in BD patients was not correlated with the co-occurrence of AITD.

Conclusion and discussion The monocytes of a considerable proportion of bipolar and AITD patients share an aberrant monocyte inflammatory gene profile, however this pro-inflammatory state can not be considered as the shared vulnerability factor for BD and AITD.

INTRODUCTION

Bipolar disorder (BD) is a complex disorder in which the core feature is pathological disturbance in mood ranging from extreme elation (mania) to severe depression. BD patients and their offspring have a raised prevalence of autoimmune thyroiditis,[1, 2] in the offspring even in the absence of a mood disorder. Also monozygotic non- bipolar co-twins of a bipolar index twin, show a raised prevalence of autoimmune thyroiditis.[3] Both these offspring and twin data refute the concept that the mood symptoms and the thyroid autoimmune response are cause or consequences of each other. They indicate that relatives of BD patients not only inherit the susceptibility to develop mood disorders,[4] but also the susceptibility to develop autoimmune thyroiditis independently from mood disorders.

Recently, we reported the presence of a pro-inflammatory state of circulating monocytes in a large proportion of BD patients and we detected the expression of a coherent set of 19 inflammation- or inflammation-related genes ("a mRNA signature") in the monocytes of up to 60% of the patients, depending on the signature definition.[5] We hypothesized that this pro-inflammatory state of monocytes and monocyte-derived cells constitutes the shared genetic susceptibility factor for BD and thyroid autoimmunity, since pro-inflammatory monocytes and monocyte-derived cells are thought to be involved in the pathogenesis of both disorders.

We here report outcomes of a search for the aberrantly expressed 19 "bipolar signature genes" in the circulating monocytes of 35 patients with autoimmune thyroid disease (AITD).

We in addition focused on finding new inflammatory genes abnormally expressed in monocytes of AITD patients. We used Affymetrix whole genome expression profiling to identify aberrantly expressed genes in the monocytes of patients with AITD and found 3 extra genes. The 3 extra found genes were also validated via Q-PCR analysis in the circulating monocytes of the AITD patients and also in the monocytes of the former series of BD patients and healthy controls.

MATERIAL AND METHODS

Patients and healthy controls

Bipolar patients

Outpatients with DSM-IV bipolar I or II disorder were recruited from two studies, i.e. the Dutch site of the Stanley Foundation Bipolar Network (SFBN), an international multi-center research program described elsewhere in detail[6, 7] (n=19) and from an ongoing Dutch twin study on bipolar disorder described in detail in Vonk *et al.*,[3] (*n*=23 index cases). A DSM-IV diagnosis of bipolar disorder was made by means of the Structured Clinical Interview for DSM-IV Axis I (SCID).

Table 1. Characteristics of bipolar patients, AITD Patients and healthy controls used for RQ-PCR, and IL-1β, IL-6 ELISAs.

		Bipolar patients	Healthy controls[1]	AITD patients	Healthy controls[1]
Group size		42	25	18 HT	28
				17 GD	
Age (years)[2]		42 (39-45)	40 (36-44)	40 (22-67)	40 (21-54)
Gender	Male	16 (38%)	11 (44%)	8 (23%)	5 (18%)
	Female	26 (62%)	14 (56%)	27 (77%)	23 (82%)
Duration of illness (years)[2]		16 (13-19)		Newly diagnosed	
Age of onset disease (years)[2]		25 (22-28)			
Mood	Euthymic	26 (62%)			
	Depressed	7 (17%)			
	Manic	7 (17%)			
	Unknown	2 (5%)			

[1] For the IL-1β, IL-6 ELISAs both control groups were combined.
[2] Mean (range).
HT = Hashimoto's thyroiditis, GD = Graves' disease, AITD = Autoimmune thyroid disease.

Autoimmune hypothyroid and hyperthyroid patients

Newly diagnosed hypothyroid Hashimoto's thyroiditis (HT) and hyperthyroid Graves' disease patients (together indicated as autoimmune thyroid disease (AITD) patients), who visited the outpatient clinic of the Department of Internal Medicine, Medical Center Rijnmond Zuid, Rotterdam, The Netherlands, were recruited. All patients with HT were clinically hypothyroid, had a lowered serum free thyroxin level (fT4, cut off levels:10.0-24.0 pmol/L) and a raised thyroid stimulating hormone level (TSH, cut off levels: 0.4-4.0 mU/L), and were positive for thyroid peroxidase antibodies (TPO-abs, cut off level: 35 IU/ml, Immulite, DPC, Breda, The Netherlands). TSH-receptor antibodies were negative (cut off level: 1.5 IU/L Brahms Diagnostics, Berlin, Germany). The hyperthyroid Graves' patients all had a raised fT4 level and a decreased TSH level. They also had a diffuse non-nodular appearance of thyroid on palpation, scan or ultrasound. In addition, patients were positive for TSH-receptor antibodies. None of the newly diagnosed HT and Graves' patients received medication (also no thyroid medication) at the time of blood collection. Of all patients serum and immune cells were collected.

Healthy controls

Age and gender matched healthy controls (HC) were recruited via enrolling laboratory staff, medical staff and medical students. The inclusion criteria for the HC were an absence of any thyroid and autoimmune disorder and an absent history of these disorders in first-degree family members. HC had to be in self-proclaimed good health and free of any obvious medical illness for at least two weeks prior to the blood withdrawal, including acute infections and allergic reactions.

The Medical Ethical Review Committee of the Erasmus MC Rotterdam approved the studies. Written informed consent was obtained from all participants after a complete description of the study was given.

Characteristics of both patients and HC are given in Table 1 (Q-PCR and IL-1β/IL-6 ELISAs) and in the legend of Figure 2 (CCL2/ CCL7 ELISAs).

Laboratory methods

Blood collection and preparation

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for immune cell preparation. From the heparinized blood peripheral blood mononuclear cell (PBMC) suspensions were prepared by low-density gradient centrifugation as described in detail before[8] within 8 hours to avoid activation of the monocytes. PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments.

Isolation of monocytes

CD14 positive monocytes were isolated from frozen PBMCs from selected subjects (Table 1) by magnetic cell sorting system (Miltenyi Biotec) The purity of monocytes was >95% (determined by morphological screening after trypan blue staining and FACS). As reported elsewhere positive versus negative selection of immune cells did not influence gene expression profiles.[9]

Affymetrix whole genome gene expression profiling

RNA was isolated from purified monocytes using RNAeasy columns as described by the manufacturer (Qiagen, Hilden, Germany) and previously.[10] Fragmented cRNA was hybridized to U95Av2 microarrays (Affymetrix, manufacturers protocol). For all experiments, the 5' / 3' ratios of GAPDH were 2 or less (usually 0.9 - 1.1).

Quantitative –PCR (Q-PCR)

RNA was isolated from monocytes as described above. To obtain cDNA for Q-PCR, we used the extensively described BIOMED-1 protocol.[11] One μg RNA was reversed transcribed using SuperscriptII (Invitrogen) and random hexamers (Amersham

Table 2. Q-PCR analysis of monocytes of BD patients, AITD patients compared to healthy controls. Fold change values.

	Fold change[1]					
	BP vs. HC[2]		HT vs. HC[3]		AITD vs. HC[3]	
Gene symbol	*n*=42	*p*[4]	*n*=18	*p*[4]	*n*=35	*p*[4]
1) Inflammation						
PDE4B	**4.92 (0.51-47.5)**	***<.001***	**2.63 (1.36-5.10)**	***.016***	**2.55 (1.57-4.13)**	***.007***
IL6	**14.40 (0.13-49.6)**	***.001***	**10.46 (2.53-43.15)**	***.006***	**11.65 (4.31-31.51)**	***.001***
Il1B	**5.82 (0.12-276)**	***.002***	3.33 (1.28-8.70)	*.068*	**3.23 (1.69-6.19)**	***.044***
PTX3	**4.56 (0.30-69.6)**	***<.001***	0.72 (0.22-2.35)	*.88*	1.04 (0.55-1.97)	*.67*
PTGS2	**3.14 (0.19-50.6)**	***.007***	2.45 (1.22-4.90)	*.063*	2.11 (1.32-3.37)	*.060*
TNF	**2.56 (0.13-49.6)**	***.01***	0.87 (0.43-1.78)	*.73*	1.21 (0.73-2.03)	*.84*
TNFAIP3	**3.17 (0.13-76.3)**	***.001***	2.12 (1.04-4.32)	*.12*	**2.20 (1.35-3.59)**	***.050***
FCAR	**3.01 (0.57-15.8)**	***<.001***	**1.85 (1.22-2.83)**	***.011***	**2.08 (1.56-2.77)**	***.001***
ADAM17	1.32 (0.39-4.54)	*.09*	1.35 (1.02-1.78)	*.11*	**1.43 (1.18-1.73)**	***.014***
EIF2S3	1.20 (0.49-2.91)	*.36*	1.05 (0.84-1.31)	*.49*	**1.22 (1.07-1.38)**	***.021***
2) Chemokinesis/ motility						
CCL7	**13.78 (0.04-4970)**	***.001***	**12.27 (2.66-56.67)**	***.001***	**11.91 (4.40-32.25)**	***<.001***
CCL20	**11.66 (0.03-5333)**	***.018***	4.44 (0.80-24.72)	*.23*	3.58 (1.17-10.96)	*.27*
CXCL2	**6.36 (0.15-272)**	***.001***	**4.56 (1.87-11.13)**	***.017***	**4.16 (2.23-7.74)**	***.006***
CCL2	**4.93 (0.06-436)**	***.004***	1.65 (0.61-4.44)	*.40*	2.12 (1.17-3.86)	*.059*
CDC42	**2.56 (0.64-10.2)**	***<.001***	**2.49 (1.57-3.96)**	***.002***	**2.63 (1.97-3.52)**	***<.001***
CCR2	**0.43 (0.05-3.48)**	***.010***	0.66 (0.43-1.00)	*.11*	0.91 (0.67-1.23)	*.85*
3) Cell survival/ apoptosis						
BCL2A1	**2.48 (0.25-24.3)**	***.001***	**2.34 (1.38-3.96)**	***.010***	**2.33 (1.58-3.42)**	***.006***
EMP1	**2.81 (0.18-43.8)**	***.005***	**1.80 (1.07-3.04)**	***.042***	**2.75 (1.87-4.05)**	***<.001***
4) Mapk pathway						
DUSP2	**4.85 (0.42-56.6)**	***<.001***	3.13 (1.46-6.69)	*.069*	**2.94 (1.69-5.12)**	***.040***
ATF3	**4.32 (0.80-23.3)**	***<.001***	**3.09 (1.94-4.90)**	***.005***	**3.31 (2.40-4.56)**	***<.001***
NAB2	**3.27 (0.12-87.6)**	***.005***	1.56 (0.95-2.55)	*.070*	**1.72 (1.22-2.43)**	***.015***
MAPK6	**2.47 (0.40-15.4)**	***<.001***	**2.21 (1.41-3.47)**	***.002***	**2.34 (1.72-3.19)**	***<.001***

[1] The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values (CT gene- CT reference gene *ABL*), by the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, Applied Biosystems, Foster City, California). Data were standardized to the HC (thus the HC were used as the calibrator). The fold change of the HC is therefore 1.

[2] Values >1: patients have a higher expression than control group.
Values<1: patients have a lower expression than control group.

[2] Healthy controls: *n*=25

[3] Healty controls: *n*=28

[4] Groups were compared using the Mann-Whitney test

BP, bipolar patients; HC, healthy controls; HT, Hashimoto's thyroiditis; AITD, autoimmune thyroid disease.

Biosciences, Roosendaal, The Netherlands) for 50 min at 42°C.

Q-PCR was performed with Taqman Universal PCR mastermix (Applied Biosystems, Foster City, CA). All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand, Appendix 1). PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and finally 1 min at 60°C. PCR amplification of housekeeping gene *ABL* was performed for each sample to allow normalization between the samples. *ABL* was chosen as housekeeping gene because it was previously shown that *ABL* was the most consistently expressed housekeeping gene in haematopoietic cells.[12] The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values (CT gene- CT housekeeping gene), by the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, Applied Biosystems, Foster City, California, see Table 2). To correct for inter-assay variance we set the mean of the studied genes found in the healthy control groups in the same assay for each gene to 1 (∑ΔCT HC=0, 2^{-0}=1). The fold change values of the genes in patient monocytes were expressed relative to this set mean of 1 (Table 2).

ELISAs

The CCL2, CCL7, IL1-β, and IL-6 levels in sera of the various patient groups were determined via commercially available ELISAs (R&D Systems, Minneapolis MN, USA) used according to the manufacturers protocol.

Statistical Analysis

Scanned micro array images were analyzed using Affymetrix Microarray Suite 4.2 software. Further analysis was performed using Rosetta Resolver (www.rosettabio.com) software and Ingenuity Systems (www.ingenuity.com) software. Statistical analysis was performed using the SPSS 11.0 package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Depending on the distribution pattern and the total number of subjects, parametric (normal distribution and ≥50 subjects) or non-parametric tests (skewed distribution or <50 subjects) were used. Levels of significance

were set at p=0.05 (2-tailed). The specific tests used are mentioned under tables and in legends.

RESULTS

Whole genome expression profiling of potential inflammatory biomarker genes in AITD monocytes and inclusion of genes previously found in monocytes of bipolar disorder

Affymetrix micro array analysis to search for aberrantly expressed genes in monocytes was performed on 2 pools of Hashimoto's thyroiditis (HT) patients, each pool to be compared to monocyte pools of age and gender-matched healthy controls (HC, pools were used for minimizing inter-individual differences in mRNA expression): Pool 1 consisted of 2 females with HT, ages 34 and 40 yrs, pool 2 consisted of 2 females, ages 49 and 54 yrs with HT, pool 1 and 2 were compared to a HC pool of 4 females, ages 33-46 yrs.

In Rosetta Resolver analysis of the expression data we took only molecules into consideration, which were more than 4 fold statistically significantly differentially expressed between patients and HC, with the purpose to identify strongly discriminating molecules. This resulted in 32 genes for HT patients (27 over- and 5 under-expressed). These genes were analyzed via Ingenuity pathway analysis software. Major functional networks/ pathways found to be involved were cell growth/differentiation, cell- to- cell interaction, and signaling and cell movement.

To select for genes which could serve as biomarkers for the monocyte inflammatory condition we took the genes in the upper or lower 33 percentile of the list, but with at least a 5 fold abnormal expression and of which the function is known and involved in inflammation or related processes such as cell movement, cell- to- cell interaction or signaling. This selection resulted in 3 genes *FCAR, EIF2S3* and *ADAM17* (all up-regulated, there were no discriminating negative genes). These 3 up-regulated genes were validated in Q-PCR in both AITD and bipolar patients in addition to the 19 genes previously found abnormally expressed in bipolar patients.[5]

Q-PCR analysis of monocytes of bipolar patients and thyroid autoimmune patients

Table 2 shows that of the 22 genes tested, the mRNA expression level of almost all genes was significantly different in the monocytes of the 42 patients with bipolar disorder as compared to HC, including *FCAR* (one of the new genes picked up in the whole genome screening in HT monocytes).

In the 18 HT patients tested 10 of the selected 22 genes were aberrantly expressed (Table 2). We decided to not only include patients with (hypothyroid) Hashimoto's thyroiditis, but also patients with (hyperthyroid) Graves' disease (GD), the other major autoimmune condition of the thyroid. This enabled us to investigate whether the thyroxine/ TSH status influenced the pro-inflammatory monocyte mRNA expression or whether

the aberrant pro-inflammatory mRNA expression was mainly due to the autoimmune condition per se. Therefore outcomes in the HT patients were compared to those of the GD patients. Virtually all of the mRNAs were similarly aberrantly expressed between these two autoimmune thyroid conditions and only 3 molecules were differentially expressed, these were *EMP1, CCR2* and *EIF2S3*. All were higher expressed in GD as compared to HT (*EMP1*: 4.21 vs. 1.80; *CCR2*: 1.26 vs. 0.66, *EIF2S3* 1.41 vs. 1.05, $p<0.05$ for all molecules, Mann Whitney test). *EMP1* and *EIF2S3* also showed a correlation with fT4 and T3 serum levels (fT4: *EMP1* r=0.41, *EIF2S3* r=0.27; T3: *EMP1* r=0.35, *EIF2S3* r=0.41, $p<0.05$ for all correlations), while *CCR2* did not show a correlation with T3, fT4 or TSH. Because of the similar aberrant expression levels of the various mRNAs in monocytes of HT and GD patients and their closely related immune-pathology, we analyzed the two clinical conditions together considering them as representing one autoimmune condition, i.e. autoimmune thyroid disease (AITD, Table 2). Doing so the statistical power increased and now 16 of the 22 aberrant "bipolar" mRNAs genes were also aberrantly expressed in monocytes of AITD patients except for the inflammatory compounds *TNF* and *PTX3* and the chemokine receptor *CCR2* (Table 2).

	PDE4B																			
DUSP2	0.83	*DUSP2*																		
ATF3	0.79	0.85	*ATF3*																	
TNFα	0.73	0.74	0.62	*TNFα*																
IL1	0.84	0.83	0.78	0.79	*IL1*															
IL6	0.63	0.53	0.50	0.47	0.73	*IL6*														
CXCL2	0.79	0.79	0.71	0.67	0.94	0.77	*CXCL2*													
CCL20	0.81	0.74	0.66	0.73	0.94	0.77	0.93	*CCL20*												
PTX3	0.79	0.71	0.73	0.70	0.85	0.81	0.83	0.87	*PTX3*											
PTGS2	0.85	0.84	0.79	0.73	0.87	0.62	0.79	0.82	0.79	*PTGS2*										
TNFAIP3	0.87	0.82	0.69	0.78	0.83	0.61	0.79	0.79	0.68	0.79	*TNFAIP3*									
BCL2A1	0.77	0.79	0.70	0.73	0.89	0.68	0.86	0.84	0.72	0.81	0.86	*BCL2A1*								
FCAR	0.74	0.70	0.75	0.60	0.77	0.66	0.76	0.73	0.82	0.78	0.67	0.77	*FCAR*							
CCL7	0.50	0.55	0.51	0.37	0.71	0.67	0.82	0.72	0.67	0.53	0.46	0.69	0.70	*CCL7*						
MAPK6	0.63	0.67	0.67	0.56	0.75	0.63	0.77	0.71	0.69	0.62	0.62	0.79	0.79	0.79	*MAPK6*					
CDC42	0.70	0.73	0.74	0.50	0.77	0.64	0.83	0.73	0.68	0.64	0.68	0.81	0.75	0.77	0.83	*CDC42*				
NAB2	0.31	0.46	0.38	0.26	0.35	0.23	0.45	0.29	0.43	0.28	0.33	0.38	0.55	0.60	0.56	0.50	*NAB2*			
CCL2	0.36	0.42	0.50	0.41	0.62	0.56	0.65	0.61	0.62	0.42	0.36	0.60	0.62	0.82	0.70	0.61	0.53	*CCL2*		
EMP1	0.37	0.38	0.50	0.16	0.44	0.47	0.55	0.45	0.55	0.34	0.27	0.36	0.65	0.71	0.57	0.55	0.58	0.68	*EMP1*	
CCR2	-0.34	-0.31	-0.25	-0.09	-0.32	-0.29	-0.47	-0.40	-0.45	-0.26	-0.13	-0.19	-0.28	-0.57	-0.37	-0.32	-0.33	-0.46	-0.42	*CCR2*
ADAM17	0.29	0.42	0.39	0.30	0.24	0.22	0.24	0.28	0.20	0.27	0.42	0.39	0.32	0.14	0.30	0.34	0.28	0.09	0.19	0.22	*ADAM17*
EIF2S3	0.20	0.22	0.32	0.21	0.28	0.30	0.27	0.29	0.37	0.23	0.33	0.47	0.55	0.34	0.51	0.52	0.27	0.32	0.36	0.33	0.40

Figure 1. Correlation of genes.
Correlation of mRNAs, data represent Pearson's correlation coefficients, tested on the relative mRNA expression of the molecules in 115 individuals (42 bipolar patients, 35 AITD patients and 53 healthy controls). Black represents: r>0.7, grey represents: r<0.5. Significance ($p<0.05$) was reached in all coloured fields, white fields are not significant.

The prevalence of the pro-inflammatory gene-expression signature in BD and AITD patients

Figure 1 shows that the expression levels of the inflammatory genes, aberrantly expressed in the two conditions, are virtually all strongly correlating in expression. The strongest correlations were found between PDE4B and the other genes (see also Padmos *et al.*[5]). In fact the genes formed a signature in which PDE4B could be viewed as an important leader gene. There is indeed reason to consider PDE4B as a key molecule in the pro-inflammatory state of the monocytes (see Padmos *et al.*[5]). We therefore tried out various definitions for the presence or absence of a PDE4B-associated pro-inflammatory mRNA signature (Table 3). Using these definitions it is evident that the monocytes of a large proportion (up to 60%) of BD patients is characterized by a "PDE4B-associated inflammatory gene expression signature". A considerable, but smaller proportion of AITD patients (up to 43%) is also characterized by such a signature.

Expression at the protein level

We measured the serum protein expression levels of the signature genes IL-1β, IL-6, CCL2 and CCL7. The serum IL-1β level was significantly higher in bipolar patients as compared to HC, however the serum IL-1β level was not significantly higher in AITD patients (Figure 2A). With regard to serum CCL2 levels, both bipolar and AITD patients had significantly higher CCL2 levels as compared to HC (Figure 2B). IL-6 and CCL7 were not different between patients and controls. Age and gender did not correlate to IL-1β and CCL2 serum levels. Hence also at the protein level there are signs of pro-inflammation in the two disorders.

Table 3. The presence of a PDE4B- associated molecular signature in healthy controls (HC), bipolar patients (BD) and autoimmune thyroid disease (AITD) patients.

Signature definitions[1]	HC[2]	BP		AITD	
	n=53	*n*=42		*n*=35	
	n (%)	n (%)	*p*[3]	n (%)	*p*[4]
PDE4B positive	11 (21)	25 (60)	<.001	15 (43)	.026
Plus ≥ 25% of genes positive	9 (17)	22 (52)	<.001	14 (40)	.016
Plus ≥ 50% of genes positive	8 (15)	17 (40)	.005	13 (37)	.018
Plus ≥ 75% of genes positive	5 (9)	6 (14)	.46	7 (20)	.16
Plus ≥ 90% of genes positive	0	2 (5)	.11	3 (9)	.030
Plus 100% of genes positive	0	1 (2)	.26	0	-

[1] Various definitions are given depending on the percentage positives of the 22 genes mentioned in Table 2. Positivity of the genes is defined as an mRNA expression + 1 SD higher than the mean level found in healthy controls.
[2] The same 53 HC were used as described in Table 1.
[3] Chi-Squared test: BP vs. HC.
[4] Chi-Squared test: AITD vs. HC.
BP, bipolar patients; HC, healthy controls; AITD, autoimmune thyroid disease.

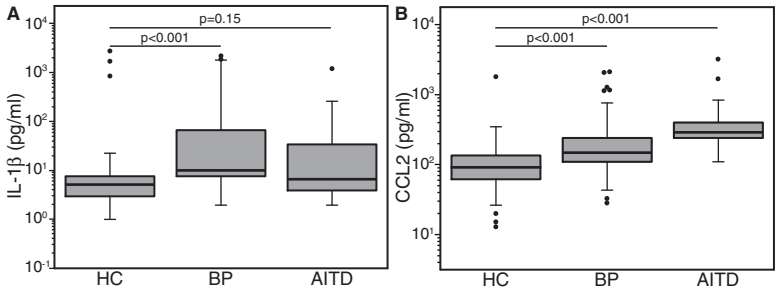

Figure 2. The protein expression of IL-1β and CCL2 in serum of bipolar and thyroid autoimmune patients.

A). and B). Boxplots of log transformed IL-1β and CCL2 serum levels are given. Data were log transformed to obtain a normal distribution. Only in the case of CCL2 this resulted in a normal distribution, hence ANOVA analysis, adjusting for age and gender could be used. For IL-1β the non-parametric Mann-Whitney test was used. The boxes indicate the lower and upper quartiles. The lines within the boxes represent the median. The whiskers extend to the 2.5 and 97.5 percentiles. The outliers are characterized by the filled dots. IL-1β was tested on the same patients as described in Table 1. CCL2 was tested on more patients and healthy controls: HC n=157, 67% females, mean age 36 years, range 19-56 years; BD n=293, 55% females, mean age 44 years, range 22-83 years; AITD n=59, 76% females, mean age 44 years, range 19-87 years.

The pro-inflammatory PDE4B-associated gene-expression signature in bipolar patients with a thyroid autoimmune response

We divided the BD patients into those with and without a positive pro-inflammatory state of their monocytes. If the pro-inflammatory monocyte state were causative (i.e. the shared vulnerability factor) for both psychiatric symptoms and AITD, one would expect a higher prevalence of positivity for TPO-abs in those bipolar patients also positive for the monocyte pro-inflammatory signature. However Table 4 shows that we did not find differences between the prevalences of TPO-abs positivity and TPO-abs negativity in signature positive and signature negative BD patients, neither was there a higher prevalence of signature positivity in BD patients with AITD (Table 5).

Table 4. TPO-abs positivity in signature negative and signature positive bipolar patients.

	Bipolar patients	
	Signature[1] positives	Signature[1] negatives
	N=22	*N*=20
	N (%)	N (%)
TPO-abs positive	5 (23%)	5 (25%)
TPO-abs negative	17 (77%)	15 (75%)
p value[2]	*.86*	

[1] Signature is here defined as PDE4B positive plus ≥25% of genes positive.
[2] Tested via chi squared test.

Table 5. Signature positivity in bipolar patients with and without thyroid autoimmunity.

	Bipolar patients	
	TPO-abs positives	TPO-abs negatives
	N=10	N=32
Signature 25 positive[1]	5 (50%)	17 (53%)
Signature 25 negative	5 (50%)	15 (47%)
p value[3]	*.86*	
Signature 50 positive[2]	3 (30%)	14 (44)
Signature 50 negative	7 (70%)	18 (56%)
p value[3]	*.44*	

[1] Signature is here defined as PDE4B positive plus ≥ 25% of genes positive.
[2] Signature is here defined as PDE4B positive plus ≥ 50% of genes positive.
[3] Tested via Chi squared test

DISCUSSION

We here confirm that monocytes of up to 60% of BD patients show a coherent expression of a set of inflammation and inflammation-related genes, but we additionally show that this coherent set also includes the 3 new genes found in whole genome screening of monocytes of patients with AITD, illustrating a relatedness of BD and AITD at the pro-inflammatory monocyte level. Also up to 43% of AITD patients shared this coherent set of aberrantly expressed inflammatory monocyte genes with BD patients. These findings strengthen the view that inflammatory monocytes and their descendent cells play a role in both mood disorders and thyroid autoimmunity. Of the 4 inflammation related genes tested at the protein level (IL-1β, IL-6, CCL2 and CCL7) 2 could be detected as raised in serum (IL-1β and CCL2) and in particular CCL2 was expressed at a higher level in both BD and AITD patients.

With regard to BD, the “macrophage-T cell theory of mood disorders” poses that inflammatory macrophages play a prominent role in mood disorders. The theory is based on reports on a raised production of IL-1β, IL-6 and TNF-α in patients with a mood disorder.[13] The theory is in addition further supported by the observation that animal models of depressive-like behavior, such as the olfactory bulbectomized rat and the gestationally stressed rat, show raised serum levels of pro-inflammatory cytokines.[14, 15] A direct effect of these cytokines on behavior is shown by the observation that administration of pro-inflammatory cytokines to cancer and hepatitis C patients is able to induce depressive or even manic symptoms,[13] suggesting a causal role for the high serum cytokine levels in mood disorder patients. Also our observation that the pro-inflammatory monocyte signature

can be detected in children of a BD parent and particularly in those later developing a mood disorder is suggestive of a causative role for pro-inflammatory monocytes in mood derangements.[5]

With regard to thyroid autoimmunity, there are several reports on an abnormal pro-inflammatory cytokine and mediator production by monocytes and macrophages in the animal models of spontaneous autoimmune thyroid disease: the NOD mouse and the BB-DP rat. In the NOD mouse a pro-inflammatory state of circulating monocytes, producing high levels of CCL2 and preferentially developing in macrophage-like cells, has been reported.[16, 17] NOD monocytes and macrophages also show a high PTGS2 gene expression levels[18] and a high pro-inflammatory cytokine production (particularly when ingesting apoptotic cells).[19, 20] In the BB-DP rat macrophages produce high quantities of TNF-α.[21] Also oxidative radicals are produced at a higher level by NOD and BB-DP macrophages.[22, 23] In the same way, there are reports on a raised production of oxidative radicals by monocytes of AITD patients.[24]

Hence it is tempting to view the here described pro-inflammatory monocyte state as the (or one of the) shared susceptibility factor(s) for BD and AITD (see objective). However our data also show that AITD is equally prevalent in BD patients with inflammatory monocytes, i.e. with a positive “signature” test, as compared to BD patients without this type of inflammatory monocytes; this observation is in contradiction to the view that pro-inflammatory monocytes act as a vulnerability factor for AITD.

Indeed, there are also reports that show that pro-inflammatory macrophages and their cytokines – although present or raised in autoimmunity - are not necessarily vulnerability factors, but that there exists a much more complicated picture and that a pro-inflammatory state can even be linked to protection from autoimmunity, depending on the phase of the autoimmune reaction. In the BB-DP rat pentoxifylline (an anti-inflammatory drug targeting phosphodiesterases like our signature gene PDE4B) clearly reduces pro-inflammatory cytokine production, but has only a beneficial effect on autoimmune destruction when given late in the process (during the effector phase, when autoreactive T cells have been formed). When given from earlier time points the anti-inflammatory action of the drug leads to a poor induction of T regulator mechanisms actually favoring the induction of T cell mediated autoimmune responses.[25] A similar but opposite paradoxical response exists in the NOD mouse: treatment schemes interfering with TNF production accelerate autoimmune disease in adult mice (by interfering with T cell deletion), while in neonatal mice similar treatments protect against autoimmune disease (probably by inducing T regulator cells).[26] Hence these observations suggest that pro-inflammatory monocytes can either be a protective or a vulnerability factor depending on the phase and the character of the autoimmune reaction.

There is evidence that anti-inflammatory treatments targeting here-described important signature genes are of benefit in patients with a mood disorder. COX-2 (PGS2) inhibitors and anti-TNF treatment have been shown to have beneficial effects in depression.[27, 28] Also the PDE4B molecule is a potential therapeutic target and PDE4 inhibitors are in a

far stage of development. These inhibitors have anti-depressive action, but also broadly inhibit functions of inflammatory and immune modulating cells.[29-31] Whether these drugs will also be beneficial to counteract the concomitant thyroid autoimmune response in the psychiatric patient is doubtful and will probably depend on the phase and character of the thyroid autoimmune reaction. Effects of these drugs on thyroid autoimmunity should be tested, when given to patients with a mood disorder.

In conclusion the monocytes of considerable proportions of BD and AITD patients show virtually the same aberrant monocyte inflammatory gene profile (signature), yet this pro-inflammatory state cannot be considered as the shared vulnerability factor for both disorders. In AITD this pro-inflammatory state of monocytes and their descendent cells may even be of benefit to counteract the thyroid autoimmune response.

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VI

Distinct Monocyte Gene-Expression Profiles in Autoimmune Diabetes

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ABSTRACT

Objective There is evidence that monocytes of patients with type 1 diabetes (T1D) show pro-inflammatory activation and disturbed migration/adhesion, but the evidence is inconsistent. Our hypothesis is that monocytes are distinctly activated/disturbed in different sub forms of autoimmune diabetes.

Methods We studied patterns of inflammatory gene expression in monocytes of patients with T1D (juvenile, *n*=30, and adult, *n*=30, onset) and latent autoimmune diabetes of the adult (LADA) (*n*=30) (controls: healthy subjects, *n*=49, type 2 diabetes patients, *n*=30) using quantitative-PCR (Q-PCR). We tested 25 selected genes: 12 genes detected in a pre-study via whole genome analyses plus an additional 13 genes identified as part of a monocyte inflammatory signature previously reported.

Results One cluster (comprising 12 pro-inflammatory cytokine/compound genes with a putative key gene *PDE4B)* was detected in LADA (60%) and adult-onset T1D (28%), but in only 10% juvenile-onset T1D. A second cluster (comprising 10 chemotaxis, adhesion, motility and metabolism genes) was detected in juvenile-onset T1D (43%) and LADA (33%) but in only 9% adult-onset T1D.

Conclusions Sub-groups of T1D patients show an abnormal monocyte gene expression with two profiles, supporting a concept of heterogeneity in the pathogenesis of autoimmune diabetes only partly overlapping with the presently known diagnostic categories.

INTRODUCTION

There is evidence that monocytes of patients with type 1 diabetes (T1D) are functionally aberrant showing a raised production of IL-1β, IL-6, superoxide anion and PTGS2,[1-3] an aberrant generation of antigen-presenting cells[4, 5] and an abnormal chemotaxis, adhesion and migratory potential.[6] These aberrancies are thought to play a role in the pathogenesis of the disease by disrupting tolerance and by aggravating the β-cell cytotoxic potential of infiltrating monocyte-derived dendritic cells and macrophages. However, these aberrant functional findings could not always be reproduced, particularly with regard to the enhanced production of PTGS2[7] and with the poor generation of antigen presenting cells from monocytes.[8] Two issues could be relevant to these discrepancies. First, raised production of pro-inflammatory monocyte-derived cytokines could be related to hyperglycemia.[9] Second, there might be heterogeneity within autoimmune diabetes, such as has been noted previously between adult and juvenile forms of T1D on the basis of genetic, immune and metabolic characteristics.[10] This possible heterogeneity in autoimmune diabetes might also become evident in different monocyte activation profiles.

To resolve these issues we here focus on patterns of inflammatory gene expression in monocytes from selected patients distinguished by clinical characteristics and age at diagnosis, as well as from control subjects. Our hypothesis is that monocytes might be distinctly activated and disturbed within the known diagnostic categories of diabetes.

Recently we reported a signature of 18 inflammation-related genes in monocytes of bipolar patients,[11] activated monocytes are thought to play a role in the pathogenesis of bipolar disorder.[12, 13] Given the reported association between bipolar disorder and autoimmune diabetes,[14] and given the possible central role of monocytes in both disorders we tested this set of 18 pro-inflammatory monocyte genes in patients with autoimmune diabetes. To these 18 monocyte genes we added 7 genes identified in a whole genome expression profile of a set of juvenile-onset T1D patients who had been compared to healthy controls (HC) and type 2 diabetes (T2D) patients (see Supplementary Figure 1). Thus, using quantitative RT-PCR (Q-PCR), we validated in this short report abnormal expression of 25 monocyte activation genes in LADA, adult-onset T1D, juvenile-onset T1D patients and in - as controls - T2D patients and healthy subjects.

MATERIAL AND METHODS

All participants were diagnosed with diabetes according to the criteria of the American Diabetes Association (ADA).[15] Patients were divided into 4 groups: 1) Type 1 diabetes (T1D), diagnosed according to the standard ADA criteria, and with a juvenile disease onset under the age of 18 yrs; 2) Type 1 diabetes, diagnosed according to the standard ADA criteria, and with a disease onset in adulthood, i.e. above the age of 18 yrs; 3) Latent autoimmune diabetes of the adults (LADA), defined as cases not needing insulin within

6 months of diagnosis, and being positive for GAD65 antibodies; and 4) Type 2 diabetes, i.e. the remaining cases. Patients were included after compensation of their metabolic derangement.

Healthy controls (HC): For each patient over 15 years, an age- and gender matched HC from the same residential area was recruited through the enrolling laboratory and medical staff at our departments, unfortunately we were not allowed to recruit healthy individuals under the age of 15 yrs. For at least two weeks prior to blood withdrawal, all HC were free of any obvious medical illness, including acute infections and allergic reactions; none had any autoimmune disorders or first-degree relatives with autoimmune disorders. Blood from patients and controls was collected at various medical centres (Rotterdam, Düsseldorf, London). The local Medical Ethical Review Committees had approved the studies performed according to the Declaration of Helsinki. Written informed consent was obtained from all participants or their parents where appropriate after a complete description of the study had been given. The characteristics of both patients and HC are shown in Tables 1 and 2.

The methods of blood collection, storage, the preparation of purified $CD14^+$ monocytes, of mRNA isolation and Q-PCR have been described in detail elsewhere.[11] All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand, see Appendix 1).

Used statistics have been given in the legends of the various tables and figures.

Techniques used for the measurement of serum concentrations of PTX3, TNFα, IL-6 and CCL2

PTX3 ELISA kits for detection of human PTX3 were used according to the protocols described earlier.[16] In short, 96 well plates (MaxiSorp; Nunc A/S) were coated with 100 μl of monoclonal rat anti-hPTX3 antibody MNB4 in coating buffer followed by overnight incubation at 4ºC. After washing, the plates were incubated for 2 h at room temperature with 5% dry milk diluted in coating buffer. Afterwards, 100 μl of either recombinant human PTX3 standards or cell supernatant was added for 2 h at 37ºC, followed by adding 100 μl biotin-labeled polyclonal rabbit anti-PTX3 antibody for 2 h at 37ºC. After washing streptavidin-HRP was added for 1 h at room temperature. In the end Chromogen TMB (tetramethylbenzidine) was added for 30 min, and 2 M H2SO4. Absorbance values were read at 450 nm by ELISA reader.

IL-6 and TNF-α were measured using Quantikine HS ELISAs from R&D Systems (Wiesbaden, Germany) as described.[17] Serum levels of CCL2 were quantified using a bead-based multiplex assay on a Luminex 100 analyzer (Luminex Corporation, Austin, TX).[17] Fluorescent xMAP COOH microspheres were purchased from Luminex Corporation. Recombinant CCL2 protein and anti-CCL2 monoclonal antibodies were obtained from R&D Systems.

Table 1. Characteristics of the patient groups used for Q-PCR analysis.

		HC	T1D juvenile onset	T1D adult onset	LADA	T2D
Group size		59	30	43	30	30
Age (years)[1]		41 (17-67)	24 (5-50)*	45 (26-71)	52 (36-70)*	50 (33-68)
Gender	Male	24 (41%)	11 (37%)	26 (60%)	12 (60%)	20 (67%)*
	Female	35 (59%)	19 (63%)	17 (40%)	18 (40%)	10 (33%)
BMI[1] (kg/m^2)		23.8 (17.5-31.0)	22.2 (15.2-36.4)	25.7 (19.8-51.4)	28.1 (19.6-48.8)*	30.5 (21.6-44.1)*
HbA1c[1] (%)		4.9 (3.1-6.0)	8.6 (4.6-12.0)*	7.1 (3.4-10.8)*	6.8 (3.8-12.1)*	7.4 (3.8-13.8)*
Duration illness (years)[1]			13 (.01-36)	9 (.04 –44)	3 (.04-28)	1 (.04-5)
Age of onset illness (years)[1]			11 (2-18)	37 (19-66)	48 (31-68)	48 (32-68)

[1] mean (range).
* $p<0.05$ versus HC, tested by the Mann-Whitney test (age, BMI, HbA1c) and the Chi Squared test (gender).
BMI, Body Mass Index

Table 2. Characteristics of the patient groups used for ELISAs.

		HC	T1D adult onset	LADA	T2D
Group size		54	43	27	73
Age (years)[1]		47 (27-67)	42 (20-67)*	52 (36-70)	51 (25-78)
Gender	Male	24 (43%)	33 (65%)*	12 (60%)	50 (67%)*
	Female	32 (57%)	17 (35%)	18 (40%)	25 (33%)
BMI[1] (kg/m^2)		24.4 (17.5-38.7)	26.7 (16.7-53.2)	28.1 (19.6-48.8)*	30.1 (19.63-44.1)*
HbA1c[1] (%)		4.6 (3.1-6.0)	7.6 (3.4-13.4)*	6.8 (3.8-12.1)*	7.1 (3.5-15.1)

[1] mean (range).
* $p<0.05$ versus HC, tested via Mann-Whitney tests (age, BMI, HbA1c) and Chi Squared tests (gender).
HC, Healthy controls; T1D juvenile onset, Type 1 diabetes patients with a juvenile (<18 yrs) onset; T1D adult onset, Type 1 diabetes patients with an adult (>18 yrs) onset, LADA, Latent autoimmune diabetes of the adults patients; T2D,Type 2 diabetes patients; BMI, Body Mass Index.

Table 3. Q-PCR analysis of monocytes of patients with various forms of diabetes compared to healthy controls. Fold change values.

	Fold change[1] (95% CI)							
	T1D juvenile onset vs. HC[2]		T1D adult onset vs. HC[2]		LADA vs. HC[2]		T2D vs. HC[2]	
Gene symbol	*n*=30	*p*[3]	*n*=43	*p*[3]	*n*=30	*p*[3]	*n*=30	*p*[3]
1) Inflammation								
PDE4B	.71 (.45-1.13)	*.31*	1.26 (.91-1.74)	*.36*	**2.86 (1.57-5.19)**	***<.001***	1.32 (.93-1.87)	*.36*
IL6	.83 (.28-2.48)	*.98*	**3.05 (1.34-6.91)**	***.013***	**17.65 (7.42-41.96)**	***<.001***	**6.82 (3.31-14.06)**	***<.001***
Il1B	.51 (.25-1.04)	*.26*	**2.46 (1.34-4.51)**	***.015***	**10.12 (4.37-23.47)**	***<.001***	**4.66 (2.68-8.10)**	***<.001***
PTX3	.95 (.61-1.48)	*.77*	1.79 (1.13-2.84)	*.13*	**4.69 (2.39-9.19)**	***<.001***	**2.23 (1.59-3.32)**	***.003***
PTGS2	**.32 (.18-.59)**	***.011***	**2.16 (1.31-3.57)**	***.006***	**8.09 (4.30-15.25)**	***<.001***	**3.98 (2.52-6.28)**	***<.001***
TNF	.57 (.32-1.03)	*.22*	1.20 (.68-2.13)	*.63*	**6.32 (2.98-13.39)**	***<.001***	**2.95 (1.79-4.87)**	***.005***
TNFAIP3	.83 (.52-1.30)	*.62*	1.24 (.81-1.92)	*.50*	**4.05 (2.34-7.00)**	***<.001***	1.71 (1.10-2.66)	*.11*
HSPA1A	**.60 (.41-.88)**	***.008***	.84 (.71-1.00)	*.36*	.95 (.56-1.59)	*.87*	.83 (.61-1.13)	*.42*
2) Chemokinesis/ motility/ adhesion								
CCL7	**22.30 (6.61-75.17)**	***<.001***	**6.36 (2.37-17.05)**	**<.001**	**22.16 (6.87-71.48)**	***<.001***	**3.63 (1.30-10.18)**	***.018***
CCL20	.80 (.26-2.48)	*.95*	**3.00 (1.14-7.87)**	**.054**	**33.17 9.21-(119.44)**	***<.001***	**7.80 (2.94-20.65)**	***.002***
CXCL2	1.50 .77-2.90)	*.27*	**2.21 (1.20-4.09)**	**.028**	**9.48 (4.00-22.48)**	***<.001***	**4.92 (2.64-9.16)**	***<.001***
CCL2	**4.19 (1.82-9.63)**	***.007***	**2.65 (1.55-4.54)**	**.001**	**4.62 (2.14-9.97)**	***<.001***	**2.53 (1.37-4.67)**	***.002***
CDC42	**1.98 (1.44-2.73)**	***.005***	1.29 (.95-1.73)	*.12*	**2.03 (1.34-3.08)**	***<.001***	**1.44 (1.13-1.84)**	***.041***
CD9	2.13 (1.32-3.44)	*.12*	**1.40 (0.98-2.01)**	***.047***	**2.04 (1.28-3.25)**	***<.001***	**2.13 (1.40-3.23)**	***.028***
STX1A	**7.31 (3.61-14.83)**	***<.001***	1.48 (.98-2.24)	*.071*	**2.89 (1.68-4.97)**	***<.001***	**1.72 (1.06-2.77)**	***.023***
3) Cell survival/ apoptosis								
BCL2A1	1.39 (.89-2.17)	*.29*	1.42 (1.01-1.99)	*.077*	**3.17 (1.87-5.37)**	***<.001***	**1.95 (1.40-2.72)**	***.005***
EMP1	**2.47 (1.49-4.10)**	***.008***	1.29 (.90-1.86)	*.18*	**3.49 (2.20-5.54)**	***<.001***	**2.13 (1.40-3.23)**	***.002***
4) Mapk pathway								
PTPN7	**2.52 (1.83-3.49)**	***<.001***	**1.42 (1.07-1.87)**	***.038***	**2.91 (1.82-4.64)**	***<.001***	**1.94 (1.42-2.64)**	***.001***
DUSP2	1.04 (.69-1.58)	*.90*	**2.26 (1.31-3.90)**	***.005***	**7.98 (4.24-15.02)**	***<.001***	**3.43 (2.00-5.88)**	***<.001***
ATF3	.88 (.60-1.29)	*.53*	**2.02 (1.38-2.96)**	***.001***	**6.07 (3.60-10.25)**	***<.001***	**2.81 (1.98-3.99)**	***<.001***
NAB2	**2.38 (1.32-4.28)**	***.025***	1.23 (.77-1.97)	*.037*	**2.37 (1.23-4.58)**	***.006***	**1.81 (1.09-3.00)**	***.043***
MAPK6	1.53 (1.09-2.16)	*.10*	1.07 (.83-1.39)	*.62*	**1.82 (1.04-3.19)**	***.005***	1.16 (.79-1.70)	*.39*
5) Metabolism								
FABP5	**2.03 (1.25-3.29)**	***.019***	.83 (.65-1.06)	*.50*	1.36 (.74-2.49)	*.16*	.92 (.59-1.45)	*.93*
DHRS3	**3.52 (1.60-7.72)**	***.001***	1.02 (.75-1.37)	*.69*	**2.26 (1.43-3.90)**	***.001***	**1.62 (1.08-2.42)**	***.031***

[1] The quantitative value obtained from Q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from normalized CT values (CT gene- CT reference gene *ABL*), by the ΔΔCT method ($2^{-\Delta\Delta CT}$, User Bulletin 2, Applied Biosystems, Foster City, California). Data were standardized to the HC (thus the HC were used as the calibrator). The fold change of the HC is therefore 1. The same data were also analyzed prior to standardization to the HC group. These analyses are demonstrated in supplementary Table B.

[2] Values >1: patients have a higher expression than control group.
Values <1: patients have a lower expression than control group.

[2] Healthy controls: *n*=59

[3] Tested by univariate analysis of covariance (ANCOVA) versus HC, age and gender included in model

HC, Healthy controls; T1D juvenile onset, Type 1 diabetes patients with a juvenile (<18 yrs) onset; T1D adult onset, Type 1 diabetes patients with an adult (>18 yrs) onset, LADA, Latent autoimmune diabetes of the adults patients; T2D,Type 2 diabetes patients

RESULTS

Q-PCR analysis

Table 3 shows the gene expression levels of 24 of 25 selected genes in the monocytes of the tested diabetes groups. These 25 genes comprised 12 genes (10 up-regulated and 2 down-regulated), which have been identified in a pre-study (Affymetrix gene expression profiling, see supplementary Figure 1) and which discriminated T1D monocytes from both T2D and HC monocytes (4 fold difference, $p<0.01$). These 12 genes were *STX1A, DHRS3 (SDR1), FABP5, CD9, CDC42, CCL2, CCL7 (MCP-3), PTPN7, NAB2, EMP1* (all up-regulated) and *BAZ1A and HSPA1A* (each down-regulated). The other 13 genes we tested (*PDE4B, IL1B, IL6, TNF, PTGS2, PTX3, CCL20, CXCL2, MAPK6, DUSP2, ATF3, TNFAIP3* and *BCL2A1)* were reported elsewhere [11] as a coherent and mutually correlating set ("signature") of 18 aberrantly expressed inflammation-related genes in monocytes of bipolar patients, of which 5 of the 18 genes, i.e. *CDC42, CCL2, CCL7, NAB2* and *EMP1*, were also detected in our Affymetrix pre-study in purified T1D monocytes.

In Table 3 data are given as relative "fold-changes", a method widely used, but with potential limitations (e.g. less accurate for genes with a large difference to the reference gene, for raw CT values see Supplementary Table A), as has our standardization of patient data to HC (which was done to correct for the observed inter-assay variation). To address this latter issue, data were also analyzed prior to standardization to HC (Supplementary Table B). In essence the same conclusions can be drawn from both analyses: a) 24/25 studied genes were validated as aberrantly expressed (*BAZ1A* was not abnormally expressed), b) although monocytes of juvenile-onset T1D, adult-onset T1D, LADA and T2D patients all showed enhanced gene expression of many of the inflammatory genes as compared to HC, they also showed differences compared to each other, and c) some of the genes were specific for a diagnostic category. Specific for LADA monocytes were the up-regulation of *PDE4B, TNFAIP3* and *MAPK6*, specific for juvenile-onset T1D monocytes was the up-regulation of *FABP5* and the down-regulation of *HSPA1A.*

Neither adult-onset T1D nor T2D had an up or down- regulation of a specific gene, yet T2D monocytes showed a clear up-regulation of many of the inflammatory genes.

The gene expression levels within each subject group did not correlate with HbA1c, Body Mass Index (BMI), age, gender, age at onset or disease duration (tested by ANCOVA). To further analyze the data we embarked on cluster analysis.

Identification of two gene-expression clusters and their presence in LADA, T1D and T2D patients

Figure 1 shows the Q-PCR data of the patients and HC in hierarchical cluster analysis. The dendrogram of average linkage showed two inter-dependent main gene clusters.

In another and different cluster analysis of the Q-PCR data we correlated the expression levels of the 24 abnormally expressed genes to the expression level of: a) *PDE4B*, since this gene is one of the genes specific for LADA and a putative key gene for cluster 1 (see discussion), and b) *FABP5*, since this cluster 2 gene is specific for juvenile-

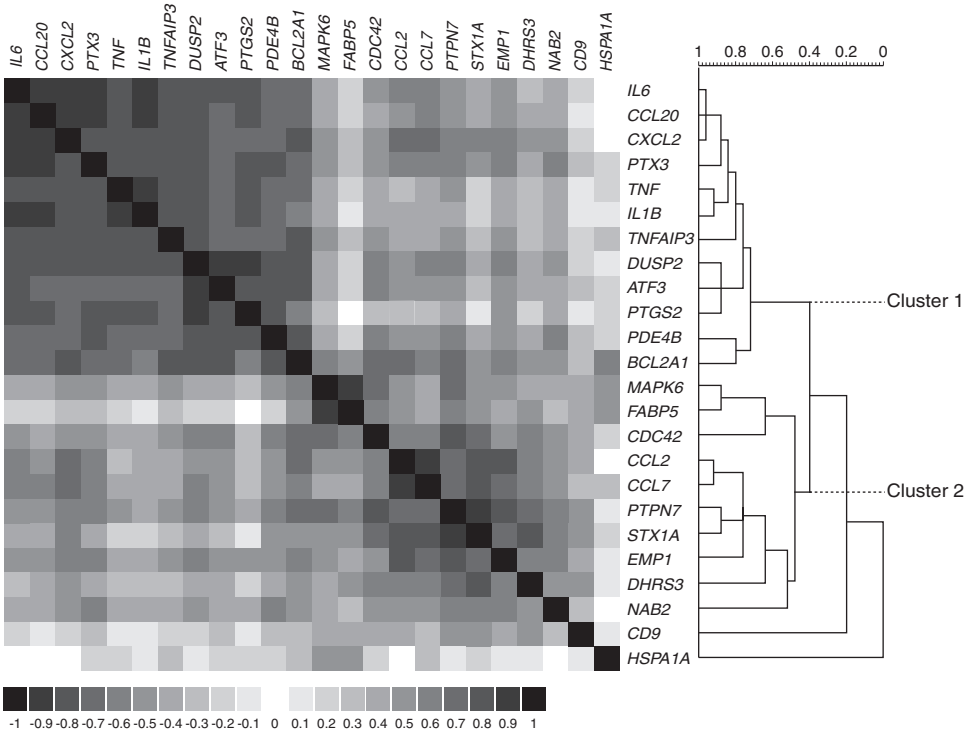

Figure 1. Hierarchical cluster analysis.
Color- coded correlation matrix illustrating pairwise correlations between the expression levels of the 24 genes aberrantly expressed in patients with various forms of diabetes (see Table 3).
The grey shade intensity codes for the strength of the correlations. Also a dendrogram is presented as a result of hierarchical cluster analysis with the use of correlation coefficients. The dendrogram shows two gene-expression clusters.

Table 4. Correlation of mRNAs.

	PDE4B			***FABP5***	
	r[1]	*P*		r[1]	*p*
PDE4B	1		*PDE4B*	.011	*.96*
CCL20	.88	*<.001*	*CCL20*	.20	*.39*
DUSP2	.88	*<.001*	*DUSP2*	.14	*.45*
IL1B	.87	*<.001*	*IL1B*	-.043	*.96*
PTGS2	.85	*<.001*	*PTGS2*	-.52	*.018*
IL6	.84	*<.001*	*IL6*	.41	*.076*
BCL2A1	.82	*<.001*	*BCL2A1*	.34	*.069*
PTX3	.78	*<.001*	*PTX3*	-.13	*.58*
ATF3	.76	*<.001*	*ATF3*	.21	*.26*
TNFAIP3	.75	*<.001*	*TNFAIP3*	.081	*.67*
NAB2	.69	*<.001*	*NAB2*	.23	*.22*
TNF	.54	*<.001*	*TNF*	-.25	*.19*
CXCL2	.87	*<.001*	*CXCL2*	.60	*<.001*
CCL7	.87	*<.001*	*CCL7*	.66	*<.001*
STX1A	.79	*<.001*	*STX1A*	.68	*<.001*
CCL2	.74	*<.001*	*CCL2*	.73	*<.001*
EMP1	.67	*<.001*	*EMP1*	.57	*.001*
CDC42	.63	*<.001*	*CDC42*	.48	*.008*
PTPN7	.60	*.001*	*PTPN7*	.66	*<.001*
MAPK6	.58	*.001*	*MAPK6*	.50	*.005*
DHRS3	.58	*.001*	*DHRS3*	.66	*<.001*
CD9	-.073	*.70*	*CD9*	.48	*.008*
HSPA1A	-.16	*.41*	*HSPA1A*	-.36	*.005*
FABP5	.27	*.15*	*FABP5*	1	
***PDE4B*- correlating set**			***FABP5*- correlating set**		

[1] Spearman's correlation co-efficient.
The *PDE4B* correlations were determined in the LADA patients (*n*=30) since *PDE4B* up-regulation was specific for that group. The *FABP5* correlations were determined in the juvenile-onset T1D patients (*n*=30) for the same reason (a specific *FABP5* up-regulation in juvenile-onset T1D).

onset T1D. Table 4 shows that around these "specific" genes two mutually correlating gene-expression sets appeared. In the *PDE4B*-correlating set all Cluster 1 and Cluster 2 genes (apart from *FABP5*) were present and correlated strongly to the gene expression

of *PDE4B*. In the *FABP5*-correlating set almost all Cluster 2 genes were present (except for *NAB2*) and in addition *CXCL2, PTGS2, HSPA1A* and *CD9.* Interestingly *PTGS2* was over-expressed in the *PDE4B* positive subjects, while its expression was reduced in *FABP5*- positive subjects (Table 4).

We next sought the relationship of different patient groups to cluster 1 and cluster 2 genes (Table 5). Cluster 1 and the *PDE4B*-correlating set were significantly more frequent in adult-onset T1D, LADA and T2D patients compared to HC; while HC and juvenile-onset T1D patients were similar. Cluster 2 and the *FABP5*-correlating set were significantly more

Table 5. The presence of cluster 1 and *PDE4B*-correlating set and cluster 2 and *FABP5*-correlating set in the monocytes of different diabetes groups and healthy controls.

Definitions	HC[1]	T1D juvenile onset	T1D adult onset	LADA	T2D
	n=94	*n*=30	*n*=43	*n*=30	*n*=30
	% (n)	% (n)	% (n)	% (n)	% (n)
Cluster 1:					
≥75% of Cluster 1 genes[2] positive[3]	10 (9)	10 (3)	**28 (12)***	**60 (18)*****	**37 (11)****
PDE4B correlating set:					
≥50% of genes[4] positive	11 (10)	13 (4)	**26 (11)***	**50 (15)*****	20 (6)**
≥75% of genes positive	3 (3)	10 (3)	**23 (10)*****	**43 (13)*****	**17(5)****
Cluster 2:					
≥75% of Cluster 2 genes[5] positive[3]	5 (5)	**43 (13)****	9 (4)	**33 (10)****	10 (3)
FABP5 correlating set:					
≥75% of genes[6] positive	2 (2)	**43 (13)****	7(3)	**13 (4)***	0

[1] For this analysis 35 extra HC were available so in total 94 HC were studied.

[2] The 12 genes belonging to Cluster 1 (see Figure 1) namely: *PDE4B*, *IL1B, IL6, TNF, PTGS2, PTX3, CCL20, CXCL2, DUSP2, ATF3, TNFAIP3* and *BCL2A1.*

[3] Positivity of the genes is defined as an mRNA expression +1 SD higher than the mean level found in the healthy controls.

[4] The 20 genes correlating with *PDE4B* (see Table 4) namely: *CCL20, DUSP2, IL1B, PTGS2, IL6, BCL2A1, PTX3, ATF3, TNFAIP3, NAB2, TNF, CXCL2, CCL7, STX1A, CCL2, EMP1, CDC42, PTPN7, MAPK6* and *DHRS3.*

[5] The 10 genes belonging to Cluster 2 (see Figure 1) namely: *CCL7, CCL2, CDC42, STX1A, EMP1, FABP5, DHRS3, PTPN7, NAB2* and *MAPK6.*

[6] The 12 genes correlating with *FABP5* (see Table 4) namely: *HSPA1A* (negative correlation), *CD9, DHRS1, MAPK6, PTPN7, CDC42, EMP1, CCL2, STX1A, CCL7, CXCL2* and *PTGS2* (negative correlation).

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus HC, tested via Chi Squared tests.

HC, Healthy controls; T1D juvenile onset, Type 1 diabetes patients with a juvenile (<18 yrs) onset; T1D adult onset, Type 1 diabetes patients with an adult (>18 yrs) onset, LADA, Latent autoimmune diabetes of the adults patients; T2D, Type 2 diabetes patients.

frequent in both juvenile-onset T1D and LADA patients compared to HC, adult-onset T1D and T2D. Neither clusters, nor specific *PDE4B* or *FABP5* gene expression were related to age, HbA1c level, glucose level or BMI within any of the groups studied.

Correlations between gene-expression levels in circulating monocytes and serum levels of cytokines

In addition to monocyte gene analysis we determined serum levels of IL-6, TNF-α, PTX3 and CCL2 in patients and controls and correlated gene expression levels to corresponding serum cytokine levels. Serum levels of CCL2, log IL-6 and PTX3 appeared associated with age (CCL2: B=5.89 pg/ml, p=0.023; log IL-6: B=6.4.10^{-3}pg/ml, p=0.028; ranks PTX3: p=0.027), while the serum level of CCL2 was higher in males than in females (B=127 pg/ml, p=0.021). Also, serum levels of IL-6 and TNF-α showed a correlation with BMI (log IL-6: B=1.3.10^{-2} pg/ml, p=0.009; log TNF-α: B=9.0.10^{-3}pg/ml, p=0.007, ANCOVA). Therefore we corrected for age, gender and BMI in further statistical analysis. HbA1c also influenced the serum level of IL-6 (B=3.7.10^{-2}, p=0.007, ANCOVA); we therefore analyzed IL-6 with and without HbA1c included in the model.

For CCL2 there were no differences between groups (Figure 2A). For IL-6 (Figure 2B) serum levels were significantly raised in adult-onset T1D, LADA and T2D patients as compared to HC, but with HbA1c included in the model these differences were lost consistent with IL-6 level being determined by chronic hyperglycaemia. With regard to TNF-α and PTX3 levels only LADA patients had (borderline) significantly raised levels as compared to HC (Figure 2C and 2D).

The monocyte gene expression levels of *PTX3* and *IL6* (PTX3: r= 0.26, p=0.004; IL-6: r=0.23, p=0.034, Spearman's correlation), but not of *TNF* and *CCL2* correlated with serum protein levels. Of note: For inflammatory cytokines gene expression and serum protein levels often do not correlate. Indeed, our study confirmed this general observation in that e.g. we detected over 4-fold raised expression of *CCL2, TNF*, *IL6* and *PTX3* at the monocyte mRNA level in LADA patients, yet only a 2-fold raised protein level for serum TNF-α, IL-6 and PTX3, and no change in serum CCL2 levels. Apparently, gene expression is influenced less than serum levels by confounders, such as body mass index (which correlated with serum TNF-α and IL-6) and blood glucose (estimated as HbA1c, which correlated with serum IL-6).

We also compared the serum cytokine levels of cluster positive and negative patients. We found higher serum levels of PTX3 in cluster 1 positive compared to cluster 1 negative patients (Figure 3), suggesting an *in vivo* relevance of at least cluster 1 gene expression. Elevated levels of serum PTX3, a novel acute phase protein, have been found in more autoimmune conditions such as rheumatoid arthritis and scleroderma.[18]

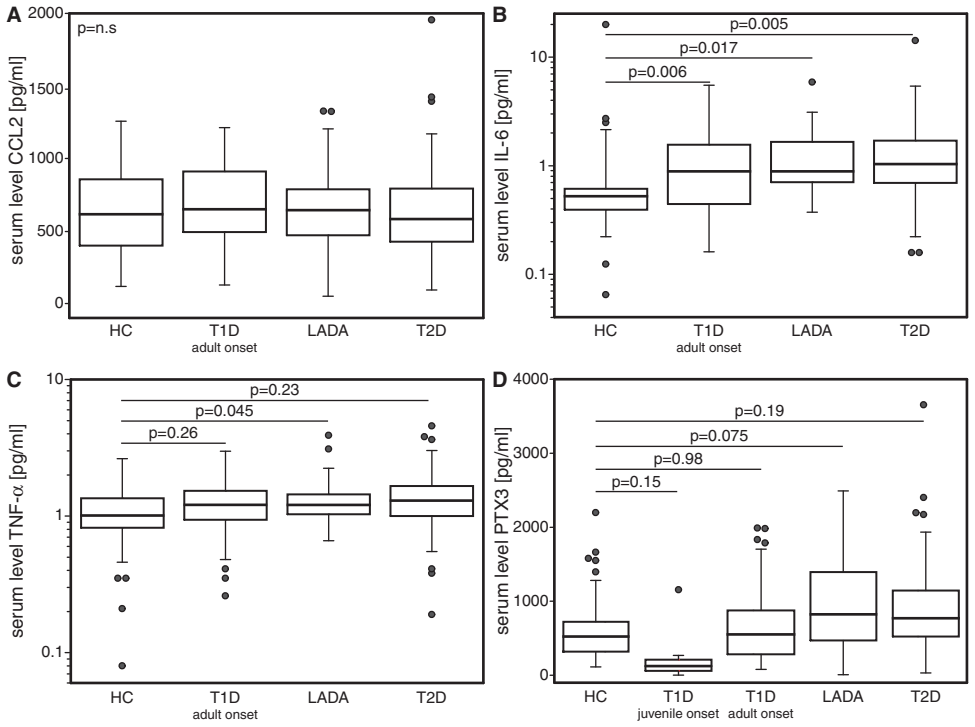

Figure 2. Serum levels of CCL2, PTX3, IL-6 and TNF-α in patient groups with various forms of diabetes and healthy controls.
The characteristics of the patients studied for serum cytokines are given in Table 2. Serum of juvenile-onset T1D patients was only available for the PTX3 ELISA (*n*=14, mean age= 12 years, range 7-17 years). IL-6 and TNF-α levels were log transformed to obtain a normal distribution. For PTX3 ranks were used in the analysis[30] because even after log transformation a normal distribution could not be obtained. Groups were compared via ANCOVA analysis with age, gender and BMI included in the model.

DISCUSSION

This study shows two distinct monocyte gene-expression profiles in autoimmune diabetes indicating different activation profiles, which suggests heterogeneity in the pathogenesis of autoimmune diabetes.

We identified one profile of mainly pro-inflammatory genes (*IL1B, IL6, TNF, PTGS2, PTX3, CCL20, CXCL2, DUSP2, ATF3, TNFAIP3* and *BCL2A1)* with a putative key gene *PDE4B. PDE4B (*phosphodiesterase 4B) is a c-AMP degrading enzyme and could be a key molecule for turning monocytes into high pro-inflammatory cytokine producing cells as targeted gene knockout studies show a crucial role for PDE4B in the cytokine production of monocytes.[19-22] A second profile consisted of genes mainly involved in chemotaxis, adhesion, motility and metabolism (*CCL7, CCL2, CDC42, STX1A, EMP1,*

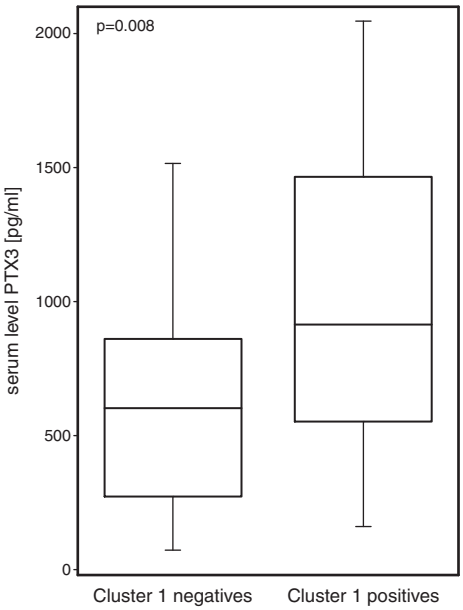

Figure 3. PTX3 serum levels of cluster 1 positives compared to cluster 1 negatives.
Serum levels of PTX3 in Cluster 1 positive (*n*=36) and negative subjects (*n*=73) (patients as well as controls). The definition was as follows: Positive: ≥75% of the Cluster 1 genes positive. Negative: <75% of the Cluster 1 genes positive. Groups were compared by ANCOVA analysis with age, gender and BMI included in the model. Because normal distribution of PTX3 could not be obtained, ranks of PTX3 were used in the analysis.[30]

FABP5, DHRS3, NAB2, PTPN7 and *MAPK6)* with a putative key gene *FABP5.*

The first profile (Cluster 1) was found in monocytes in LADA (60%) and adult-onset T1D patients (28%) more than in juvenile-onset T1D and controls (each 10%). The second profile (Cluster 2), on the other hand, was found in 43% of juvenile-onset T1D and in 33% of the LADA patients, but in less than 10% of adult-onset T1D patients and controls. These different frequencies of the two activation clusters in the known diagnostic categories of diabetes are consistent with the view that the categories are pathologically different such that LADA and adult-onset T1D have similar immune characteristics, distinct from juvenile-onset T1D.[10]

We also found many of the inflammatory genes up-regulated in T2D monocytes, supporting the view that inflammatory monocytes are involved in the pathogenesis of T2D.[23] However, most (83%-100%) T2D patients had a normal expression of the key genes *PDE4B* and *FABP5,* which resulted in their monocyte gene cluster being distinct from that in the majority of LADA and juvenile-onset T1D patients.

Since the monocytes appear to be distinctly activated and disturbed in LADA, adult-onset, juvenile-onset T1D and T2D, it is possible that these profiles can be used to identify

sub forms of diabetes within the known diagnostic categories of diabetes. This subdivision could improve outcome prediction and gene-association studies, may lead to more consistent reports on immune aberrancies in autoimmune diabetes and could result in new intervention strategies by providing new targets for treatment. PDE4B, in particular, might be such a target as inhibitors are in development[19-22] and rolipram, an archetypical PDE4 inhibitor, reduced insulitis and prevented diabetes in the NOD mouse.[24] Another potential target for drug intervention is prostaglandin-endoperoxide synthase 2 (PTGS2), a key enzyme in the biosynthesis of prostanoids. COX-2 (PTGS2) inhibitors are well known for their anti-inflammatory functions,[25] but there are no studies of them in diabetes patients. Of note is, that both a raised and normal basal PTGS2 have been described in T1D;[3, 7] we here find that *PTGS2* is raised in Cluster 1, but down-regulated in Cluster 2 positive T1D patients. Thus, COX-2 inhibitors might alter monocyte activation in Cluster 1 positive patients (i.e. many LADA and adult-onset T1D patients), but not in Cluster 2 positive patients (i.e. many juvenile-onset T1D patients).

A recent study [26] showed that factors in serum of T1D patients could induce inflammatory genes (*CCL2, CCL7, IL1B*) in peripheral blood mononuclear cells (PBMC). Another gene expression study on PBMC of T1D patients[27] also detected over-expression of inflammatory genes (amongst others *IL1B* and *PTGS2*) without evidence of the extended signatures as described here. In this latter study investigators used non-fractionated PBMC. Indeed an important issue is the cell collection, preservation and separation used in our study. We used frozen-stored PBMC and positive CD14 MACS separation. Specifically, freeze-storing might induce differences in gene expression, however positive MACS separation does not influence gene expression.[28] Alternative monocyte separation techniques do modify gene expression profiles e.g. we found plastic adhered monocytes to down-regulate PDE4B gene expression (as is known when monocytes change into macrophages.[29] However, despite these limitations, the differences we describe cannot be due to freeze storage since all monocytes are handled similarly. Further investigations are needed to establish consistency and diagnostic and prognostic consequences of monocyte inflammatory profiles under various storage and isolation conditions.

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Supplementary Table A. Q-PCR analysis of monocytes of patients with various forms of diabetes compared to healthy controls. Raw CT values.

	CT (mean ± sd)[1]				
	HC	T1D Juvenile onset	T1D adult onset	LADA	T2D
Gene symbol	*n*=59	*n*=30	*n*=43	*n*=30	*n*=30
Reference gene					
ABL	23.87 ± 1.78	25.55 ± 2.35	23.64 ± 1.26	23.84 ± 2.26	23.86 ± 1.85
1) Inflammation					
PDE4B	23.07 ± 1.44	24.11 ± 2.41	22.97 ± 1.49	22.91 ± 1.72	23.77 ± 2.24
IL6	30.66 ± 3.63	28.42 ± 4.07	29.00 ± 4.72	28.80 ± 3.58	30.22 ± 3.10
IL1B	20.51 ± 2.46	20.41 ± 3.13	19.16 ± 2.62	19.12 ± 2.98	19.82 ± 2.76
PTX3	24.67 ± 1.99	25.23 ± 2.33	24.01 ± 1.87	23.27 ± 2.41	24.63 ± 2.53
PTGS2	22.76 ± 2.08	23.37 ± 3.57	21.80 ± 2.20	21.73 ± 2.37	22.94 ± 2.92
TNF	21.51 ± 1.79	22.13 ± 3.03	21.05 ± 2.25	20.34 ± 2.83	21.57 ± 2.87
TNFAIP3	22.19 ± 1.39	23.18 ± 1.95	22.03 ± 1.84	20.99 ± 2.42	22.65 ± 2.83
HSPA1A	19.93 ± 1.74	22.36 ± 1.89	20.05 ± 1.63	19.94 ± 1.22	20.30 ± 1.79
2) Chemokinesis/ motility/ adhesion					
CCL7	31.70 ± 2.54	27.46 ± 5.29	29.41 ± 4.06	29.28 ± 4.06	31.43 ± 4.36
CCL20	29.90 ± 3.91	28.30 ± 4.82	27.76 ± 4.82	27.28 ± 4.84	29.26 ± 4.05
CXCL2	22.84 ± 2.49	21.64 ± 2.98	22.01 ± 3.25	21.36 ± 3.34	22.63 ± 2.94
CCL2	26.14 ± 2.34	24.45 ± 3.80	29.94 ± 2.25	25.26 ± 2.62	25.81 ± 2.73
CDC42	23.90 ± 1.13	23.42 ± 1.93	23.86 ± 1.14	23.67 ± 1.04	23.94 ± 1.28
CD9	23.37 ± 2.31	23.77 ± 3.04	22.76 ± 1.82	22.62 ± 1.74	22.99 ± 2.28
STX1A	30.32 ± 1.58	28.22 ± 1.93	30.07 ± 1.55	29.74 ± 1.80	30.33 ± 2.28
3) Cell survival/ apoptosis					
BCL2A1	19.32 ± 1.75	19.31 ± 2.30	18.48 ± 1.44	18.33 ± 1.50	18.64 ± 1.36
EMP1	25.27 ± 2.32	25.00 ± 3.27	24.66 ± 1.70	24.15 ± 2.01	24.77 ± 2.73
4) Mapk pathway					
PTPN7	25.84±1.42	25.65 ± 2.28	25.45 ± 1.25	24.70 ± 1.39	25.28 ± 1.98
DUSP2	24.41 ± 1.63	24.41 ± 2.02	23.64 ± 2.21	23.17 ± 2.47	24.39 ± 2.77
ATF3	23.57 ± 1.47	23.99 ± 2.54	22.84 ± 1.41	22.58 ± 1.68	23.51 ± 2.35
NAB2	25.99 ± 1.93	26.24 ± 3.24	25.94 ± 1.93	25.25 ± 2.14	26.22 ± 2.41
MAPK6	22.62 ± 1.50	23.09 ± 2.21	22.12 ± 0.98	21.67 ± 0.89	22.06 ± 0.81
5) Metabolism					
FABP5	23.03 ± 3.79	25.14 ± 4.67	22.05 ± 2.15	20.91 ± 0.84	21.20 ± 0.77
DHRS3	26.29 ± 1.50	25.88 ± 4.24	26.17 ± 1.31	25.37 ± 1.32	25.87 ± 1.86

[1] The quantitative value obtained from Q-PCR is a cycle threshold (CT). A low CT value corresponds with a high gene expression.

CT, Cycle threshold; HC, Healthy controls; T1D juvenile onset, Type 1 diabetes patients with a juvenile (<18 yrs) onset; T1D adult onset, Type 1 diabetes patients with an adult (>18 yrs) onset, LADA, Latent autoimmune diabetes of the adults patients; T2D,Type 2 diabetes patients.

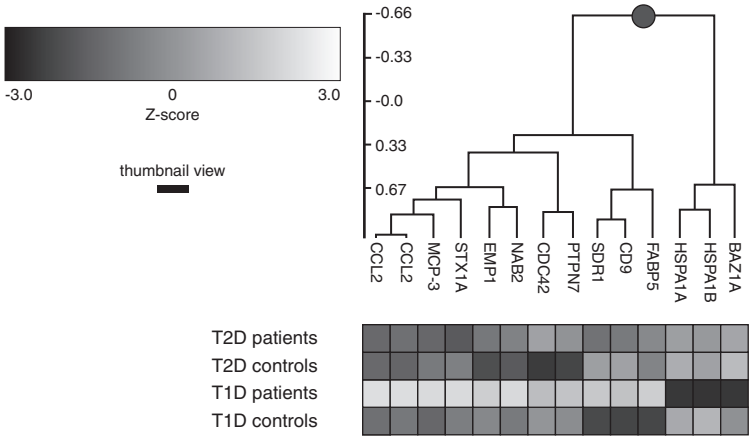
Supplementary Table B. Q-PCR analysis of monocytes of patients with various forms of diabetes compared to healthy controls. ΔCT values.

	ΔCT (mean ± sd)[1]								
	HC	T1D juvenile onset		T1D adult onset		LADA		T2D	
Gene symbol	*n*=59	*n*=30	p^2	*n*=43	p^2	*n*=30	p^2	*n*=30	p^2
1) Inflammation									
PDE4B	-.80 ± 1.80	-1.44 ± 2.56	*.25*	-.67 ± 1.68	*.42*	**-.94 ± 2.21**	***.046***	-.10 ± 1.26	*.51*
IL6	7.30 ± 4.40	3.94 ± 4.12	*.88*	**5.35 ± 5.70**	***.024***	**4.96 ± 3.55**	***<.001***	**6.35 ± 2.88**	***.002***
IL1B	-3.34 ± 3.56	-4.98 ± 3.73	*.41*	**-4.48 ± 3.51**	***.008***	**-4.73 ± 3.24**	***<.001***	**-4.05 ± 2.26**	***<.001***
PTX3	1.31 ± 2.17	.75 ± 1.83	*.96*	.37 ± 2.53	*.058*	**-.58 ± 2.55**	***.007***	**.76 ± 1.60**	***.046***
PTGS2	-.60 ± 2.60	**-1.10 ± 3.50**	***.001***	**-1.84 ± 2.98**	***.007***	**-2.11 ± 2.34**	***<.001***	**-.92 ± 1.81**	***<.001***
TNF	-2.34 ± 2.42	-3.27 ± 2.72	*.32*	-2.59 ± 2.95	*.51*	**-3.50 ± 2.84**	***<.001***	**-2.29 ± 1.95**	***.008***
TNFAIP3	-1.68 ± 1.87	-2.37 ± 2.12	*.75*	-1.61 ± 2.18	*.35*	**-2.87 ± 2.22**	***<.001***	**-1.22 ± 1.92**	***.017***
HSPA1A	-3.94 ± 0.77	**-3.19 ± 1.63**	***.021***	-3.59 ± .88	*.51*	-3.91 ± 1.94	*.49*	-3.56 ± 1.21	*.67*
2) Chemokinesis/ motility/ adhesion									
CCL7	7.83 ± 2.91	**1.91 ± 4.90**	***<.001***	**5.77 ± 4.66**	***<.001***	**5.44 ± 4.09**	***<.001***	**7.57 ± 3.71**	***.025***
CCL20	6.54 ± 4.74	3.82 ± 4.87	*.87*	**4.12 ± 5.76**	***.041***	**3.44 ± 4.80**	***<.001***	**5.40 ± 3.73**	***.008***
CXCL2	-1.03 ± 3.70	-3.91 ± 3.74	*.41*	**-1.63 ± 4.15**	***.028***	**-2.50 ± 3.47**	***<.001***	**-1.23 ± 2.47**	***.003***
CCL2	2.27 ± 2.34	**-1.10 ± 3.25**	***.016***	**1.30 ± 2.62**	***<.001***	**1.42 ± 2.88**	***.001***	**1.95 ± 2.31**	***.004***
CDC42	.032 ± 1.68	**-2.13 ± 1.79**	***.006***	.22 ± 1.87	*.19*	**-.17 ± 1.80**	***.023***	.07 ± 1.19	*.074*
CD9	-.49 ± 1.43	-1.79 ± 1.92	*.076*	-.88 ± 1.68	*.086*	**-1.22 ± 1.83**	***.031***	-.88 ± 1.34	*.20*
STX1A	6.45 ± 1.70	**2.67 ± 3.17**	***<.001***	6.42 ± 1.94	*.085*	**5.90 ± 2.07**	***.004***	6.47 ± 1.78	*.093*
3) Cell survival/ apoptosis									
BCL2A1	-4.55 ± 2.27	-6.24 ± 2.29	*.28*	-5.17 ± 2.36	*.060*	**-5.53 ± 2.17**	***.004***	**-5.22 ± 1.37**	***.003***
EMP1	1.34 ± 1.58	**-.55 ± 1.89**	***.025***	1.02 ± 1.73	*.15*	**.31 ± 1.75**	***.001***	**.90 ± 1.57**	***.006***
4) Mapk pathway									
PTPN7	1.97 ± 1.06	**.10 ± 1.50**	***<.001***	**1.81 ± 1.42**	***.022***	**.86 ± 1.85**	***.001***	**1.42 ± 1.21**	***.001***
DUSP2	.54 ± 2.39	-1.14 ± 2.42	*.98*	**.00 ± 2.76**	***.005***	**-.67 ± 2.36**	***<.001***	**.53 ± 2.07**	***.001***
ATF3	-.30 ± 1.95	-1.56 ± 1.96	*.32*	**-.80 ± 1.93**	***.001***	**-1.28 ± 1.89**	***<.001***	**-.35 ± 1.31**	***<.001***
NAB2	2.12 ± 1.45	**.69 ± 2.58**	***.052***	2.30 ± 2.17	*.57*	1.41 ± 2.40	*.21*	2.36 ± 1.72	*.94*
MAPK6	-1.25 ± 0.93	**-2.30 ± 1.17**	***.012***	-1.52 ± 1.36	*.31*	-2.17 ± 2.23	*.10*	-1.80 ± 1.54	*.32*
5) Metabolism									
FABP5	-.83 ± 2.55	**-.41 ± 3.14**	***.004***	-1.60 ± 1.52	*.56*	-2.94 ± 2.42	*.77*	-2.67 ± 1.85	*.72*
DHRS3	2.43 ± 1.11	**.33 ± 3.06**	***.005***	2.53 ± 1.35	*.55*	**1.53 ± 1.92**	***.036***	2.00 ± 1.53	*.31*

[1] The quantitative value obtained from Q-PCR is a cycle threshold (CT). A low CT value corresponds with a high gene expression. ΔCT=CT target gene - CT reference gene *ABL*.

[2] Tested by univariate analysis of covariance (ANCOVA) versus HC, with "assay" included in the model as a variable (together with age and gender) since we had observed inter-assay variations.

CT, Cycle threshold; HC, Healthy controls; T1D juvenile onset, Type 1 diabetes patients with a juvenile (<18 yrs) onset; T1D adult onset, Type 1 diabetes patients with an adult (>18 yrs) onset, LADA, Latent autoimmune diabetes of the adults patients; T2D,Type 2 diabetes patients



Supplementary Figure 1. Affymetrix analysis of T1D patients, T2D patients and their sex- and age-matched controls.

Affymetrix analysis was performed on 3 pools of CD14 MACS purified monocytes of juvenile-onset type 1 diabetes (T1D) patients. One T1D monocyte pool consisted of the monocytes of 5 males and 3 females (mean age 15 yrs), a second T1D pool of 7 males and 1 female (mean age 13 yrs) and a third T1D pool of 4 males and 5 females (mean age 16 yrs), (3 T1D micro-arrays). Data were controlled by Affymetrix analysis on 3 pools of MACS purified monocytes of healthy controls, i.e. a pool of 5 young males and 3 young females (mean age 14 yrs), a pool of 4 males and 4 females (mean age 16 yrs) and a third pool of 2 adolescent males and 5 adolescent females (mean age 25 yrs) (3 Co-T1D micro-arrays). In addition data were also controlled by T2D data and for this we used 1 pool of MACS purified monocytes of T2D patients (2 males, 5 females, mean age 56 yrs) (1 T2D micro-array) and a pool of 1 middle-aged healthy male and 6 middle-aged healthy females (mean 49 yrs) (1 co-T2D micro-array).

The purified and pooled monocytes were analyzed for gene expression using DNA microarrays. Image analysis was performed with Microarray Suite, version 5.0 (Affymetrix). To facilitate comparison between samples and experiments, the trimmed mean signal of each micro-array was scaled to a target intensity of 2500. The expression profiles of T1D and T2D micro-arrays were compared to expression profiles of the CoT1D and CoT2D micro-arrays, respectively. Metric files from comparison analysis were exported to Microsoft Excel software package for further filtering and analysis. Genes called significantly changed were those that possessed 1) a reliably detectable signal in control and/or patient sample and 2) were determined by the statistical algorithm to be changed 2-fold or greater. To increase stringency, genes meeting the above criteria were filtered further to include only those that 1) were significantly changed between T1D and CoT1D but not changed between T2D and CoT2D or changed in the opposite direction (increased in T1D versus decreased in T2D, and vice versa) and 2) were consistently differently expressed between T1D and CoT1D in the two chips. The expression profiles were also exported to the Rosetta Resolver Expression Data Analysis System (Rosetta Inpharmatics, Kirkland, Washington, USA) to perform cluster analysis. To increase confidence, the two T1D profiles and two CoT1D profiles were combined in an error weighted fashion into a single T1D experiment and CoT1D experiment, respectively. The data of these T1D and CoT1D experiments were used to create an intensity based ratio experiment. The genes that were changed at least 4-fold and had a $p<0.01$ in this ratio experiment were grouped together as a bio-set. We then clustered data of the T1D-, CoT1D, T2D, and CoT2D experiments restricted to the genes in the bio-sets employing the agglomerative clustering algorithm. The results of this 2D cluster were displayed in a heat map in which each cell represents a log (ratio). Intensities were normalized across all intensity experiments and expressed on a gray scale by use of the software Rosetta Resolver. The increase (white) and decrease (black) in expression level relative to the median value are shown. Rows represent the relative intensity in the four groups. Columns represent the genes. Each cell in the heat map represents a Z-score. This heat map represents the 12 T1D-specific genes, that is the genes that were up or down-regulated in T1D patients only, but not in T2D patients nor in CoT1D and CoT2D. These genes were further validated in Q-PCR.

All raw data obtained by Affymetrix are available as MIAMExpress submission E-MEXP-1516 (http://www.ebi.ac.uk/miamexpress/).

VII

Conclusions and General Discussion

7.1 CONCLUSIONS

The main conclusions of this body of work are:

1) Bipolar disorder is associated with organ-specific autoimmunity, since not only the prevalence of TPO-antibodies (associated with AITD), but also the prevalences of antibodies to GAD65 (associated with autoimmune diabetes) and antibodies to H^+/K^+ ATPase (associated with atrophic gastritis) were higher in bipolar patients than in healthy controls (**Chapter 2**).

2) The monocytes of a large proportion of bipolar patients are in a specific pro-inflammatory state as evidenced by a typical inflammatory gene-expression profile (a "signature", comprising of 19 pro-inflammatory cytokine/compound genes with as putative key gene PDE4B). This phenomenon is in accord with the view of the existence of an activated inflammatory response system in bipolar patients (at least in part of the patients) and provides possible biomarkers for diagnosis and new targets for treatment (such as the putative key gene PDE4B for the inflammatory signature) (**Chapter 3**).

3) The specific pro-inflammatory state of monocytes was also present in the offspring of bipolar parents especially in the offspring developing a mood disorder indicating a possible prognostic value of the determination of the gene-expression signature in monocytes of family members of bipolar patients **(Chapter 3)**.

4) The specific pro-inflammatory state of monocytes in bipolar disorder is not the result of genetic factors as was shown in a twin study, but primarily the result of a common environmental factor. However, although this holds true for the majority of individual "true inflammatory" signature genes, there was a small sub-cluster of chemotaxis/motility genes where genetic influences could dominate (**Chapter 4**).

5) The bipolar-associated pro-inflammatory monocyte state ("the monocyte signature", here defined as PDE4B positivity plus 50% of the other genes positive) was also detected in 1. 50% of LADA and 26% of adult-onset T1D patients (**Chapter 6**), and

2. 37% of patients with AITD.

However, the pro-inflammatory monocyte state could not be the shared vulnerability factor of bipolar disorder and AITD, because bipolar patients with the pro-inflammatory monocyte state did not have a higher risk for thyroid autoimmunity as compared to bipolar patients without the pro-inflammatory monocyte state (**Chapter 5**).

6) In the monocytes of patients with autoimmune diabetes two main correlating sub-clusters were indentified. One sub-cluster (comprising 12 pro-inflammatory cytokine/compound genes with as putative key gene PDE4B) was detected in LADA (60%) and adult-onset T1D (28%), but in only 10% juvenile-onset T1D. A second sub-cluster (comprising 10 chemotaxis, adhesion, motility and metabolism genes) was detected in juvenile-onset T1D (43%) and LADA (33%) and in only 9% adult-onset T1D.

The different prevalences of the 2 subsets of aberrantly expressed inflammatory genes in monocytes of different groups of T1D patients indicates a heterogeneity in

the pathogenesis of autoimmune diabetes, which only partly overlaps with the known diagnostic categories (**Chapter 6**).

Also interesting, some sub-cluster 2 genes were found in the twin study mentioned above to be special in that they were not that tightly influenced by common environmental factors but also by genetic influences (**Chapter 4**).

Taken together, all these conclusions strongly support the concept that an activated inflammatory response system is linked to the pathogenesis of bipolar disorder and to that of endocrine autoimmune disease.

7.2. LIMITATIONS OF OUR STUDIES

Patients and controls

Our studies are based on comparisons between patients and controls. To be able to do this, patients and controls must resemble each other as much as possible, ideally just differing in the presence or absence of disease. However this was not always the case and potential confounding factors influencing results were age, gender, body mass index, smoking behaviour and medication use. In our studies we corrected, whenever possible, for these confounding factors with various statistical methods. However sometimes we could not, because of small sample size or the lack of data on the possible confounding factor. Hence, these factors could have influenced our results to some extent.

Laboratory techniques

The lead player in this thesis is the blood monocyte. Due to its nature (a ”danger” sensing cell) the monocyte is quite sensitive to changes in environment meaning that virtually every manipulation of the cell will set in motion a gene activation program. To perform our tests we had to do quite a few manipulations: the actual drawing of the blood into a collection tube, the density gradient separation, the freeze storage and the $CD14^+$ MACS separation, all on the living cells. One could assume that the found pro-inflammatory profiles are just the result of these manipulations. However, the observed differences between patients and controls are real in our view, since monocytes of patients and controls were manipulated in exactly the same way. However further study is clearly needed.

Analyses methods

Most likely none of the expression profiles described (the PDE4B-associated inflammatory gene-expression signature, sub-cluster 1 and sub-cluster 2) are complete as genes were selected on the basis of arbitrary chosen cut off levels in Affymetrix analysis also taking into account the character of the genes (on the first sight being involved in inflammation or inflammation-related processes). However we justified the selection of genes with a high possible discriminative function only, since we did not aim at getting “fully complete signatures”, but “discriminative signatures”. Consequently the PDE4B-

Table 1. Pro-inflammatory gene-expression profiles in bipolar disorder, AITD and autoimmune diabetes.

Bipolar PDE4B-related signature		Thyroid PDE4B-related signature		Autoimmune diabetes PDE4B-related signature		Autoimmune diabetes	
						Sub-cluster 1	Sub-cluster 2
PDE4B		*PDE4B*		*PDE4B*		*PDE4B*	
IL1B		*IL1B*		*IL1B*		*IL1B*	
IL6		*IL6*		*IL6*		*IL6*	
PTX3		*PTX3*		*PTX3*		*PTX3*	
PTGS2		*PTGS2*		*PTGS2*		*PTGS2*	
TNF		*TNF*		*TNF*		*TNF*	
TNFAIP3		*TNFAIP3*		*TNFAIP3*		*TNFAIP3*	
CCL2		*CCL2*		*CCL2*			*CCL2*
CCL7	"bipolar" genes	*CCL7*	"bipolar" genes	*CCL7*	"bipolar" genes		*CCL7*
CCL20		*CCL20*		*CCL20*		*CCL20*	
CXCL2		*CXCL2*		*CXCL2*		*CXCL2*	
CCR2		*CCR2*		*nd*		*nd*	*nd*
CDC42		*CDC42*		*CDC42*			*CDC42*
BCL2A1		*BCL2A1*		*BCL2A1*		*BCL2A1*	
EMP1		*EMP1*		*EMP1*			*EMP1*
DUSP2		*DUSP2*		*DUSP2*		*DUSP2*	
ATF3		*ATF3*		*ATF3*		*ATF3*	
NAB2		*NAB2*		*NAB2*			*NAB2*
MAPK6		*MAPK6*		*MAPK6*			*MAPK6*
		FCAR	"thyroid genes""	*STX1A*	"T1D" genes		*STX1A*
		ADAM17		*PTPN7*			*PTPN7*
		EIF2S3		*DHRS3*			*DHRS3*
							FABP5

associated inflammatory signature found in bipolar patients (Chapter 2) differs to some extent from PDE4B-associated inflammatory signature mentioned in AITD (Chapter 3) and autoimmune diabetes (Chapter 4). In Table 1 an overview is given on the present state of the art of the various signature genes.

7.3 THE RELATIONSHIP BETWEEN THE VARIOUS PRO-INFLAMMATORY GENE-EXPRESSION PROFILES

Examining in retrospect the PDE4B-associated inflammatory profile found in monocytes of bipolar patients, a similar "pro-inflammatory sub-cluster 1" and "motility/ chemotaxis sub-cluster 2" grouping can be observed as was later found in autoimmune diabetes (see Chapter 3, Figure 1), but less obvious: *IL1B, IL6, PTX3, PTGS2, TNF,*

TNFAIP3, CCL20, CXCL2, BCL2A1, DUSP2 and *ATF3* (sub-cluster 1 genes according to Chapter 5) are found highly correlated with *PDE4B* and with each other, while *CCL2, CCL7, CDC42, EMP1, NAB2* and *MAPK6* (sub-cluster 2 genes according to Chapter 5) are only moderately correlated with *PDE4B* and the other sub-cluster 1 genes. In the same way, the two sub-clusters in autoimmune diabetes are not two independent profiles but are correlating with each other forming a profile very similar to the PDE4B-associated profile of bipolar disorder (Chapter 5, Figure 1). This entire profile of sub-cluster 1 plus sub-cluster 2 is predominantly present in LADA patients. How and why these two sub-clusters are associated needs further investigation.

Figure 1 gives the possible relationships between the various genes found in the above mentioned profiles. All is based on literature data.

7.4 THE ORIGIN OF THE INFLAMMATORY GENE-EXPRESSION PROFILES

From our twin study it became clear that the bipolar-associated inflammatory gene-expression profile is almost entirely the result of shared environmental factors between the twins. This paragraph will discuss some environmental factors that are reported as being associated with inflammatory monocyte activation, bipolar disorder, AITD and type 1 diabetes.

Infections

Infections are first possible candidates. Next to having a clear effect on the immune system, they have also been implicated in the pathogenesis of all three disorders.

To begin with bipolar disorder, studies have reported that being born or raised in a city is a risk factor for developing bipolar disorder. This is thought to be due to household crowding, and the consequent high exposure to infectious agents.[1] Also relating bipolar disorder to infections are the reports on an excess of winter and spring births in bipolar disorder as winter and spring borne children are thought to be more prone to develop perinatal infections.[2] Of the infectious agents, especially viruses are likely candidates[3]. First, they are known for their neurotropism and latency. Second, virus infections can be accompanied by depressive symptoms and manic behaviour. And third, the mood stabilizers lithium and valproate have been described to have antiviral effects.[4, 5] The viruses mentioned in the literature as being associated with bipolar disorder are Herpes Simplex virus (HSV), Cytomegalovirus (CMV) and Borna virus.[3, 6, 7] However, data are very inconsistent with regard to the presence of virus-specific antibodies in serum as well as with regard to detecting virus RNA in brain or in peripheral blood mononuclear cells of bipolar patients.[3, 6, 7] With regard to bacterial infections, Borrelia Burgdorferi is known to be able to induce symptoms reminiscent of those seen in bipolar disorder;[8] unfortunately no systematic research has been done on the prevalence of this bacterium in bipolar disorder. Toxoplasmosis, an intracellular protozoan parasite, is capable of latency and

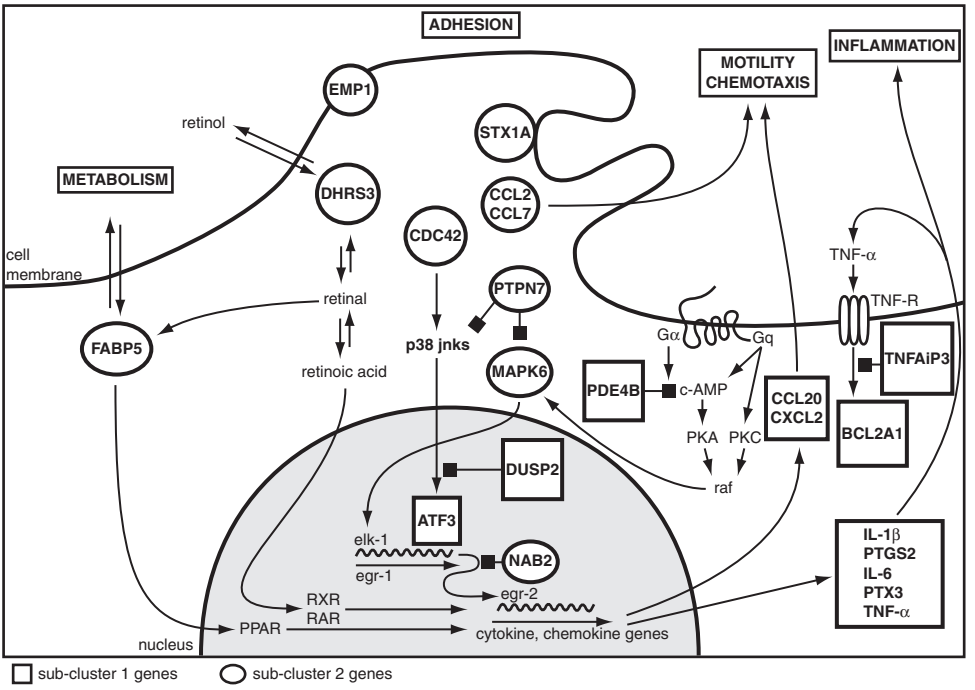


Figure 1. The possible relationships between the various genes of the inflammatory gene-expression profiles.

The possible function of the 19 "bipolar" genes has been explained in Chapter 3 (legend Figure 2) in a similar, but less extensive figure. The remaining ones will be shortly discussed here. All is based on literature data.

Fatty acid binding proteins (FABPs) are thought to be at the interface of the metabolic and inflammatory systems and play a role in the binding and intracellular transport of long fatty acids,[87] including metabolites of the cyclooxygenase pathway and the storage of lipids. Mice deficient for FABPs display protection from atherosclerosis, improved glucose and lipid metabolism, an increased survival, but also a reduced sensitivity to experimentally-induced autoimmunity,[88] while their DC are poor producers of the pro-inflammatory Th1 skewing cytokine IL-12 (although this has also been disputed.[89] When FABP5 is knocked out in macrophages the expression of CCL2 (a sub-cluster 2 gene) is (amongst other cytokines) significantly reduced.[90] Interestingly FABP5 also shuttles retinoic acid (the active metabolite of Vitamin A) to PPARβ/δ regulating survival pathways in the cell[91], whilst DHRS3 (SDR1) is an important enzyme in the Vitamin A metabolism catalyzing the transition from retinol to retinal.[92] Retinoids are able to influence integrin expression, Rac/Rho proteins and paxillin[93] and to modulate the gene expression of CCL2.[94] Disturbances in the retinoid metabolism have been described in T1D patients and BB-DP rats.[95]

The SNARE molecule STX1A plays a role in the fusion of secretory vesicles to the outer membrane[96] particularly in lipid rafts.[97] Interestingly STX1A plays a key role in the secretion of insulin from β-cells[98] and a polymorphism in the gene (D68D, T to C) correlates to the age of onset and the insulin requirements of T2D patients.[99] PTPN7 is a DUSP and predominantly blocks the ERKs and p38.[100] The molecule itself is activated by downstream molecules in the ERK pathway and this activation thus forms a negative feedback mechanism.

FABP, fatty acids binding protein; PPAR, peroxisome proliferator-activated receptor; DHRS3, dehydrogenase/reductase (SDR family) member 3; SDR1, short-chain dehydrogenase/reductase 1; SNARE, soluble NSF attachment receptor; STX1A, syntaxin 1A; PTPN7, protein tyrosine phosphatase, non-receptor type 7; DUSP, dual specificity phosphatases; ERK, extracellular signal-regulated kinase; DC, dendritic cells

brain infiltration[9] and is therefore an interesting candidate to study in psychiatric diseases. In schizophrenia many studies have reported a correlation to Toxoplasmosis infection,[10] especially with prenatal exposure. However, for bipolar disorder (to our knowledge) only one study is available, and this study reports a negative result with regard to the presence of T. gondii sequences in post mortem brains.[11] Nevertheless, valproate does inhibit T. gondii development,[12] suggesting that more extensive research is needed to determine whether or not Toxoplasmosis plays a role of significance in bipolar disorder as it is been suggested to do in schizophrenia.

The period of time in which both twins have the highest chance to be exposed to the same pathogens is in uteri. Prenatal infections mentioned to be correlated with psychiatric diseases include influenza,[13] rubella, Toxoplasmosis and HSV.[14] However, most correlations have been found with schizophrenia. Again no extensive studies have been conducted in bipolar disorder.

(Perinatal) infections are thought to be important in mood disorder development via a direct damaging effect on the brain in genetically at risk individuals.

With regard to autoimmune thyroid disease and type 1 diabetes the infections associated with their pathogenesis are mentioned in Table 4 of Chapter 1. Notably and surprisingly there is only little overlap with the micro-organism thought to play a role in mood disorders. The mechanisms behind the involvement of infections in autoimmunity include direct β-cell lysis, bystander activation of autoreactive T cells, loss of regulatory T cells and molecular mimicry.

Stress

As indicated above the prenatal period is an interesting period to study for the interpretation of our data, as the environmental factors experienced by the twins in uteri are strongly shared. One of the next possible environmental factors that is experienced in uteri and can influence both the immune system as well as the brain, is prenatally experienced stress.

Literature describes that the effect of prenatal stress on the immune system mostly leads to a reduction of immune function.[15] However, a few studies report on an exaggeration of inflammatory function after prenatal stress. Hashimoto *et al*. showed that prenatal stress in rats led to an increased fever response to LPS.[16] In addition, Laviola *et al.* demonstrated an increase of spleen and brain frontal cortex levels of IL-1β in prenatally stressed rats.[17] Possible mechanisms behind prenatal-stress-induced immune alterations are thought to be 1) a direct influence of maternal hormones and neurotransmitters on the ontogeny of immune cells, 2) an indirect effect via deregulation of the HPA-axis in the prenatally stressed offspring and 3) a via stress mediators induced change in placental function.[15]

With regard to the effect of prenatal stress on the brain, there is increasing evidence suggesting that exposure to prenatal stress is a risk factor for psychopathology. Prenatally stressed rats for instance show higher emotional reactivity, higher levels of anxiety and a depressive-like behaviour.[18] In humans, a low birth weight is considered an index of prenatal

stress, and indeed low birth weight has been shown a risk factor for later development of mood symptoms.[19] Also, the amount of stress experienced by the mother during pregnancy was positively correlated to emotional, cognitive and behavioural problems of the offspring.[20] It is suggested that stress exposure at critical time points during foetal development may 1) influence the HPA-axis,[21] leading to glucocorticoid resistance and hypercortisolism, 2) alter brain development, and 3) change neurotransmitter systems.[18, 20-22]. All these events have been implicated in the pathogenesis of mood disorders.

The impact of stress on the immune system in *adulthood* has extensively been researched. It is a complex interaction in which the HPA-axis and the sympathetic nervous system play pivotal roles, especially their neuroendocrine products cortisol and catecholamines. These two main mediators of stress effects can regulate a variety of immune functions such as cytokine and chemokine production, the trafficking of immune cells and their proliferation, differentiation and effector functions. The final outcome is, although dependent on the quantity and quality of stress and on coping strategies, an increased susceptibility to infection and inflammatory and autoimmune diseases.[23, 24]

Stress experienced later in life is able to induce mood symptoms. In rats it was shown that chronic mild stress experienced during adulthood elicited depressive-like behaviour.[25] And, in children of bipolar patients, major life events increased the risk to develop a mood disorder.[26]

With regard to type 1 diabetes, study has demonstrated that more negative life events were experienced by patients prior to disease than by healthy controls and that experiencing negative life events increased the risk for type 1 diabetes.[27] Also, prenatal stress seems to influence beta cell autoimmunity as Lernmark *et al.* showed that islet antibodies in core blood was positively correlated with the experienced stress of the mother during pregnancy.[28] The current idea about this relationship between psychological stress and T1D development is based on the “β-cell stress hypothesis” of type 1 diabetes which suggests that any event that increases demand on the β-cell should be considered a possible risk factor for type 1 diabetes. Life events are known to reduce insulin sensitivity and insulin secretion and hence increase pressure on the beta cell. This could accord the β-cell stress hypothesis lead to type 1 diabetes.

Also for AITD, literature predominantly shows a positive relationship between negative life events and the development of disease, especially for GD.[29-32] However one recent report contradicted these results by showing that experienced stress did not correlate with TPO-antibodies.[33] The idea here is that stress influences AITD development primarily via its effect on the immune system.

Diet

Omega-3 fatty acids

Diet and in particular the consumption of omega-3 fatty acids (present in marine life and various plants)[34] is a third possible shared environmental factor connecting bipolar disorder, autoimmune diabetes and AITD with each other and the immune system.

The immune system is known to be shaped and regulated by polyunsaturated fatty acids. Omega-6 fatty acids are recognized to potentiate inflammatory responses while omega-3 fatty acids are known to have anti-inflammatory effects. Studies reporting on effects of omega-3 fatty acids on monocyte activation have been inconsistent, with data demonstrating a reduction of IL-1, IL-6 and TNF-α production but also many reports showing no effect on monocyte activation.[35]

Various epidemiological studies have reported an inverse relation between fish consumption and mood disorders.[36] In addition, Tiemeier *et al.* showed that elderly with depressive symptoms had lower serum levels of omega-3 fatty acids than control subjects without depressive symptoms.[37] Furthermore, treatment of bipolar patients with omega-3 fatty acids decreased affective symptoms and prolonged remission periods.[38-41] This positive effect of omega-3 fatty acids on mood disorder development is amongst others thought to be due to its effect on brain plasticity via brain derived neurotrophic factor. In addition, omega-3 fatty acids are abundantly present in cell membranes. Changes in lipid concentrations could change structure and function of various cell membrane proteins such as receptors and enzymes changing in this way cell signalling in brain tissue. And finally, the positive effect of omega-3 fatty acids could be achieved via the immune system.

As for diabetes, a recent study illustrated that consumption of omega-3 fatty acids was inversely associated with the development of islet autoimmunity in children genetically at risk for type 1 diabetes.[42] In addition, a case control study demonstrated that children with type 1 diabetes had been given less often cod liver oil (which contains omega-3 fatty acids) than children without diabetes.[43] In the same way, long chain fatty acids prevented chemically-induced diabetes in an animal model.[44] With regard to AITD no data is available on its association with omega-3 fatty acids.

Fatty acids probably are beneficial in autoimmunity via its effect on the immune system. However, it must be noted that omega-3 fatty acids improve insulin resistance and this could subsequently reduce β-cell stress[45] suggesting that also the β-cell stress hypothesis could apply here.

Gluten

Another dietary factor that could explain the correlation of monocyte activation, bipolar disorder, AITD and T1D is gluten. Gluten has been demonstrated to induce maturation and chemokine/cytokine secretion of dendritic cells (which are -as said- descendents of monocytes).[46] Furthermore, it reduced the number of intestinal regulatory T cells.[47] Both effects will lead to an enhanced immune response.

As for bipolar disorder, some studies observed an improvement of depressive disorders in patients with celiac disease after the introduction of a gluten free diet.[48] The mechanisms behind this are unclear.

In animal models of T1D it was reported that introduction of gluten in the diet accelerated disease progression. Similarly, infants who had been given a diet containing gluten before

the age of 3 months, had a higher risk to develop islet cell autoimmunity than children who had only been breastfed or children who had been exposed to gluten at the age of 4 and 6 months. Furthermore, a gluten-free diet in high risk individuals improved insulin response during the glucose tolerance test.[49] With regard to AITD an improvement of thyroid abnormalities (thyroid antibodies, thyroid function) after gluten free diet have been reported but rather inconsistent.[50] A reason for this could be that -as with T1D- gluten should be avoided before the development of autoimmunity. Once autoimmunity is established, removing gluten from the diet has only limited effects.

Two possible mechanism behind this correlation of gluten introduction and autoimmunity are (next to the above mentioned effects on the immune system) 1) a by gluten induced higher permeability of the intestine[49] and 2) the exposure of cryptic antigens in the intestine by an gluten-induced immune response.[51]

Vitamin D

Another possible candidate that might influence all three diseases and the immune system is Vitamin D. The primary source of Vitamin D is sunlight but it can also be taken up from food (e.g. fatty fish and their oils). Vitamin D interacts with the immune system and has multiple effects. However its main effect is tolerogenic.[52]

Vitamin D deficiency is reported to be associated with mood disorders.[53, 54] A possible mechanism behind this association is the effect of Vitamin D on neurotrophins (which play a role in neuroprotection) and neurotransmitters.[55]

Vitamin D deficiency has been associated with various autoimmune diseases as well such as systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis but also T1D and AITD. With regard to the latter two, vitamin D administration in animal models prevented the development of insulitis, diabetes and thyroiditis.[56] Regulatory intake of Vitamin D in childhood reduced the risk of T1D development. Low serum levels of Vitamin D have been found in T1D and AITD patients.[57] And, AITD as well as T1D have been associated with polymorphisms in the Vitamin D receptor and binding protein.[58-63] In addition, a higher intake of Vitamin D during pregnancy reduced β cell autoimmunity.[63, 64] No other mechanisms than the effect of Vitamin D on the immune system has been suggested to play a role in the observed association of Vitamin D and autoimmunity.

Taken together, infections, stress and dietary components are all factors mentioned in literature to be associated with the immune system, bipolar disorder and related endocrine autoimmune diseases.

7.5 CAUSALITY

In theory the association of monocyte activation with bipolar disorder, AITD and T1D can be explained in three ways. To determine which causative pathway is correct more extensive research is needed but three options are tempting to speculate on.

First, the presence of pro-inflammatory monocytes (due to for instance an intrinsic abnormality) could lead to bipolar disorder (macrophage theory of depression, see Chapter 1.3) and to autoimmunity (Chapter 1.7). In our studies we did not find evidence that refutes the first causal relationship (pro-inflammatory monocytes give mood disorders, Chapter 3), however our data do not directly lend support to the second causal relationship (pro-inflammatory monocytes give endocrine auto-immunity) as in bipolar patients monocyte activation was not correlated to the presence of concomitant thyroid autoimmunity (Chapter 5).

Second, there could be a set of mutually dependent underlying factors that induce monocyte activation, bipolar disorder as well as endocrine autoimmune disease but independently from each other. Possible underlying factors have been mentioned above, such as infections, stress, diet and Vitamin D status, and various theories about these factors causing mood disorders and endocrine disease separate from an activation of monocytes/macrophages have been mentioned throughout the text. A putative other example of an underlying co-factor could be an activation of the T cell system or an activation of other parts of the immune system independently from the here described monocyte activation, yet having its own effect on the induction of mood disorders and endocrine autoimmunity. However, it can also be proposed that (one of) these factors is/are the initiator(s) of a cascade with pro-inflammatory monocytes being an early component in this, bringing the causative pathway back to option 1.

Third, bipolar disorder and its associated autoimmune diseases could cause monocyte activation. However this option is, especially with regard to bipolar disorder, theoretically more difficult to comprehend, since children of a bipolar parent and co-twins of a bipolar index case show monocyte activation in the absence of mood symptoms (Chapters 3 and 4).

7.6 IMPLICATIONS FOR FURTHER RESEARCH AND THE CLINICAL PRACTICE

Diagnosis

The diagnosis of bipolar disorder is complicated. Under-diagnosis is a frequent problem (see introduction). This leads to under-treatment of many bipolar patients. The here found pro-inflammatory monocyte signature might facilitate this difficult diagnoses of bipolar disorder. Especially for patients free of lithium and antipsychotic treatment this new diagnostic tool could be helpful. The ROC (receiver operating characteristic) curve showed a discriminating ability of 82%, which means that 82% of those with and without disease were classified correctly using the pro-inflammatory signature (defined as PDE4B positive + 25% of the other genes) as diagnostic test in bipolar patients who were free of lithium and antipsychotics. The measured sensitivity and specificity were both 82%. These figures look promising, however one must keep in mind that relatively small numbers of patients and controls were investigated. Furthermore, our data show

that the signature is able to discriminate bipolar patients from healthy controls. But what about its ability to discriminate bipolar disorder from unipolar depression? As the main reason for under-diagnosis of bipolar disorder was misdiagnosis with unipolar depression, answering this question is a priority. It is already clear that the PDE4B-associated gene-expression profile is not bipolar-specific since also many AITD and autoimmune diabetes patients are positive.

Prognosis

Since the pro-inflammatory monocyte gene-expression signature was present in offspring of bipolar patients, especially in those developing a mood disorder, it is tempting to speculate that the signature has prognostic value. If this holds true after testing larger patient samples it could have major implications for prediction of disease progression and treatment strategies (anti-inflammatory agents, diet, stress-coping strategies) in affected families.

Treatment

Bipolar disorder is difficult to treat using the present therapies (see introduction). The detection of the signature may open new avenues for innovative forms of treatment by providing new drug targets.

PDE4B for instance could be such a possible target for therapy. PDE4B is essential for monocyte activation[65] and is also known to play a role in mood.[66] PDE4B blockers were used in the early nineties for unipolar depression and although quite efficient in treating the mood disturbances,[67] clinical trials were stopped because of the accompanying side effects (nausea). Currently, new, less nauseatic PDE4B blockers are in far stage of development.[68, 69]

Another possible target is PTGS2 (COX2). One study reported beneficial effects in unipolar depression of additional use of a COX2 inhibitor next to the conventional therapy of raboxetine.[70] With regard to AITD and autoimmune diabetes, new and existing therapies targeting the pro-inflammatory state of monocytes/macrophages might be useful in patients suffering from AITD and diabetes together with mood disturbances. And, perhaps providing this treatment to individuals at risk might even prevent development of thyroid and islet autoimmunity. Indeed, in animal models it was demonstrated that PDE4 blockers[71, 72] and anti-TNF therapy reduced autoimmunity.[73] However, timing of this therapy seems to be important factor for success.

7.7 THE PRESENCE OF TWO MONOCYTE GENE-EXPRESSION SUB-PROFILES IN AUTOIMMUNE-MEDIATED DIABETES

With regard to a pro-inflammatory state of monocytes and monocyte-derived cells in patients with T1D the literature is inconsistent.[74-86] Our data illustrating the presence of two inflammatory monocyte gene expression sub-profiles in T1D could provide a possible

explanation for this inconsistency. Sub-cluster 1 primarily involves cytokine/inflammatory compound genes, while sub-cluster 2 involves genes of chemotaxis, adhesion, motility and metabolism. Examining diabetes patients with a sub-cluster 1 profile would lead to findings of raised levels of cytokines and inflammatory compounds, whereas studying patients with a sub-cluster 2 profile only would lead to negative results for these cytokines/compounds. This implies that more consistent reports can be expected if patients are before hand divided in sub-cluster 1 or sub-cluster 2 positives. Our detected profiles thus provide a new way of classifying autoimmune diabetes which only partly overlaps with the existing diagnostic categories.

7.8 FUTURE DIRECTIONS

As is usual with research, the ending of one study, is the beginning of many others, the answer to one question raises a dozen more.
The following research issues are essential to make further progress:

1. Confirming the presence of the pro-inflammatory profiles in larger patient samples.
2. Verifying the possible prognostic value of the inflammatory monocyte gene-expression signature for bipolar disorder by adding a fourth round to the bipolar offspring study to see whether or not the adolescents with a positive monocyte signature expression have developed mood symptoms more often than those without the monocyte signature. A same prognostic value could be suggested for the pro-inflammatory profiles in type 1 diabetes and tested by studying individuals at risk for autoimmune diabetes development.
3. Establishing a correlation between the pro-inflammatory profiles with various biological and clinical parameters of disease (islet antibodies, TPO-antibodies, brain abnormalities, HPA-axis abnormalities, genetic polymorphisms).
4. Determining correlations of the aberrant gene expressions with functional abnormalities. Several approaches come to mind.
   First, via usage of *in vitro* studies. Possible objectives are to determine the actual PDE4B activity in the PDE4B signature positive monocytes, to find an abnormal high production of cytokines that belong to PDE4B signature and sub-cluster 1 positive monocytes, and to find motility and adhesion abnormalities in sub-cluster 2 postive monocytes.
   Second, animal models could be used. The NOD mouse has primarily been used as a model for autoimmune diabetes and thyroiditis, but NOD mice also have an altered behaviour, indicating that perhaps these mice could be studied in the context of mood disorders as well. This needs to be further examined, while also the presence of the pro-inflammatory profile needs to be confirmed in NOD mice. If such a profile is present in the NOD mouse, it can be correlated to the found functional aberrancies of the monocytes in these animals especially with regard to

pro-inflammation, migration and adhesion (see introduction). By silencing possible key genes from the profiles (such as PDE4B) with for instance siRNA it is possible to investigate whether subsequently the functional aberrancies will improve or disappear.

A third approach is the determination of signature and sub-cluster molecules (such as certain cytokines and chemokines) in the serum of patients.

5. Establishing possible environmental factors that cause the observed aberrantly expressed gene-expression profiles. This issue can be addressed via *in vitro* and animal studies by introducing the environmental factor (infection, prenatal stress, a special diet).
6. Testing the possible new therapeutic interventions in animal models. Will treatment of NOD mice with f.i. a PDE4B inhibitor or a COX-2 inhibitor lead to reduced insulitis, diabetes development and thyroiditis? Is there time dependency? Will the gene-expression profiles change in monocytes after targeting the profile genes with drugs? If behavioural changes have been confirmed in the NOD mouse, will PDE4B inhibitor treatment alter these?

7.9 FINAL REMARK

The results presented in this thesis have provided further evidence regarding the association between bipolar disorder and autoimmunity. They have confirmed the presence of an activated inflammatory response system in bipolar disorder and the association of bipolar disorder with endocrine autoimmune diseases. Additionally, by showing the presence of two inflammatory monocyte sub-profiles in type 1 diabetes, it has suggested heterogeneity in the pathogenesis of T1D.

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SUMMARY

Bipolar disorder, previously known as manic-depressive illness, is a mood disorder in which patients suffer from severe and extreme mood swings. The etiology is far from unraveled but is thought to involve multiple genes as well as environmental factors. Recently the immune system has been implicated in the pathogenesis of bipolar disorder. Various abnormalities have been reported, indicating the presence of an activated inflammatory response system. Furthermore, data revealed a higher susceptibility for autoimmunity in these patients since they had higher serum levels of TPO-antibodies (associated with autoimmune thyroid disease) and antinuclear antibodies (associated with systemic autoimmune diseases). The higher susceptibility for thyroid autoimmunity was also seen in first-degree relatives of bipolar patients, independent from any mood disturbances. To further explore this association of bipolar disorder with autoimmunity, we investigated the prevalence of autoantibodies associated with type 1 diabetes, the GAD65-abs in bipolar patients. We also measured serum levels of autoantibodies associated with atrophic gastritis (H/K ATPase-Abs). For both autoantibodies the prevalence of positivity was found higher in bipolar patients than in healthy controls (GAD65: 11.7% versus 6.1%, H/K ATPase: 11.3 versus 2.6%), strengthening the concept that patients with bipolar disorder have a higher risk for autoimmune diseases. The data are presented in **Chapter 2**.

In **Chapter 3** we describe a study in which we examined one particular cell of the inflammatory response system, the circulating monocyte, in more detail, since especially this cell and its descendents are important in the activation of the inflammatory response. We studied the gene expression levels in these monocytes via the advanced technique of Affymetrix whole genome screening. The most discriminative genes with a known function in inflammation were chosen for further analysis. Via this method we detected an inflammatory gene-expression signature comprising of 19 pro-inflammatory cytokine/compound genes with a possible key gene PDE4B in the monocytes of a large proportion of bipolar patients. We discussed that this signature might be helpful in the diagnosis of bipolar disorder and could also lead to new therapeutic strategies by providing new targets for treatment (such as the possible key molecule PDE4B). Furthermore, the gene-expression profile was also found in the monocytes of offspring of bipolar patients, especially in those developing a mood disorder. This might indicate that a positive monocyte signature test could have prognostic value.

Subsequently, we wanted to investigate whether the inflammatory state of monocytes found in bipolar disorder was the result of genes or environmental factors. Therefore we conducted a twin study including monozygotic and dizygotic bipolar and healthy twin pairs. The data (presented in **Chapter 4**) showed that the inflammatory gene-expression signature was mainly the result of common environmental influences. The same could be concluded for the association of the gene-expression profile with bipolar disorder. This means that similar environmental factors influence the expression of bipolar disorder as well as the inflammatory signature. However, although this holds true for the majority

of the “true inflammatory” signature genes, there was a small subcluster of chemotaxis/ motility/ cell survival genes where genetic influences could dominate.

Monocytes and monocyte-derived cells are not only involved in the pathogenesis of bipolar disorder but are also considered prime suspects in the pathogenesis of type 1 diabetes and autoimmune thyroid disease (AITD). Given this central role of monocytes in the etiology of bipolar disorder, type 1 diabetes and AITD, and given the previously found association of bipolar patients and their first-degree relatives with organ-specific autoimmunity, we proposed pro-inflammatory monocytes as being the shared vulnerability factor for bipolar disorder and organ-specific autoimmunity. Therefore we investigated the presence of the in **Chapter 3** described monocyte gene-expression signature in AITD patients (**Chapter 5**) and T1D patients (**Chapter 6**).

In **Chapter 5** we reported the presence of the monocyte inflammatory gene-expression profile in about a third of AITD patients. However, we had to reject our hypothesis that these pro-inflammatory monocytes are the shared vulnerability factor between bipolar disorder and thyroid autoimmunity since bipolar patients with the pro-inflammatory monocyte state did not have a higher risk for thyroid autoimmunity as compared to bipolar patients without the pro-inflammatory monocyte state.

The “bipolar” inflammatory gene-expression signature was also found in the monocytes of patients with autoimmune diabetes, i.e. in 50% of LADA patients and 26% of T1D patients with an adult onset, but in only 13% juvenile-onset T1D (the latter not different from healthy controls). This is presented in **Chapter 6.** Next to analyzing the bipolar genes in diabetes monocytes, we also included new genes found via Affymetrix whole genome analysis performed on the monocytes of young T1D patients. This resulted in the recognition of two monocyte sub-clusters in patients with autoimmune diabetes. One sub-cluster (comprising of 12 pro-inflammatory cytokine/compound genes with as putative key gene PDE4B) was detected in LADA (60%) and adult-onset T1D (28%), but in only 10% juvenile-onset T1D. A second sub-cluster was comprised of 10 chemotaxis, adhesion, motility and metabolism genes and was detected in juvenile-onset T1D (43%) and LADA (33%) but hardly (9%) in adult-onset T1D. The presence of these two pro-inflammatory states of monocytes in type 1 diabetes implies heterogeneity in autoimmune diabetes only partly overlapping with the known diagnostic categories.

In **Chapter 7** (the general discussion) we discuss the various pro-inflammatory monocyte profiles. We show that sub-cluster 1 and sub-cluster 2 (found in diabetes patients) can (in retrospect) also be discovered in the bipolar inflammatory profile. Furthermore, sub-cluster 1 and 2 correlated with each other forming a profile, which closely resembles the “entire” PDE4B-associated signature found in a considerable proportion of bipolar and LADA patients. Why these (sub-clusters of) genes correlate in such a specific way in various diseases is not yet clear and needs further investigation.

In addition, we suggest in **Chapter 7** that infection, stress and dietary components are possible candidates for the shared environmental influences on monocyte activation and bipolar disorder (and possibly also autoimmune diabetes and AITD), as they are

known from literature to be associated with monocyte activation, bipolar disorder, AITD and autoimmune diabetes. Furthermore, we state that especially the prenatal period is interesting to study since most environmental influences experienced in uteri will be shared between twins.

SAMENVATTING

De bipolaire stoornis, ook bekend als de manische- depressieve stoornis, is een stemmingsstoornis waarbij patiënten ernstige stemmingswisselingen hebben. De etiologie is nog steeds niet duidelijk, maar zowel meerdere genen als omgevingsfactoren worden verondersteld een rol te spelen. Recent is het immuunsysteem geïmpliceerd in de pathogenese van de bipolaire stoornis, omdat bij bipolaire patiënten verschillende afwijkingen in het immuunsysteem zijn gerapporteerd. Zo zijn in bipolaire patiënten sommige cellen van het immuunsysteem meer actief dan in gezonde controles en hebben ze een verhoogde gevoeligheid voor het ontwikkelen van autoimmuniteit. Dit is gebleken uit rapportages over verhoogde serumconcentraties van TPO-autoantistoffen (geassocieerd met schildklier-autoimmuniteit) en antinucleaire antistoffen (geassocieerd met gegeneraliseerde autoimmuniteit). Deze hogere kwetsbaarheid voor het ontwikkelen van schildklier-autoimmuniteit is ook geobserveerd in eerstegraads verwanten van bipolaire patiënten, onafhankelijk van stemmingsstoornissen. Om deze associatie van de bipolaire stoornis met autoimmuniteit verder te analyseren hebben we onderzoek gedaan naar de prevalentie van autoantistoffen geassocieerd met type 1-diabetes (GAD65-antistoffen) en met atrofische gastritis (H/K ATPase- antistoffen) bij bipolaire patiënten. We vonden voor beide autoantistoffen een hogere prevalentie bij bipolaire patiënten dan in gezonde controles (GAD65: 11.7% vs 6.1%, H/K ATPase: 11.3 vs 2.6%). Deze resultaten bevestigen de observatie dat bipolaire patiënten gevoeliger zijn voor autoimmuniteit. Dit onderzoek wordt gepresenteerd in **Hoofdstuk 2**.

In **Hoofdstuk 3** beschrijven we een studie waarin we een bepaalde cel van het immuunsysteem, de monocyt, nader onderzocht hebben. Dit is een belangrijke cel van het niet-specifieke immuunsysteem. Met name in deze cel (en zijn afstammelingen) zijn afwijkingen gevonden bij patiënten met een bipolaire stoornis. We hebben in de monocyten van bipolaire patiënten gezocht naar afwijkende genexpressies via de geavanceerde methode van de Affymetrix- analyse. Dit is een methode waarbij de genexpressie van duizenden genen tegelijkertijd kan worden gemeten. Deze analyse resulteerde in een lange lijst van genen die afwijkend tot expressie kwamen bij patiënten. Uit de betreffende genen werden die gekozen, welke het meest discriminerend waren (dus die het meest in waarde verschilden van de gezonde controlewaarde) en die een bekende functie hadden in inflammatie. Deze manier van gen-selectie resulteerde in de ontdekking van een gen-expressie signatuur bestaande uit 19 genen die allemaal een rol spelen in inflammatie. De signatuur was aanwezig in de monocyten van een groot deel van de bipolaire patiënten (tot 60%) en heeft mogelijk PDE4B als sleutelgen. PDE4B is namelijk een molecuul dat essentieel is voor inflammatie, maar ook bekend staat als zijnde betrokken bij de regulatie van stemming aangezien PDE4B-blokkers vroeger gebruikt werden bij patiënten met een depressieve stoornis. De “bipolaire” pro-inflammatoire gen-expressie signatuur is ook gevonden in kinderen van bipolaire patiënten, met name bij degenen die later ook een stemmingsstoornis ontwikkelden. Dit zou kunnen betekenen dat een positieve monocyten signatuurtest prognostische waarde heeft.

Vervolgens wilden we onderzoeken of de hierboven genoemde bipolaire monocyten gen-expressie signatuur het resultaat was van genetische dan wel omgevingsinvloeden. Om hier een antwoord op te kunnen vinden, hebben wij een tweelingstudie verricht, welke gepresenteerd wordt in **Hoofdstuk 4**. In deze studie hebben we gebruik gemaakt van eeneiige en twee-eiige gezonde en bipolaire tweelingparen. Aangezien de eeneiige tweelingparen alle genen delen en de twee-eiige tweelingparen maar de helft, zal een hogere concordantie van een bepaalde variabele (zoals de monocyten signatuur) bij eeneiige tweelingparen dan bij twee-eiige tweelingparen een genetische invloed op die bepaalde variabele betekenen. Echter, als beide concordanties even hoog zijn, betekent dit een invloed vanuit de omgeving, aangezien die voor zowel eeneiige tweelingparen als twee-eiige tweelingparen dezelfde zal zijn. Wij vonden dat de inflammatoire gen-expressie signatuur het gevolg was van met name omgevingsfactoren. Ditzelfde gold voor de relatie van de signatuur met bipolariteit. Dit betekent dat dezelfde omgevingsfactoren invloed hebben op monocyten en op bipolariteit.

Monocyten en hun afstammelingen zijn niet alleen belangrijk in de pathogenese van de bipolaire stoornis. Ze zijn namelijk ook hoofdverdachten in de pathogenese van autoimmune schildklierziekten en type 1-diabetes. Gegeven deze centrale rol van de monocyten in de etiologie van de bipolaire stoornis, AITD (autoimmune thyroid disease, autoimmuun schildklierziekte) en type 1-diabetes en de hierboven genoemde associatie van bipolariteit met autoimmuniteit, hebben we de pro-inflammatoire monocyt verondersteld als de gedeelde kwetsbaarheidsfactor. Daarom hebben wij gezocht naar de in **Hoofdstuk 3** beschreven “bipolaire” monocyten gen-expressie signatuur in de monocyten van AITD (**Hoofdstuk 5**) en T1D-patiënten (**Hoofdstuk 6**).

In **Hoofdstuk 5** rapporteren we dat in ongeveer een derde van de AITD-patiënten de monocyten inflammatie gen-expressie signatuur aanwezig is. Echter, we hebben toch onze hypothese moeten verwerpen dat deze pro-inflammatoire monocyt de gedeelde kwetsbaarheidsfactor is tussen de bipolaire stoornis en endocriene autoimmuniteit, aangezien bipolaire patiënten met een positieve signatuur geen hoger risico bleken te hebben op het ontwikkelen van schildklierautoimmuniteit dan de bipolaire patiënten zonder de pro-inflammatoire monocyten status.

De “bipolaire” inflammatoire gen-expressie signatuur werd ook gevonden in de monocyten van patiënten met autoimmuun diabetes. Dit zijn niet alleen de type 1-diabeten, maar ook de patiënten die niet voldoen aan de criteria om een type 1-diabeet genoemd te worden, maar die wel autoantistoffen tegen de pancreas hebben, de zogenoemde LADA-patiënten (LADA=Latent Autoimmune Diabetes of the Adults). LADA-patiënten zijn vaak ouder dan type 1 diabetes-patiënten en zijn in het algemeen niet meteen insulineafhankelijk. Vijftig procent van deze LADA-patiënten was positief voor de “bipolaire” monocyten signatuur, alsmede 26% type 1-diabeten bij wie de ziekte manifest was geworden op volwassen leeftijd. Van de type 1-diabeten bij wie de ziekte zich al presenteerde op jonge leeftijd, was maar 13% positief, niet verschillend van de gezonde controles. Deze bevindingen zijn gepresenteerd in **Hoofdstuk 6**. Naast het

bestuderen van de aanwezigheid van de “bipolaire” genen hebben we ook gezocht naar nieuwe genen, weer via de geavanceerde methode van Affymetrix-analyse, maar nu bij de monocyten van kinderen met type 1-diabetes. Dit resulteerde in de ontdekking van twee subclusters in autoimmuun diabetes. Het eerste subcluster bestaat uit 12 pro-inflammatoire genen met weer als mogelijk sleutelgen PDE4B. Het tweede subcluster bestaat uit 10 genen die met name betrokken zijn bij de migratie,adhesie en metabolisme van cellen. Subcluster 1 was aanwezig in 60% van de LADA-patiënten, 28% van type 1-diabeten met een manifestatie van ziekte op volwassen leeftijd, maar in slechts 10% van type 1-diabeten bij wie de ziekte vroeg aanwezig was. Echter, in deze groep patiënten was 43% positief voor sub-cluster 2, terwijl maar 9% van de type 1-diabeten met een volwassen manifestatie hier positief voor was. In de LADA-groep was 33% positief voor sub-cluster 2. De aanwezigheid van verschillende monocyten activatie profielen (de twee sub-clusters) in type 1-diabetes, veronderstelt heterogeniteit in de pathogenese van autoimmuun diabetes. Deze heterogeniteit overlapt maar ten dele de bekende diagnostische categorieën.

In **Hoofdstuk 7** (de algemene discussie) bespreken we de verschillende pro-inflammatoire profielen die we gevonden hebben in de monocyten. We laten zien dat sub-cluster 1 en sub-cluster 2 (gevonden bij de diabeten) ook retrospectief kunnen worden gevonden in de “bipolaire” gen-expressie signatuur. Bovendien correleren de twee sub-clusters met elkaar en vormen zo een profiel dat erg lijkt op de “bipolaire” gen-expressie signatuur. Waarom deze (subclusters van) genen op deze specifieke manier met elkaar correleren in verschillende ziektebeelden is nog niet duidelijk en zal verder onderzocht moeten worden.

In **Hoofdstuk 7** noemen we infectie, stress en dieet als omgevingsfactoren die invloed kunnen hebben op zowel de gen-expressie van monocyten als de bipolaire stoornis (en misschien ook op AITD en autoimmuun diabetes). Deze factoren zijn alle genoemd als zijnde geassocieerd met de bipolaire stoornis, AITD en autoimmuun diabetes. Ook brengen we in de discussie naar voren dat met name de prenatale periode interessant is om te bestuderen, aangezien met name in de baarmoeder de omgevingsinvloeden dezelfde zullen zijn voor de tweelingen. Uiteraard is ook hier verder onderzoek geïndiceerd.

Appendix 1. Primers and probes used in Q-PCR.

Public ID	Gene Symbol	Applied Biosystems gene expression assays (Hs)/ Primer/ Probe 5'-FAM-3'
NM_001037339	*PDE4B*	Hs00387320_m1
NM_000600	*IL6*	Hs00174131_m1
NM_000576	IL1B	Hs00174097_m1
NM_002852	*PTX3*	Hs00173615_m1
NM_000963	*PTGS2*	Hs00153133_m1
NM_000594	*TNF*	Hs00174128_m1
NM_006290	*TNFAIP3*	Hs00234712_m1
NM_013448	*BAZ1A*	Hs00203772_m1
NM_003183	*ADAM17*	Hs00234221_m1
NM_006273	*CCL7*	Hs00171147_m1
NM_004591	*CCL20*	Hs00355476_m1
NM_002089	*CXCL2*	Hs00236966_m1
NM_002982	*CCL2*	Hs00234140_m1
NM_044472	*CDC42*	Hs00741586_mH
NM_000647	*CCR2*	Hs00174150_m1
NM_001337	*CX3CR1*	Hs00365842_m1
NM_001769	*CD9*	Hs00233521_m1
NM_004603	*STX1A*	Hs00270282_m1
NM_004049	*BCL2A1*	Hs00187845_m1
NM_001423	*EMP1*	Hs00608055_m1
NM_002832	*PTPN7*	Hs00160732_m1
NM_004418	*DUSP2*	Hs00358879_m1
NM_001030287	*ATF3*	Hs00231069_m1
NM_005967	*NAB2*	Hs00195573_m1
NM_004753	*DHRS3*	Hs00191073_m1
NM_001444	*FABP5*	Hs02339439_m1
NM_002748	*MAPK6*	Hs00833126_g1
NM_005345	*HSPA1A*	Probe 1. TTCGAGAGTGACTCCCGTTGTCCCA FPrimer 2. GCTGCGACAGTCCACTACCTT RPrimer 3. GGTTCGCTCTGGGAAGCC
NM_005157	*ABL*	Probe 1. TGGAGATAACACTCTAAGCATAACTAAAGGT FPrimer 2. CCATTTTTGGTTTGGGCTTCACACCATT RPrimer 3. GATGTAGTTGCTTGGGACCCA

ABBREVIATIONS

5-HT	Serotonin
Abs	Antibodies
ACTH	Adrenocorticotrophin hormone
ADA	American diabetes association
AITD	Autoimmune thyroid disease
Alpha 1-AT	Alpha-1 antitrypsin
ANA	Antinuclear antibodies
ANCOVA	Analysis of covariance
APC	Antigen presenting cells
APS	Autoimmune polyendocrine syndrome
ATF3	Activating transcription factor 3
ATPase	Adenosine triphosphatase
BB-DP	Bio breeding diabetes prone
BBB	Blood-brain-barrier
BCL2A1	BCL2-related protein A1
BD	Bipolar disorder
BDNF	Brain derived neurotrophic factor
BMI	Body mass index
BP	Bipolar patients
cAMP	Cyclic adenosine monophosphate
CCL2	Chemokine (CC-motif) ligand 2
CCR2	Chemokine (CC-motif) ligand 2 receptor
CD	Cluster of differentiation
CGI-BP	Clinical global impressions scale-bipolar version
CMV	Cytomegalovirus
CRH	Corticotrophin-releasing hormone
CRP	C-reactive protein
Csk	C-terminal Src tyrosine kinase
CTLA4	Cytotoxic T-lymphocyte antigen 4
CXCL2	Chemokine (C-X-C motif) ligand 2
DC	Dendritic cells
DHRS3	Dehydrogenase/reductase (SDR family) member 3
DSM-IV	Diagnostic and statistical manual of mental disorders
DUSP	Dual specificity phosphatases
DZ	Dizygotic
ECM	Extra-cellular matrix
Egr-1	Early growth response 1
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3
ELISA	Enzyme-linked immunosorbent assay

EMP1	Epithelial membrane protein 1
ERK	Extracellular signal-regulated kinase
FABP	Fatty acids binding protein
Fb	Fibrinogen
FCAR	Fc fragment of IgA receptor
FN	Fibronectin
GABA	γ-aminobutyric acid
GAD65	65 kDA glutamic acid decarboxylase
GC	Glucocorticoids
GD	Graves' disease
GM-CSF	Granulocyte monocyte colony stimulating factor
HLA	Human leukocyte antigen
Hp	Haptoglobin
HPA-axis	Hypothalamic-pituitary-adrenal axis
HPT-axis	Hypothalamic-pituitary-thyroid axis
Hpx	Hemopexin
HT	Hashimoto's thyroiditis
IA-2	Insulinoma-associated protein 2
ICA69	69 kDa islet cell antigen
IDO	Indoleamine-2,3-dioxygenase
IDS	Inventory of depressive symptomatology
IFN	Interferon
IIF	Indirect immunofluorescence
IL	Interleukin
IRS	Inflammatory response system
Jnk	Jun N terminal kinases
LADA	Latent autoimmune diabetes of the adults
Li	Lithium
LPS	Lipopolysaccharide
MACS	Magnetic cell sorting system
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
MZ	Monozygotic
NA	Noradrenalin
NAB2	NGFI-A binding protein
NK cell	Natural killer cell
NOD	Non obese diabetic
NOS	Not otherwise specified

PBMC	Peripheral blood mononuclear cell
PDE4B	Phosphodiesterase 4B
PPAR	Peroxisome proliferator-activated receptor
PTPN7	Protein tyrosine phosphatase, non-receptor type 7
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PTX3	Pentraxin 3
PVN	Paraventricular nucleus
Q-PCR	Quantitative polymerase chain reaction
ROC	Receiver operating characteristic
SCH	Schizophrenia
SCID	Structured clinical interview for DSM-IV axis I
SDR1	Short-chain dehydrogenase/reductase 1
SEM	Structural equation modelling
SFBN	Stanley foundation bipolar network
sIL-2R	Soluble IL-2 receptor
SMAC	Supra-molecular activation clusters
SNARE	Soluble NSF attachment receptor
SNS	Sympathetic nervous system
STX1A	Syntaxin 1A
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Tg	Thyroglobulin
TGF	Tumor growth factor
Th	T helper cell
TNF	Tumor necrosis factor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TPO	Thyroid peroxidase
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
VNTR	Variable number tandem repeats
YMRS	Young mania rating scale

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Werk is leuk, maar het kan ook behoorlijk veeleisend en stressvol zijn, niets is dan fijner om met vrienden en familie te kunnen lachen en relativeren. Lieve familie en vrienden, bedankt voor al jullie gezelligheid en interesse. En lieve volleybal- en tennisvrienden, bedankt voor de sportieve uitlaatklep die ook altijd gepaard gaat met veel gezelligheid. Zonder iemand te kort te willen doen, wil ik toch een paar vrienden in het bijzonder noemen.

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Marijke, mijn herinneringen aan jou zijn me enorm dierbaar. Ik zal je nooit vergeten.

CURRICULUM VITAE

Roos Padmos was born on December 18th, 1978 in Rotterdam. She grew up with two brothers in a small town called Puttershoek. She attended secondary school at the Willem van Oranje in Oud- Beijerland. She began to study medicine in September 1997 in Antwerp, Belgium, where she obtained her three candidatures (of which two cum laude). She continued her medical career at the Medical School of the Erasmus University in Rotterdam. At the same time she followed a Master of Science program in Clinical Epidemiology of which she graduated in August 2003. In that same year she received the Gerrit Jan Mulder price (a price given to Erasmus students performing promising research) for her research on the prevalence of organ-specific autoantibodies in bipolar patients. Two years later she obtained her medical degree. In September 2005 she started her PhD at the Department of Immunology of the Erasmus MC, University Medical Center Rotterdam, under the guidance of Prof. Dr. H.A. Drexhage. During her medical education and PhD training the author of this thesis had been given several opportunities to combine her work with one of her greatest passions, travelling. She has spent several months at the University of Washington in Seattle, at the University of Florida in Gainesville, at the Harvard school of Public Health in Boston and in the Algemeines Krankenhaus in Vienna. In September 2008 she started as a resident Internal Medicine at the Harbour Hospital and Institute for Tropical Diseases in Rotterdam (supervisors Dr. P.J. Wismans, Harbour Hospital, and Prof. Dr. J.L.C.M. van Saase, Erasmus MC).

She lives happily together with Kees Klerk at the city center of Rotterdam.

LIST OF PUBLICATIONS

Padmos RC, Bekris L, Knijff EM, Tiemeier H, Kupka RW, Cohen D, Nolen WA, Lernmark Å, Drexhage HA. A High Prevalence of Organ-Specific Autoimmunity in Patients with Bipolar Disorder.
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Padmos RC, Hillegers MHJ, Knijff EM, Vonk R, Bouvy A, Staal FJT, Ridder D, Kupka RW, Nolen WA, Drexhage HA. A Discriminating mRNA Signature for Bipolar Disorder formed by an Aberrant Expression of Inflammatory Genes in Monocytes.
Archives of General Psychiatry 2008;65:395-407.

Drexhage RC, **Padmos RC**, de Wit H, Versnel MA, Hooijkaas H, van der Lely AJ, van Beveren N, de Rijk RH, Cohen D. Patients with Schizophrenia show Raised Serum Levels of the Pro-inflammatory Chemokine CCL2: Association with the Metabolic Syndrome in Patients?
Schizophrenia Research 2008 Jul;102(1-3):352-355.

Padmos RC, Schloot NC, Beyan H, Ruwhof C, Staal FJT, Ridder D, Aanstoot HJ, Lam-Tse WK, de Wit H, Herder C, Drexhage RC, Menart B, Leslie D, Drexhage HA. Distinct Monocyte Gene-Expression Profiles in Autoimmune Diabetes.
Diabetes 2008;57(10):2768-2773.

Padmos RC, van Baal GCM, Vonk R, Wijkhuijs AJM, Kahn RS, Nolen WA, Drexhage HA. A twin study: Pro-inflammatory Monocytes in Bipolar Disorder Mainly the Result of a Common Environmental Factor.
Re-submitted to *Archives of General Psychiatry.*

PHD PORTFOLIO SUMMARY

Summary of PhD training activities

'Inflammatory monocytes in bipolar disorder and related endocrine autoimmune diseases' Roos Padmos, February 25th, 2009

PhD training

	Year	ECTS
Research skills		
Principles of Research in Medicine and Epidemiology, NIHES	2001	1.0
Clinical Decision Analysis, NIHES	2001	1.0
Methods of Clinical Research, NIHES	2001	1.0
Data Collection in Epidemiologic Research, NIHES	2001	1.0
Study Design, NIHES	2001	3.0
Introduction to Data-Analysis, NIHES	2002	2.0
Regression Analysis, NIHES	2002	2.0
Survival Analysis, NIHES	2002	2.0
Clinical Trials, NIHES	2003	1.0
Topics in Meta-Analysis, NIHES	2003	1.0
Bayesian Analysis, NIHES	2003	1.0
Analysis of Repeated Measurements, NIHES	2003	1.0
General academic skills		
Working with SPSS for Windows, NIHES	2002	0.3
Introduction to Medical Writing, NIHES	2003	2.0
Biomedical English Writing and Communication	2006	3.0
In-depth courses		
Addiction and Substance Use: Epidemiology and HSR, NIHES	2002	1.0
Planning and Evaluation of Screening, NIHES	2002	2.0
Pharmaco-Epidemiology and Drug Safety, NIHES	2002	1.0
Medical Demography, NIHES	2003	2.0
Molecular Immunology, Molmed	2006	2.0
Neuro-Immuno-Endocrinology, Molmed	2006	1.5

International courses

Principles of Epidemiology, Harvard School of Public Health, Boston, USA	2003	4.0
Management of Health Care Organisations, Harvard School of Public Health, Boston, USA	2003	4.0

Symposia and conferences

Annual Symposium Dutch Thyroid Club, Amsterdam	2006+2007	0.6
NVVI course, Lunteren	2006	0.6
NVVI year conference, Noordwijkerhout	2007	0.3
Symposium Systemic Diseases, Groningen	2007	0.3
Molecular Medicine Day, Rotterdam	2006+2007+2008	1.0
Symposium Clinical Immunology, Amsterdam	2008	0.3

Presentations

Endo-Neuro-Psychiatry meeting, Doorwerth	2006	0.3
Merck European Thyroid Symposium, Noordwijk	2006	0.6
Psycho-Immunology Expert Meeting, Günzburg, Germany	2007	1.0
International Congress of the Immunology of Diabetes Society and American Diabetes Association, Miami Beach, USA	2007	1.5
Seminar, German Diabetes Center, Düsseldorf, Germany	2008	0.1