Aus der Klinik für Psychiatrie und Psychotherapie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Neuro-immune correlates of stress-related symptoms in multiple sclerosis

Identifizierung von neuroimmunologischen Korrelaten stress-bedingter Symptome bei Multipler Sklerose

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List of abbreviations

CNS	Central nervous system
EDSS	Expanded Disability Status Scale
GC	Glucocorticoid
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
MADRS	Montgomery-Åsberg depression rating scale
MDD	Major depressive disorder
MS	Multiple sclerosis
PBMC	Peripheral blood mononuclear cell
SDMT	Symbol Digit Modality Test
TCM	T central memory

Abstract

Major depressive disorder (MDD) is one of the most common mental disorders affecting people worldwide. With the risk to develop depression 2-5 times higher than the general population and a lifetime prevalence of 50% it is the most common comorbidity in multiple sclerosis (MS), a chronic autoimmune and neurodegenerative CNS disease. Over the last decades, evidence has been highlighting the role of inflammation in developing depressive symptoms. It is also plausible that different inflammatory profiles are associated with different subtypes of depression. Therefore, studying depressed patients with a comorbid inflammatory condition could provide a good example to investigate the inflammatory subtype of depression. Besides its well-established role in the pathophysiology of MDD, the stress in MS has also been discussed since the disease was described and there has been growing evidence that disease activity in MS is associated with stress.

This PhD thesis investigated the immune correlates of depression in the context of background inflammation in MS patients with depression and the association between the cellular measure of immune system responsivity to stress hormones and neural stress processing in MS.

We conducted a cross-sectional case-control study that enrolled MS patients with and without depression (N=45) as well as healthy controls (N=30). The study design combined clinical assessments, immunophenotyping, cell-specific quantification of stress hormone signalling in the immune system as well as brain imaging, which included an fMRI stress task to quantify central stress processing.

Using clustering analysis of immunophenotyping data our results identified decreased CD4+CCR7low TCM cell frequencies as an immune correlate of MS-associated depression. These cell frequencies correlated with clinical measures of depression severity, specifically with affective symptoms of depression such as sadness, but not with MS severity, measured as disability or cognitive impairment. We also found that lower peripheral CD4+CCR7low TCM cell frequencies were associated with lesion load, a measure of increased neuroinflammation. CD4+CCR7low TCM cell frequencies, however, were not associated with HPA axis activity, suggesting that identified immune correlate of depression is not secondary to stress system alterations. Further examination of cellular and CNS stress processing revealed that the activity of a network comprising the right anterior

insula, right fusiform gyrus, left midcingulate and lingual gyrus was differentially associated with T cell glucocorticoid signalling across groups of MS patients and healthy controls and linked to disease severity indicating the importance of CNS-immune system crosstalk in MS.

Zusammenfassung

Depressionen (Major Depression = MDD) gehören zu den weltweit häufigsten psychischen Störungen. Mit einem 2- bis 5-mal höheren Risiko, an einer Depression zu erkranken und einer Lebenszeitprävalenz von 50 % ist die Depression die häufigste Komorbidität bei Multipler Sklerose (MS), einer chronischen Autoimmun- und neurodegenerativen ZNS-Erkrankung. In den letzten Jahrzehnten wurde deutlich, dass Entzündungen bei der Entwicklung depressiver Symptome eine Rolle spielen könnten. Es wurde auch gezeigt, dass unterschiedliche Entzündungsprofile mit unterschiedlichen Subtypen von Depressionen assoziiert sind. Daher könnte die Untersuchung depressiver Patienten mit einer komorbiden entzündlichen Erkrankung wertvolle Erkenntnisse zum entzündlichen Subtyp der Depression liefern. Darüber hinaus wird Stress, neben seiner etablierten Rolle in der Pathophysiologie von MDD, auch als Einflussfaktor bei MS diskutiert und es gibt zunehmend Hinweise, dass die Krankheitsaktivität bei MS mit Stress assoziiert ist.

Diese Doktorarbeit untersuchte die Immunkorrelate von Depressionen vor dem Hintergrund des Entzündungsgeschehens bei MS-Patienten mit Depressionen sowie den Zusammenhang zwischen der zellulären Stressreaktion des Immunsystems und der neuronalen Stressverarbeitung bei MS.

Wir führten eine Fall-Kontroll-Querschnittsstudie durch, in die MS-Patienten mit und ohne Depression (N=45) sowie gesunde Kontrollpersonen (N=30) eingeschlossen wurden. Das Studiendesign kombinierte klinische Bewertungen mit Immunphänotypisierung, zellspezifischer Quantifizierung der Stressreaktion im Immunsystem sowie mit einem bildgebenden Verfahren, welches eine fMRI-basierte Stressaufgabe zur Quantifizierung der zentralen Stressverarbeitung im Gehirn beinhaltete.

Clustering-Analysen der Immunphänotypisierungsdaten konnten eine verringerte CD4+CCR7low-TCM-Zellfrequenz als Immunkorrelat von MS-assoziierter Depression identifizieren. Diese Zellfrequenz korrelierte mit der Depressionsschwere, insbesondere

mit affektiven Symptomen wie Traurigkeit, aber nicht mit dem Schweregrad der MS-Erkrankung oder der kognitiven Beeinträchtigung. Niedrigere periphere CD4+CCR7low-TCM-Zellfrequenzen waren mit der Läsionslast assoziiert, ein Maß für eine erhöhte Neuroinflammation. CD4+CCR7low-TCM-Zellfrequenzen waren jedoch nicht mit der Aktivität der HPA-Achse assoziiert, was darauf hindeutet, dass dieses Immunkorrelat der Depression nicht sekundär zu Veränderungen im Stresssystem auftritt. Eine weitere Untersuchung der zellulären und der ZNS-Stressverarbeitung ergab, dass die Aktivität eines Gehirn-Netzwerks, das die rechte vordere Insula, den rechten Gyrus fusiformis, den linken mittleren cingulären Kortex sowie den lingualen Gyrus umfasst, bei MS-Patienten und gesunden Kontrollpersonen unterschiedlich mit der T-Zell-Glukokortikoid-Signalübertragung assoziiert und zudem mit der Schwere der Erkrankung verknüpft ist, was die Bedeutung der ZNS-Immunsystem-Interaktion bei MS unterstreicht.

1 Introduction

Major depressive disorder (MDD) is one of the most common mental disorders affecting people worldwide with an overall prevalence of 6% (Malhi & Mann, 2018). It is characterized by a heterogeneous pathobiology and is caused by a complex interaction between genetics and the environment, with stress being a major risk factor (Wang et al., 2015; Fakhoury et al., 2016; Otte et al., 2016; Shadrina et al., 2018). Pathophysiological features of MDD have been identified in the central nervous system (CNS) and in the major stress response systems, that are the hypothalamic-pituitary-adrenal (HPA) axis, the autonomic nervous system, and the immune system (Otte et al., 2016). They include smaller hippocampal volumes, changes in neural networks, monoamine depletion, resistance of glucocorticoid receptor (GR), alterations of corticotropin-releasing hormone levels, changes in cortisol levels, as well as excess glutamate (Otte et al., 2016; Boku et al., 2018; Seki et al., 2018; Bruno et al., 2020).

1.1 Inflammation and depression

Over the last decades, evidence has been emphasizing a crucial role inflammation plays in the development of depressive symptoms (Beurel et al., 2020), suggesting that proinflammatory cytokines contribute to stress-induced mood disturbances and MDD (Duarte-Silva et al., 2019), and indicating a bidirectional link between peripheral markers of inflammation and depression (Köhler et al., 2017; Giollabhui et al., 2021). Moreover, cytokines released in the periphery during systemic inflammatory diseases get to the brain where they promote fatigue, anhedonia, sleep disturbances, social withdrawal, and anxiety (Raison et al., 2006; Dantzer et al., 2008; Miller et al., 2009). Therefore, chronic inflammatory diseases, such as cardiovascular disease, type 2 diabetes, cancer, psoriasis, rheumatoid arthritis and the inflammatory autoimmune disease multiple sclerosis (MS), have been associated with a high incidence of depressive symptoms and an up to 3-4-fold higher risk of MDD compared with the general population (Raison et al., 2006; Finnell and Wood, 2016; Poole and Steptoe, 2018; Gold et al., 2020). Depressed patients are not only more vulnerable to these and other disorders, but the treatment outcomes for these medical disorders are also poorer (Katon et al., 2011; McKay et al., 2018). For that reason, an association of changes in immune markers with the antidepressant treatment

response may also have therapeutic implications (Liu et al., 2020). It has been demonstrated that treatments with anti-inflammatory drugs can improve depression severity (Köhler-Forsberg et al., 2019) and cytokine inhibitors showed anti-depressive effects in clinical trials of patients with underlying inflammatory conditions (Kappelmann et al., 2018). It is also possible that different inflammatory profiles are associated with different subtypes of depression (Kaestner et al., 2005; Karlovic et al., 2012; Dunjic-Kostic et al., 2013; Milaneschi et al., 2019). Using cellular phenotyping in the peripheral immune system, Lynall et al. (2020) identified a subgroup of MDD patients with an “inflammatory” type of depression indicating that inflammation likely plays a role in a subset of MDD patients. Consequently, studying depressed patients with a comorbid inflammatory condition would possibly facilitate the investigation of the inflammatory subtype and identify the immune correlates of MDD.

1.2 Multiple sclerosis and depression

With a 2-5 times higher risk to develop depression when compared to the general population and a lifetime prevalence of 50% (Feinstein et al., 2011), the chronic autoimmune disease multiple sclerosis (MS) may serve as a good example of depressed patients with a comorbid inflammatory condition. Even though psychiatric comorbidity is common in MS, it is often overlooked and undertreated (Marrie et al., 2009). One argued key question is whether psychiatric symptoms represent a primary symptom of the disease or only a psychological reaction to being diagnosed with MS and side effects of therapies. A higher frequency of MDD diagnosis that has been reported up to 10 years before the first records of MS speak in favour of the first hypothesis (Feinstein et al., 2014; Disanto et al., 2018). Evidence from preclinical and clinical studies have been supporting the immune-mediated hypothesis of depression in MS (Bruno et al., 2020). Besides its well-established causal role in MDD, the role of stress in MS has also been discussed since the disease was described (Charcot, 1877). Indeed, there has been growing evidence that disease activity in MS is associated with stress (Mohr et al., 2004; Mohr et al., 2007) and that stressful life events precede the appearance of new lesions (Mohr et al., 2004; Golan et al., 2008; Yamout et al., 2010). On the other hand, stress-management techniques have been useful in the prevention of new brain lesions (Mohr et al., 2012). Moreover, studies have found an impaired regulation of the HPA axis in MS (Schumann et al., 2002; Gold et al.,

2005b) as well as reduced sensitivity to stress hormone regulation in T cells (Wüst et al., 2008; Ysrraelit et al., 2008; Gold et al., 2012), which may contribute to neuroinflammation.

All the previously mentioned findings indicate that MS-associated depression could be more closely related to the underlying biology rather than simply representing a psychological response to chronic disease. However, the immune cell correlates of MS-associated depression are unknown and a link between peripheral markers of inflammation with MS disease severity or neuroinflammatory activity has not been explored in this patient group. Moreover, even though the importance of stress in MS onset and relapses has been shown, it has not been investigated if there is an interplay between the peripheral immune response to stress and central stress processing in the brain and how it can be related to disease severity.

This PhD thesis aimed to investigate the immune cell correlates of MS-associated depression as well as the relationship between the cellular measure of immune system responsivity to stress hormones and neural stress processing in MS. A cross-sectional case-control study that combined clinical assessments, in-depth immunophenotyping, cell-specific quantification of stress hormone signalling in the immune system as well as multimodal neuroimaging, which included task fMRI to quantify central stress processing and structural MRI to assess markers of neuroinflammation and neurodegeneration was used in this work.

Results of the study were reported in two peer-reviewed papers for this publication-based PhD thesis (Brasanac et al., 2022a; Brasanac et al., 2022b). The methodology and results for both are summarized below.

2 Methods

This section describes the study design and main techniques used in this PhD thesis. More details of the methods used for each study, the elaborated inclusion and exclusion criteria, outcome measures and statistical analysis can be found in the respective publications (Brasanac et al., 2022a; Brasanac et al., 2022b).

2.1 Participants

This PhD work was embedded in the DFG-funded study “Neural mechanism of cognitive and emotional processes in MS and their relevance for disease severity” (COGEMS), conducted at the NeuroCure Clinical Research Center (NCRC), Charité Universitätsmedizin Berlin. The study complied with relevant guidelines (Helsinki Declaration of 1975) and was approved by the ethics committee of Charité – Universitätsmedizin Berlin (EA1/208/16). Written informed consent prior to study enrolment was provided by all participants who also received financial reimbursement for their time and efforts. Participants had two study visits within a two-week period, first for clinical assessments and blood withdrawal and the second time for neuroimaging.

The publication “Immune signature of multiple sclerosis-associated depression” additionally used data from participants of the “Depression and Immune Function” (DENIM) study conducted at the Institute of Neuroimmunology and Multiple Sclerosis (INIMS), Universitätsklinikum Hamburg-Eppendorf, which has been approved by the Hamburger Ärztekammer ethics committee (PV3792).

Both case-control studies enrolled patients with relapsing-remitting MS with and without comorbid depression and non-depressed healthy controls. To explore the specificity of the immune correlates of MS-associated depression using a new bioinformatics tool, we additionally re-analysed data from two case-control studies previously published on idiopathic MDD, without comorbid MS. Details on inclusion and exclusion criteria can be found in the respective publications (Hasselmann et al., 2018; Patas et al., 2018).

Inclusion criteria for MS patients were (1) diagnosis of relapsing-remitting MS (Polman et al., 2011), (2) immunomodulatory drugs or no disease-modifying treatment for the last 3 months and (3) older than 18 years. In MS-associated depression group MS patients

were diagnosed with a current major depressive episode in the Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1997) (COGEMS) or scored > 14 on Beck Depression Inventory (BDI)-II (DENIM) which is the established threshold of clinically relevant depressive symptoms (Sullivan et al., 1995; Gold et al., 2010).

Exclusion criteria for MS patients were (1) pregnancy, (2) diagnosis of psychiatric disorders other than major depression or anxiety disorders, (3) diagnosis or history of neurological disorders other than MS, (4) MS relapse or steroid treatment in the last 4 weeks, or (5) contraindications for MRI scanning. Apart from MS diagnosis, treatments and relapses, controls had the same inclusion and exclusion criteria.

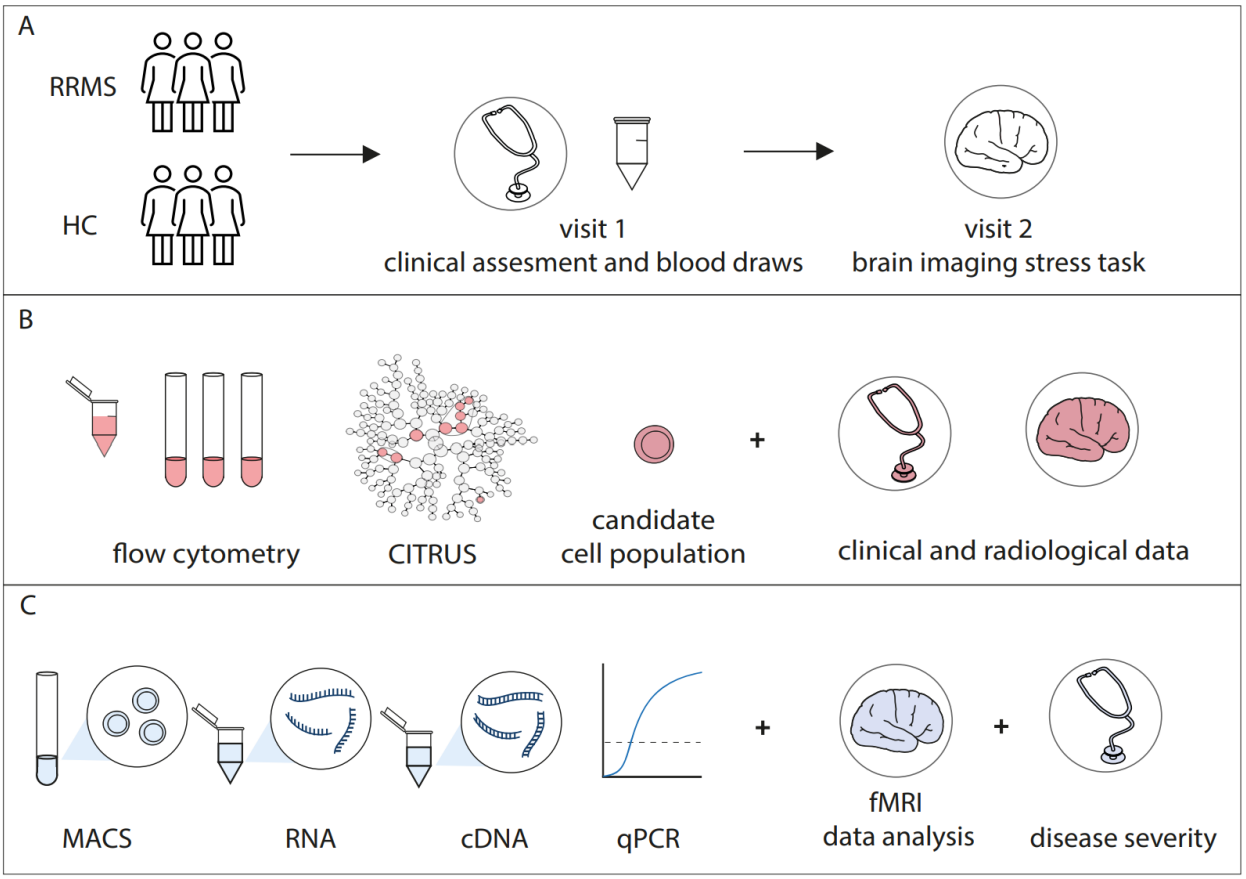

Figure 1: Study design

The COGEMS study enrolled patients with relapsing-remitting multiple sclerosis (RRMS) and healthy controls (HC). Both groups had two study visits, visit 1 for a clinical assessment and taking blood samples and visit 2 for brain imaging that included fMRI stress task (A). The first set of experiments involved immunophenotyping by flow cytometry. Flow cytometry data were analysed using the CITRUS clustering algorithm to identify candidate cell population of MS-associated depression, which was then correlated with clinical and radiological data (B). The second set of experiments included the separation of CD4+ and CD8+ T cell fractions using magnetic-activated cell sorting (MACS) technology, RNA isolation, cDNA synthesis, and gene expression using qPCR. These data then entered interaction analysis with fMRI data from the stress task. The brain

network identified in the interaction analysis was tested for an association with MS disease severity markers (C). Own representation: Jelena Brasanac

2.2 Clinical assessments and blood draws

On the first day of the study visit participants underwent clinical assessment and blood draws (Figure 1A). The clinical assessment comprised the neurological examination, the assessment of neurological disability via the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983), an in-depth psychiatric evaluation using a structured clinical interview (MINI, version 5), the Montgomery-Åsberg depression rating scale (MADRS) (Montgomery & Asberg, 1979) as well as self-reported questionnaire for depressive symptoms Beck Depression Inventory (BDI)-I (Beck et al., 1961). The Symbol Digit Modality Test (SDMT) (Smith et al., 1982) in its oral version was used to screen for cognitive impairments. On the same day, a venous blood sample was collected between 8-11 a.m. from the participants after overnight fasting and processed within 2h. Protocol for isolation and processing of peripheral blood mononuclear cells (PBMCs) followed established lab procedures (Hasselmann et al., 2018) that use density-gradient centrifugation. After isolation, PBMCs were cryopreserved in liquid nitrogen (-196°C) until further analysis.

2.3 Flow cytometry and CITRUS analysis

To characterize the cellular composition of the peripheral immune system as well as the cellular phenotype we used multiparametric flow cytometry of cryopreserved PBMC samples (Figure 1B).

The project used antibody panels designed to characterise T cell differentiation and polarization, B cells and different innate immune cell populations. The first panel was a T cell differentiation panel used to distinguish naive, central memory, effector memory and effector memory re-expressing CD45RA (TEMRA) CD4+ and CD8+ T cells which included anti-CD3, -CD4, -CD8, -CD45RA and -CCR7 antibodies, as well as anti-CD25 and -CD127 antibodies for defining T regulatory cells (Treg). The second panel, the T cell polarization panel, used anti-CD3, -CD4, -CXRC3, -CCR4 and -CCR6 antibodies to identify T helper (TH) cell subsets. A third panel, a non-T cell panel, including anti-CD3,-CD20, -CD16, -CD14, -HLA-DR and -CD56 antibodies was used to analyse natural killer, B cells, dendritic cells and monocytes.

A data-driven approach using the CITRUS (cluster identification, characterization, and regression) algorithm within the cloud-based Cytobank software (Cytobank Inc. USA) was applied for analysing flow cytometry data. CITRUS is an unsupervised clustering algorithm developed to automatically find stratifying signatures from within multidimensional cytometry data sets that explain differences between groups of samples (Mair et al., 2014; Bruggner et al., 2014). To identify robust immune correlates of MS-associated depression we were looking for differences in cell frequencies that 1) could be identified in both independent case-control studies COGEMS and DENIM and 2) exhibited correlations with depression severity markers as well as neuroinflammation.

CITRUS was run for each antibody panel using 10.000 live cell events per sample, with all makers of a panel selected as clustering channels and a minimum cluster size of 1%. To detect associations between the relative cluster abundance of each sample and the study groups (HC, MS, MSD) we used the correlative model SAM. Cluster abundances with a false discovery rate of 1% were used for further statistical analysis. CITRUS runs were repeated three times to check for consistency of results. For cell populations that were robustly identified to be differentially abundant between the study groups by the CITRUS algorithm in both studies, we next exported the corresponding cell frequencies from Cytobank for correlational analyses with clinical variables.

2.4 CD4+ and CD8+ T cell separation and GC-related gene expression

In order to quantify stress hormone signalling in the adaptive immune system, we assessed gene expression of four key components of the glucocorticoid (GC) signalling pathway in CD4+ and CD8+ T cells: glucocorticoid receptor (*GR*), Glucocorticoid-induced leucine zipper (*GILZ*), FK506-binding protein 4 (*FKBP4*) and FK506-binding protein 5 (*FKBP5*). The GR is the main intracellular receptor for GCs and has a crucial role in regulating immune response (Cain and Cidlowski, 2017). FKBP5 is a co-chaperon and modulator of GR activity via a short negative feedback loop and is a known regulator of the stress response (Zannas et al., 2016). When GCs bind to GR, FKBP4 is replacing FKBP5 in the chaperon complex and facilitates translocation of GR to the nucleus where GR acts as a transcription factor. *GILZ* is one of the genes regulated by GR and has a role in mediating anti-inflammatory activity of GCs, especially in T cells (Cannarile et al., 2019).

Using magnetic-activated cell sorting (MACS) we sorted populations of CD4+ and CD8+ T cells. MACS is separating cells based on surface antigens (Figure 1C) and the use of

CD4 and CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). We have followed the manufacturer's instructions and per 10 million cells, 80µl of MACS buffer (phosphate-buffered saline, 0.5% BSA and 2mM EDTA) was added together with 20µl of CD4 MicroBeads. Cells with beads were then incubated for 15min in the dark at 4°C. After that cells were washed for 5min at 350 × g (4°C), resuspended in 500µl MACS buffer and sorted on MACS LS columns. Next, using CD4 negative fraction we sorted CD8+ T cells, following the same protocol, labelling cells with CD8 MicroBeads, incubating at 4°C for 15min and separating on MACS MS columns. Purity of cell fractions was checked by flow cytometry, and it was 91.67% + 6.74 SD (0.89 SEM) for CD4+ T cells and 95.15% + 5.73 SD (0.76 SEM) for CD8+ T cells.

Using Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and following the manufacturer's instructions we have isolated total RNA from CD4+ and CD8+ T cell fractions. The purity and concentration were evaluated by NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Berlin, Germany). RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Berlin, Germany) was used to transcribe the complementary DNA (cDNA) from RNA following the manufacturer's protocol. cDNA was stored at -20°C until further analysis.

TaqMan Gene Expression Assays (Thermo Fisher Scientific, Berlin, Germany) for GR (Hs00353740_m1), GILZ (Hs00608272_m1), FKBP4 (Hs00427038_g1) and FKBP5 (Hs01561006_m1) were used to amplify cDNA on a StepOne real-time PCR System (Thermo Fisher Scientific, Berlin, Germany). Real-time PCR reactions were all performed in triplicates. TATA Box Binding Protein (TBP; Hs00427620_m1) and Importin 8 (IPO8; Hs00183533_m1) were used as housekeeping genes as they have been shown to be reliable referent genes for gene expression analysis in T cells (Ledderose et al., 2011). The expression of genes of interest was normalized to the geometric mean of these two housekeeping genes. Calculated delta cycle threshold (ΔCT) values were used to compute the T cell GC summary marker of gene expression which then entered further statistical analyses.

2.5 Assessment of daily salivary cortisol levels

To assess the daily levels of salivary cortisol we have collected saliva from participants in the morning and in the evening. On the day of the first visit, participants were instructed how to collect saliva at home for two days before the next visit for MRI scanning when

they returned samples. Samples were centrifuged for 5min at room temperature at 1000 x g, aliquoted and stored at -20°C until analysis. Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (IBL, Germany) was used to measure daily levels of cortisol.

2.6 MRI scanning and processing

On the second day of the study visit which happened around 1 week after the clinical examination, participants had MRI scanning at the Berlin Center for Advanced Neuroimaging (Figure 1A). We obtained a T1-weighted sagittal 3D magnetization prepared rapid gradient echo (MPRAGE) sequence used in spatial normalization of Arterial-Spin-Labeling (ASL) images. We also acquired a sagittal T2-weighted fluid-attenuated inversion recovery (FLAIR) sequence used in the manual mapping of focal hyperintense lesions with ITK-SNAP software (http://www.itksnap.org). A widely established ASL fMRI stress task that consisted of mental arithmetic and performance feedback was used in COGEMS (for details see Weygandt et al. (2016)) as a stress paradigm. The stress paradigm had five stages, with three of them being Rating periods (stages 1, 3, and 5) where participants were asked how stressed, anxious, frustrated, or relaxed they were feeling. The second stage was Rest and resting state ASL fMRI scans were acquired. The Stress stage was the fourth stage and there participants underwent a stress task performing a series of subtraction tasks with two operands X and Y as fast as possible. A pseudo-continuous ASL EPI sequence (Wu et al., 2007) with 22 ascending transversal slices that covered the whole brain was used to measure functional ASL brain images. SPM12 toolboxes (Wellcome Trust Centre for Neuroimaging, Institute of Neurology, UCL, London UK - http://www.fil.ion.ucl.ac.uk/spm) were used in fully automatized pipelines for pre-processing of the fMRI scans.

2.7 Differential associations of brain activity, gene expression and salivary cortisol in MS and healthy controls

In order to investigate if there is an altered interplay between central stress processing in the brain and peripheral stress processing in the immune system in multiple sclerosis, we performed an interaction analysis where we tested whether stress-induced neural network activity is differentially associated with T cell glucocorticoid signalling in multiple sclerosis patients in comparison to healthy people. Specifically, one factorial analysis was

computed with robust regression for each of the 59 networks based on the data of 26 patients with multiple sclerosis and 18 healthy controls. In the next step, the activity of the network that turned out significant in the first interaction analysis was related to disease severity markers (MS disability measured as EDSS, cognitive impairment measured as SDMT, grey matter fraction, and T2-weighted lesion load) in patients in independent (regression) analyses. The same type of analysis, robust linear regression, was performed to test for differential associations between daily levels of salivary cortisol and the T-cell GC summary marker or brain activity across groups.

More details regarding the analyses can be found in Brasanac et al., 2022b.

3. Results

3.1 MS-associated depression shows a decrease in circulating CD4+CCR7low TCM cell frequencies

To investigate cellular correlates of MS-associated depression, we were looking to detect candidate cell populations which showed a significant difference in their frequencies between groups in both cohorts, COGEMS and DENIM. Analysing flow cytometry data on T cell differentiation with unsupervised clustering by CITRUS resulted in the detection of cluster J in COGEMS and cluster A in DENIM which were identified as CD4+ T central memory (TCM) cells with low expression of CCR7 according to the heatmap (Figure 2C). The CD4+TCM cell frequencies were decreased in depressed MS patients compared to MS patients without depression and healthy controls in COGEMS as well as in DENIM (Figure 2A). Therefore, we considered the lower frequency of CD4+ CCR7low TCM cells as a (candidate) cellular correlate of MS-associated depression that was detected in both studies. Using the same method, we then re-analyzed two datasets from previously published cohorts with MDD patients without any comorbid medical/autoimmune disorders. Those results showed that CD4+CCR7low TCM cell frequencies (clusters K1 and L1) did not differ between MDD patients and healthy controls in idiopathic MDD (Figure 2B). This indicates that the identified immune correlate could be specific to depression with a background inflammation.

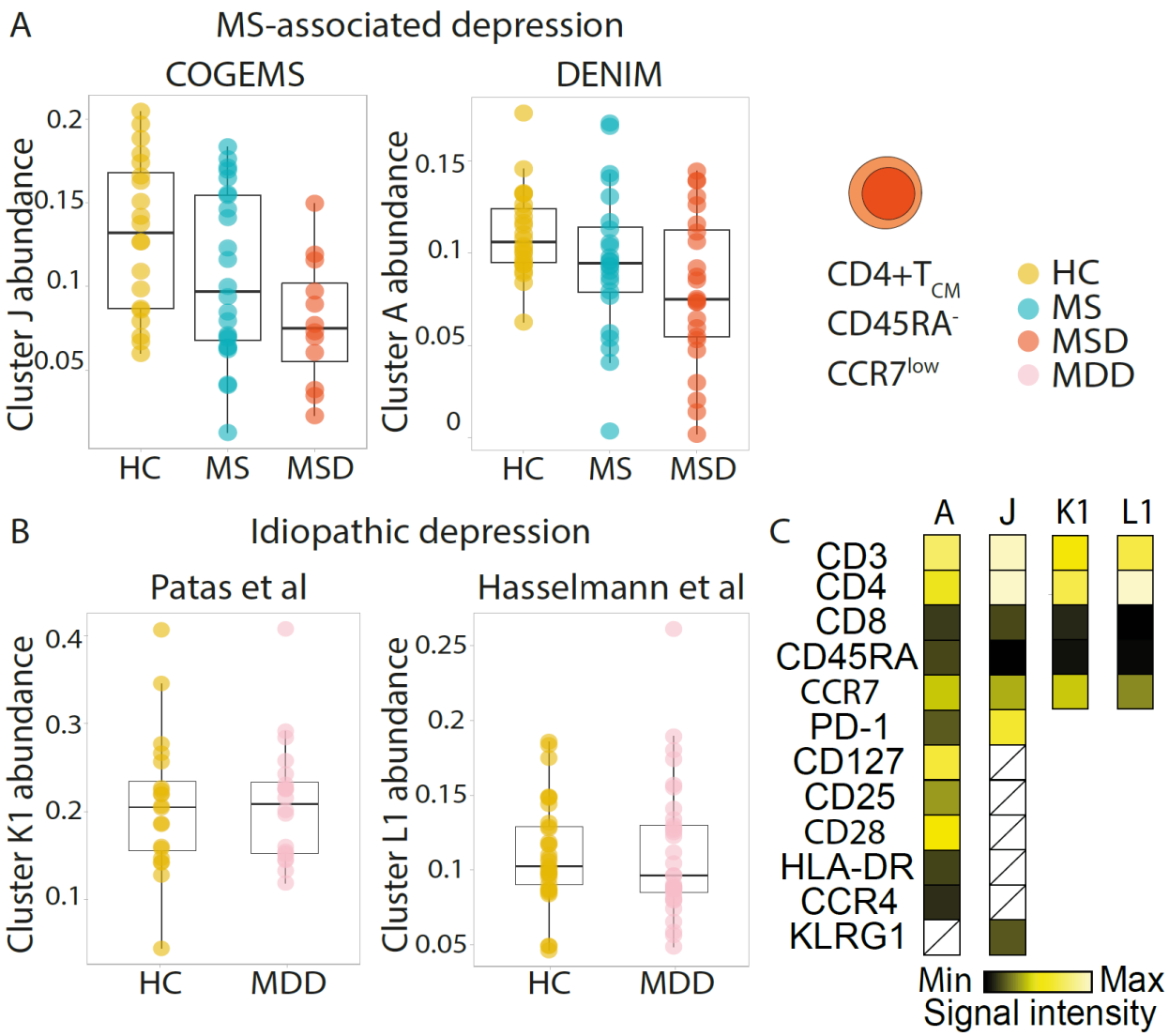

Figure 2: The decreased CD4+CCR7low TCM cell frequencies in MS-associated depression, but not idiopathic depression, as identified by clustering analysis of immunophenotyping data

The unsupervised clustering algorithm CITRUS identified clusters with differential abundance in healthy controls (HC), MS patients (MS), and MS patients with comorbid depression (MSD) in MS-associated depression cohorts, COGEMS and DENIM (A). Cluster J in COGEMS and cluster A in DENIM were identified as CD4+CCR7low T central memory (TCM) cells based on the expression of the surface markers displayed in the heatmap (C). CITRUS analysis of two idiopathic MDD cohorts did not show a difference in abundance between groups in clusters K1 and L1 corresponding to CD4+CCR7low TCM cells (B). The abundance of the corresponding clusters is represented as median and interquartile range, overlaid with data points that are representing individuals. Modified from Brasanac et al., 2022a.

3.2 CD4+CCR7low TCM cell frequencies correlate with depression severity, affective symptoms and lesion load

Having detected the frequency of the CD4+CCR7low TCM cell population as an immune cellular correlate of MS-associated depression we then tested its association with clinical variables of MS and MDD disease severity. Our results showed no significant association between CD4+CCR7low TCM cell frequencies and MS disease severity markers: EDSS, a measure of MS disability ($r = 0.044$, $p = 0.69$) (Figure 3A) or SDMT, a marker of cognitive impairment ($r = 0.0299$, $p = 0.75$) (Figure 3B). Interestingly, a significant negative correlation was shown for T2 lesion load ($r = -0.273$, $p = 0.049$) which is an estimate of MS-related neuroinflammation (Figure 3D).

On the other hand, correlation analysis of CD4+CCR7low TCM cell frequencies and MDD disease severity markers revealed a significant negative correlation ($r = -0.256$, $p = 0.0038$) with depression severity measured as patient-reported BDI theta scores (Figure 3C), as well as with psychiatrist-reported MADRS items that code for depression severity. MADRS items that showed a significant negative correlation were items coding for affective symptoms such as apparent and reported sadness and inner tension. In contrast, somatic and vegetative, or cognitive symptoms did not show significant correlations (Figure 3E).

Taken together, our results imply that CD4+CCR7low TCM cell frequencies may specifically correlate with core depressive symptoms and neuroinflammation in MS.

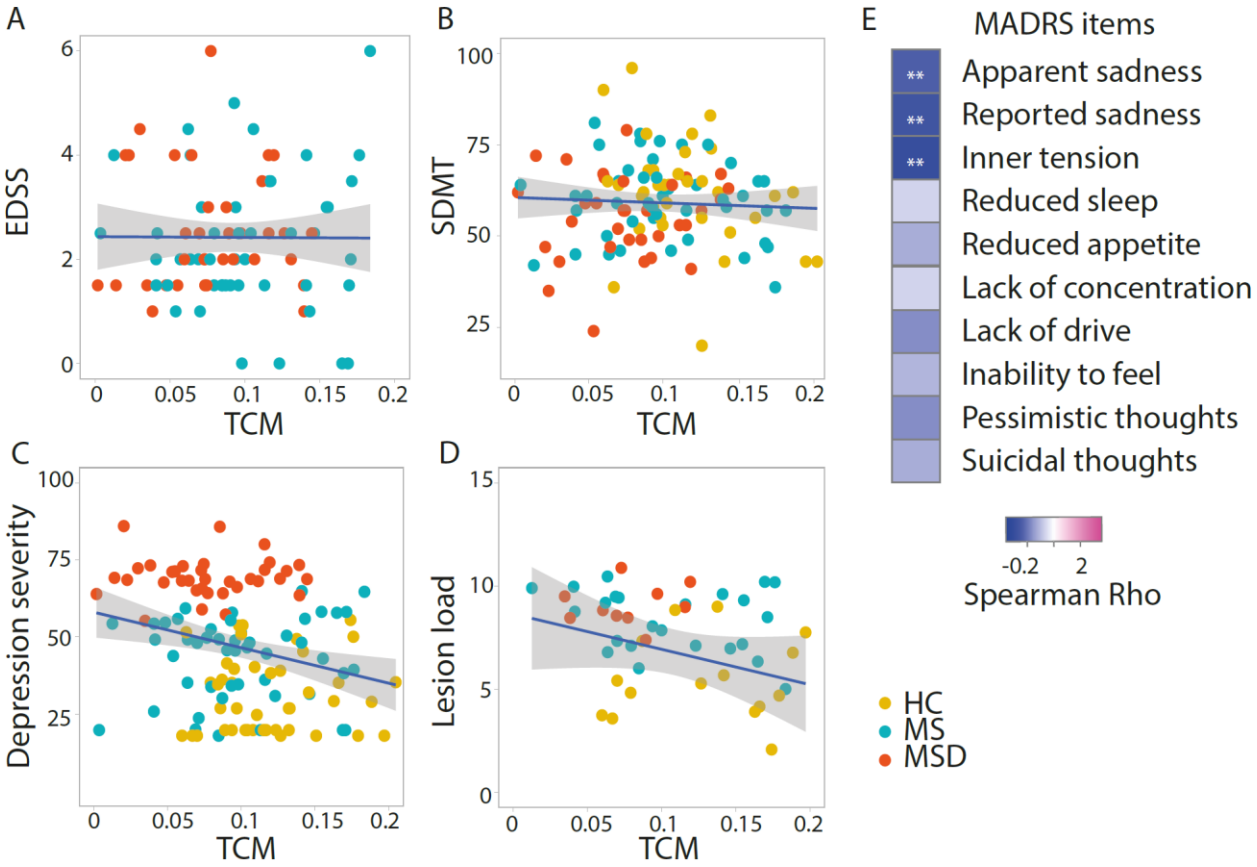

Figure 3. CD4+CCR7low TCM cell frequencies correlate with depression severity and lesion load but not with disability in MS or cognitive impairment

CD4+CCR7low TCM cell frequencies showed no significant correlation with Expanded Disability Status Scale (EDSS) score that measures disability in MS (A) or Standard Digit Modality Test (SDMT) score, a measure of cognitive impairment (B). CD4+CCR7low TCM cell frequencies negatively correlated with depression severity presented as BDI theta score (C). Items from Montgomery-Åsberg Depression Rating Scale (MADRS) coding for sadness and inner tension showed a negative correlation with CD4+CCR7low TCM cell frequencies (E) (colour code of Spearman's rho values is depicting the strength and direction of the correlation, * represents $p < 0.05$, ** $p < 0.01$). CD4+CCR7low TCM cell frequencies showed a significant negative association with T2-weighted lesion load in COGEMS (D). Modified from Brasanac et al., 2022a.

3.3 CD4+CCR7low TCM cell frequencies do not correlate with HPA axis activity or intracellular stress hormone signalling

Considering the pivotal role of stress in the pathogenesis of MDD, as well as its significant role in MS relapses, we further went on to explore whether there is an association between CD4+CCR7low TCM cell frequencies and markers of HPA axis activity. Our analysis showed no significant correlation between cell frequencies and average daily cortisol levels (r = -0.007, p = 0.9) or circadian slope (r = - 0. 242, p = 0. 083) (Figure 4A). Correlation analysis between CD4+CCR7low TCM cell frequencies and glucocorticoid-related CD4+ T cell gene expression were also not significant for any of the genes investigated:

GR (r = 0.109, p = 0.422), *FKBP4* (r = -0.1, p = 0.461), *FKBP5* (r = 0.02, p = 0.882) and *GILZ* (r = -0.176, p = 0.193) (Figure 4B). All of this indicates that even though stress signalling plays an important role in both MDD and MS, HPA axis activity and intracellular stress hormone signalling are not coupled with the immune cellular correlate of MS-associated depression.

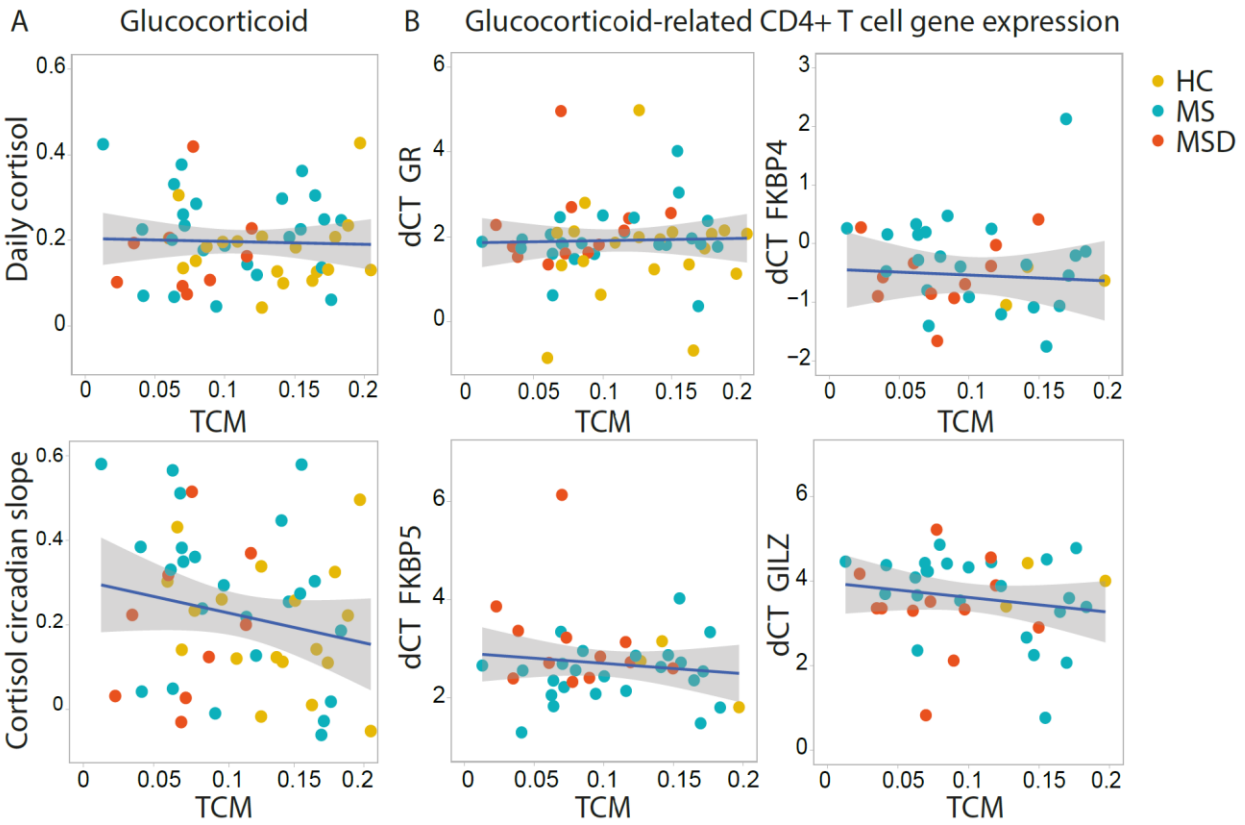

Figure 4. CD4+CCR7low TCM cell frequencies do not correlate with daily cortisol levels or glucocorticoid-related CD4+ T cell gene expression

CD4+CCR7low TCM cell frequencies showed no association with cortisol markers, daily cortisol level or circadian slope (A). No significant correlation was observed with cellular stress processing measured as glucocorticoid-related gene expression of the glucocorticoid receptor (*GR*), glucocorticoid-induced leucine zipper (*GILZ*), FK506-binding protein 5 (*FKBP5*) and FK506-binding protein 4 (*FKBP4*) in CD4+ T cells (B). Own representation: Jelena Brasanac

3.4 Stress processing brain network shows differential association with T cell GC signalling gene expression in MS and healthy controls and is linked to MS disease severity markers

In the next set of analyses, we wanted to investigate the interplay between different stress processing systems in MS versus HCs in the COGEMS dataset. We first tested if there is a stress processing brain network that is differentially associated with the T cell stress response captured as a summary marker of CD8+ and CD4+ T cells gene expression of

GR, *FKBP4*, *FKBP5* and *GILZ* across groups. Our analysis identified a brain network (#58) consisting of the right anterior insula, right fusiform gyrus, left midcingulate and lingual gyrus (t = -4.49, p = 0.0004, β = -0.76) (Figure 5A) that was differentially associated with T cell gene expression in MS and HCs. This brain network was negatively correlated with the T cell summary marker in MS and showed no correlation in HC (Figure 5A). Interestingly, the activity of this brain network also showed a significant association with three out of four MS disease severity markers: T2-weighted lesion load (t=2.70, W=7.28, p=0.011, β=0.43) (Figure 5E), clinical disability measured as EDSS (t=2.09, W=4.35, p=0.042, β =0.36) (Figure 5D), and information processing speed measured as SDMT (t=-2.49, W=6.20, p=0.019, β =-0.33) (Figure 5F), but not with grey matter fraction (t=-0.79, W=0.62, p=0.341, β =-0.10) (Figure 5C).

We then went on to investigate if daily levels of the stress hormone cortisol and its circadian slope were associated either with the T cell gene expression or brain activity. Results showed that cortisol markers did not differ between groups nor were associated with T cells gene expression (average diurnal salivary cortisol: t = 1.50, p = 0.129, β = 0.27; circadian slope: t = -1.62, p = 0.112, β = -0.28). However, the results showed a differential association between circadian slope and brain activity in frontal poles in the left and right hemispheres (stress processing brain network #7) (t = -4.11, p = 0.0002, β = -0.40) (Figure 5B). Specifically, the circadian slope of salivary cortisol was positively correlated with the stress processing brain network in HCs but not MS patients (Figure 5B).

In summary, our results show that central stress-processing and T cell stress hormone sensitivity are differentially associated in MS patients in comparison to HCs and are related to key disease severity markers.

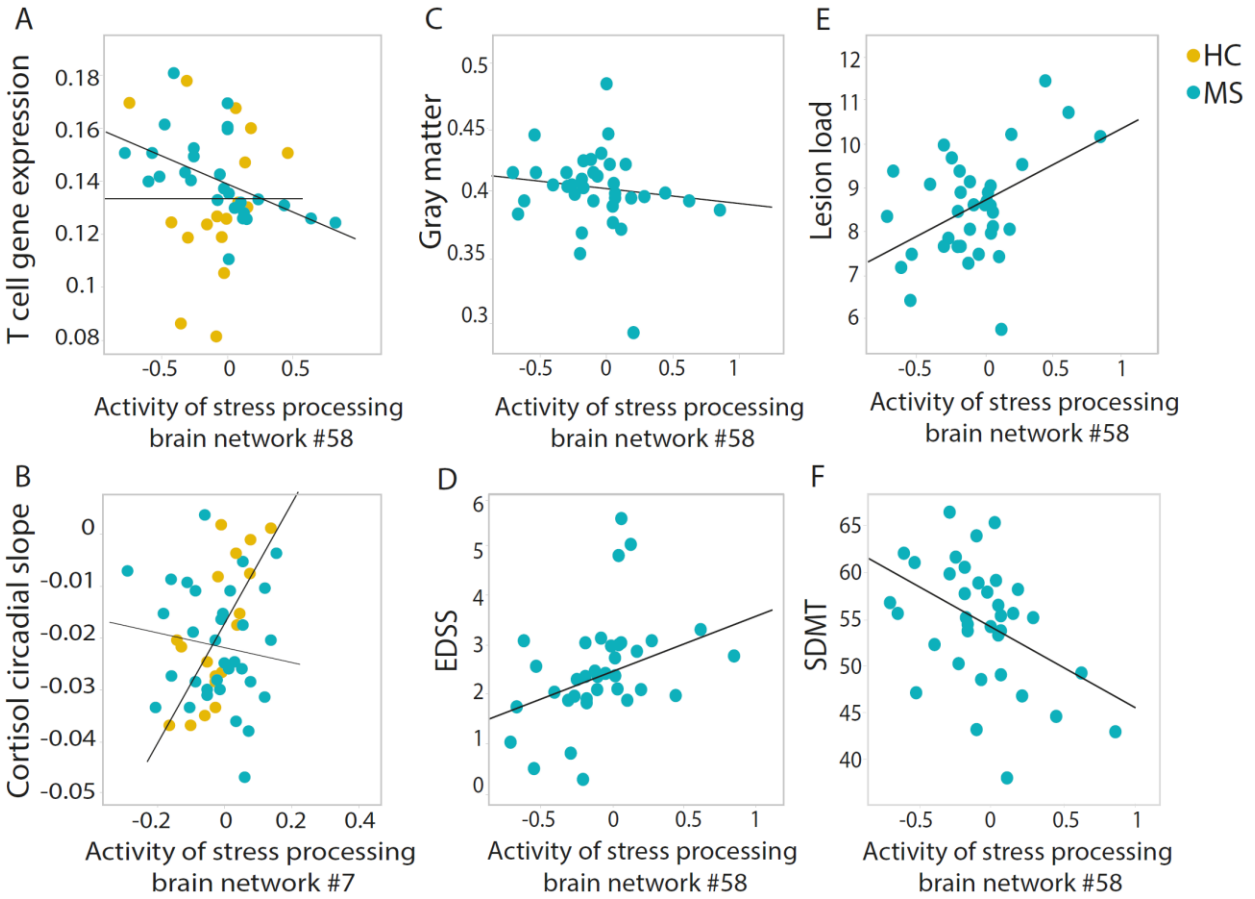

Figure 5. Stress processing brain network showing differential association with T cell gene expression in MS patients compared to healthy controls is linked to markers of disease severity

Robust linear regression analysis showed a negative association of fMRI brain activity in the #58 brain network consisting of the right anterior insula, right fusiform gyrus, left midcingulate and lingual gyrus, with GC related gene expression in T cells of MS patients versus healthy controls (HCs) (A). The activity of the identified brain network was significantly associated with T2-weighted lesion load (E), Expanded Disability Status Scale (EDSS) score that measures MS disease disability (D) and Symbol Digit Modality Test (SDMT) score, a measure of cognitive impairment (F), but not with grey matter fraction (C) in MS patients. The activity of the #7 stress processing brain network was differentially associated with a circadian slope of salivary cortisol across groups (B). Modified from Brasanac et al., 2022b.

4. Discussion

4.1 Short summary of results

This PhD thesis investigated the cellular correlates of depression (MDD) in the context of background inflammation in patients with MS-associated depression (MSD). A decreased frequency of CD4+CCR7low TCM cells in the periphery was identified in depressed MS patients in comparison to MS patients without depression and healthy people using clustering analysis of immunophenotyping data. These cell frequencies showed a correlation with depression severity, specifically with MADRS items coding for core affective symptoms of depression like sadness, but not with MS severity, measured as disability or cognitive impairment. Furthermore, our results showed an association of lower peripheral CD4+CCR7low TCM cell frequencies with lesion load, a measure of increased neuroinflammation. These cell frequencies, however, were not associated with HPA axis activity measured as daily cortisol levels or glucocorticoid-related T cell gene expression. Further examination of cellular and CNS stress processing revealed that the activity of a network consisting of the right anterior insula, right fusiform gyrus, left midcingulate and lingual gyrus was differentially associated with T cell GC signalling across groups of MS patients and healthy controls and linked to disease severity indicating the importance of neuroimmune crosstalk in MS.

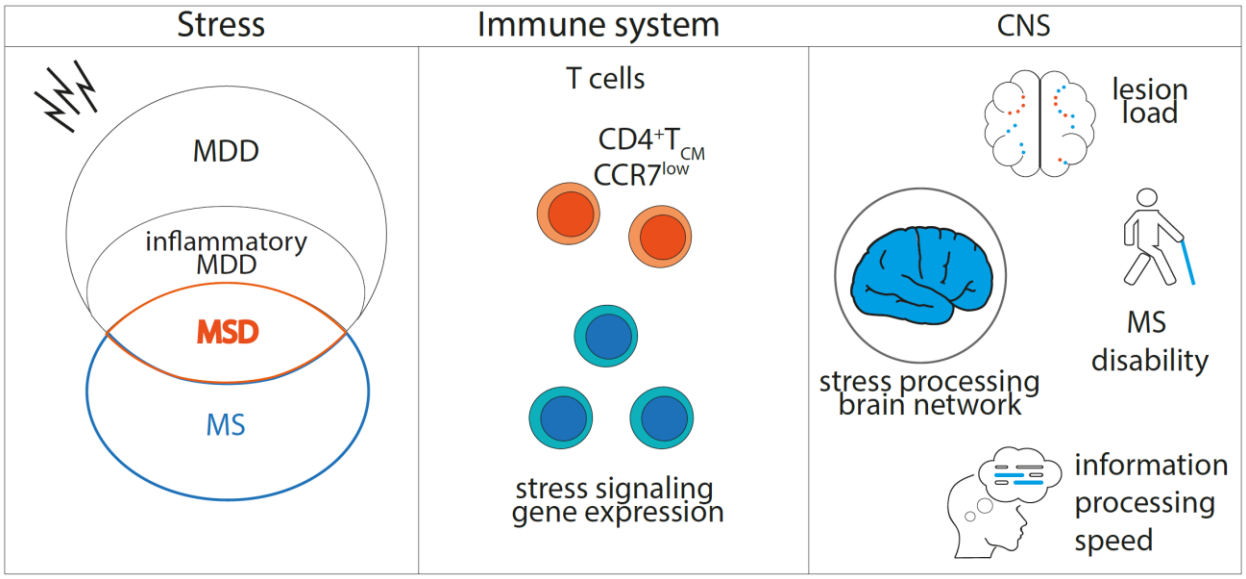

Figure 6. Graphical summary of results

This PhD thesis investigated the immune cellular correlates of depression (MDD) in the context of background inflammation in patients with MS-associated depression (MSD). Clustering analysis of immunophenotyping data identified decreased frequencies of T cell subset, CD4+CCR7low TCM cells, as a robust correlate of depressive symptoms in MSD. These cell frequencies were also correlated with T2-weighted lesion load, a measure of neuroinflammation. Further examination of cellular and CNS stress processing revealed a differential association between glucocorticoid-related T cell gene expression and stress processing brain activity in MS patients versus healthy people. The brain network that was differentially related to T cell gene expression in MS patients in comparison to healthy controls was also linked to disease severity markers: T2-weighted lesion load, MS disease disability and cognitive impairment. Modified from Brasanac et al., 2022b.

4.2 Interpretation of results

Looking for biological correlates of MS and MDD, large scale studies have not identified genetic risk factors that are common for MDD and MS (The Brainstorm Consortium, 2018), suggesting that presumed biological pathways that are connecting the two disorders may be intersecting further downstream. The cellular correlate of MS-associated depression identified in this thesis, decreased CD4+CCR7low TCM cell frequencies correlated with clinical and radiological markers may help us understand the intersection of biological pathways linking mood and autoimmune disorders. CD4+T central memory cells are known as a significant player in MS pathobiology since most T cells identified in the cerebrospinal fluid of patients with MS belong to the central memory subpopulation. Studies have been suggesting that these cells become pathogenic when entering the CNS parenchyma after reactivation (Kivisäkk et al., 2004). It is, however, difficult to determine if decreased cell frequencies in the peripheral circulation represent a loss of these

cells and/or their infiltration into the CNS. We can speculate that the latter can be the case in our study as our results showed lower circulating levels of CD4+TCM cells were associated with a higher neuroinflammatory T2-weighted lesion load in the brain. The role of neuroinflammation in MS-associated depression has been previously reported in studies looking at the activation of microglia in MS patients with depression (Colasanti et al., 2016) as well as studies demonstrating that MS patients with an ongoing neuroinflammation have a higher incidence of depressive symptoms (Rossi et al., 2017).

A correlation between the occurrence of stressful life events and the following development of MS disease activity indicated by both clinical exacerbations and neuroimaging markers of brain inflammation has been shown in the literature (Gold et al., 2005; Mohr et al., 2004; Mohr et al., 2000). Studies have also reported a positive correlation between HPA axis activity and global atrophy (Schumann et al., 2002), as well as HPA axis hyperactivity and disability in patients with progressive MS disease (Heesen et al., 2002; Then Berg et al., 1999). Alterations in the stress response systems have been evident in MDD for decades and the HPA axis is among the most researched biological systems in MDD (Holsboer and Ising, 2010). Reduced glucocorticoid receptor function, GC resistance, and HPA axis hyperactivity are present in individuals showing increased activity of the immune system, which is under the physiological inhibitory control by the glucocorticoid hormone cortisol (Otte et al., 2016). Therefore, we also set to examine the relationship between the identified immune cellular correlate and stress processing/regulating mechanisms as stress is a factor contributing to the pathobiology of both MDD and MS. Our results, however, showed no significant association between CD4+CCR7low TCM cell frequencies and HPA axis activity measured as daily cortisol levels or glucocorticoid-related gene expression indicating that the immune cellular correlate of MS-related depression is unlikely to be secondary to putative central or peripheral stress system alterations. On the other hand, our analyses identified a stress processing brain network consisting of the right anterior insula, right fusiform gyrus, left midcingulate and lingual gyrus that was differentially associated with T cell GC signalling across groups of MS patients and healthy controls and linked to MS disease severity indicating the importance of neuro-immune crosstalk in MS. The neuro-immune communication occurs through humoral and neuronal pathways and is a basis for the immunoregulatory role of the brain and a sensory function of the immune system (Dantzer, 2018). We speculate that an altered link between stress processing brain activity and immune cell sensitivity to glucocorticoids might

be an indicator of an impaired regulation of cellular immunity through the CNS in MS. The identification of the anterior insula as a part of a brain network differentially related to T cell stress signalling supports that hypothesis as the insula is a brain region which acts as a signalling hub for bidirectional brain-immune system interactions (Gogolla, 2017). A recent study established clear evidence for communication between the insular cortex and the peripheral immune system where the insular cortex was shown to encode specific information about the inflammation in the body and regulate peripheral immune responses (Koren et al., 2021). Taking into account the role of psychological factors and stress in the onset and progression of various diseases, including autoimmune disorders (Dantzer, 2018), it is intriguing to speculate if stress activation of the insula may trigger the reactivation of immunological memories (Gogolla, 2021) and elicit relapses in MS.

4.3 Embedding the results into the current state of research

Until now there was no study investigating alterations in immune cell populations in MS-associated depression (MSD). Our study, using two independent case-control cohorts, identified a new immune cellular correlate of MSD and pointed to the shared biology of these two diseases. Moreover, it explored the association between the peripheral immune response and neuroinflammation, supporting the findings of the role of neuroinflammation in MDD (Rossi et al., 2017; Colasanti et al., 2016).

Furthermore, our study is supporting the validity of studying subtypes of depression based on similar biology (Felger and Miller, 2020) rather than just diagnostic categories which include a heterogeneous population of patients. In this regard, interest has been growing to identify subgroups of patients with MDD associated with blood biomarkers of peripheral inflammation (Kiecolt-Glaser et al., 2015; Otte et al., 2016; Milaneschi et al., 2021; Frank et al., 2021). Over the past years, the literature on immunological correlates of MDD has substantially increased with numerous studies investigating frequencies of immune cells (Grosse et al., 2016; Suzuki et al., 2017; Hasselmann et al., 2018; Patas et al., 2018; Becking et al., 2018; Mohd Ashari et al., 2019; Nowak et al., 2019; Lynall et al., 2020). However, even though there has been a significant number of studies, replications of immunological markers have not been successful. The reason can be an inconsistent use and definition of the cellular markers, but it could also be a consequence of biological heterogeneity in MDD. It is also possible that MS-associated depression represents a

more homogeneous population of MDD patients than idiopathic MDD, at least from the point of view of immunology. Therefore, studying immune correlates of depression in MDD patients with a comorbid inflammatory disease can be suitable to “dismantle the monolith” of MDD biopathology (Felger and Miller, 2020).

The importance and role of central stress processing and immune cell sensitivity to stress hormones (cellular stress processing) in MS have been investigated only separately either in neuroendocrine or neuroimaging studies (Schumann et al., 2002; Gold et al., 2005b; Wüst et al., 2008; Ysrraelit et al., 2008; Gold et al., 2012; Weygandt et al., 2016; Meyer-Arndt et al., 2020). In our study, we investigated the interplay between these stress processing systems and showed that their relationship may be clinically meaningful. A stress processing brain network consisting of the right anterior insula, right fusiform gyrus, left midcingulate and lingual gyrus was differentially associated with T cell GC signalling across groups of MS patients and healthy controls and linked to MS disease disability, lesion volume and information processing speed. These results are in line with the findings from a recent study showing that neural and psychological stress processing predicted grey matter atrophy in MS (Meyer-Arndt et al., 2020) and together point to stress processing as a potential intervention target in MS (Sinha et al., 2016; Mohr et al., 2012).

4.4 Strengths and weaknesses of the study(s)

This section provides an overview of the strengths and weaknesses of the studies that the thesis is based on. Detailed descriptions can be found in respective papers (Brasanac et al., 2022a; Brasanac et al., 2022b). The biggest strength in our approach to studying MS-associated depression was analysing data from two independent studies (COGEMS and DENIM) which amounted to the overall sample size of n = 132. The methodology used in both studies was mostly overlapping, but also had dissimilarities in participants matching, inclusion criteria and flow cytometry antibody panels. On top of that, data from two previously published studies on idiopathic MDD were available for clustering re-analysis. All of this strengthens the robustness of our results as well as the specificity of the detected immune cellular correlates of MS patients with depression. Another strength is having both cellular and brain imaging data from the COGEMS study that allowed a multidisciplinary approach and exploration of the relationship between stress processing in

the peripheral circulation and centrally in the brain. Only by integrating results from different biological systems, we can try to understand the whole picture of MS and MDD. On the other hand, there are some limitations that have to be considered, especially when it comes to medications. The differences in MS disease modifying therapies (DMTs) between MS patients with and without depression were not statistically significant, however we cannot completely dismiss that they might have had an impact on the immunophenotyping analyses. We have performed additional sensitivity analyses in participants without DMTs and results have shown the same pattern of decreased CD4+TCM cell frequencies in MS-associated depression in comparison to MS patients and healthy people. Nevertheless, most MS patients were on DMTs, as expected, and the sample size for sensitivity analysis was small and not powered enough to detect any differences in this reduced subgroup of patients and it did not reach the threshold for statistical significance (supplement Brasanac et al., 2022a). It is also possible that treatment with DMTs covered up some additional correlates of MS-associated depression that will be discovered in future studies with a larger sample size of MS patients without DMTs. Another potential issue could be the influence of DMTs on GC-related gene expression in subpopulations of T cells. However, human studies investigating the influence of DMTs on gene expression did not show significant changes for our genes of interest *GR*, *GILZ*, *FKBP4* or *FKBP5* (Henig et al., 2013; Thamilarasan et al., 2013; Friess et al., 2017; Gafson et al., 2018). In addition, we can rule out the interference of steroid treatment since MS patients taking steroids in a period of four weeks before potentially participating in our study were not included. When it comes to antidepressants, only a few participants were taking antidepressants likely due to a combination of factors. For example, it is well documented that depression in MS is generally underdiagnosed and – as a result - undertreated (Marrie et al., 2009). Also, in many of our cases, MDD was formally diagnosed for the first time as part of the thorough psychiatric assessment in our studies. Taken together, it seems that the effects of medication are unlikely to have a significant impact on the observed immune cellular correlate of MS-associated depression.

Further, it should be noted that we accessed glucocorticoid-related T cell signalling as a summary marker of T cell gene expression which was measured by quantitative PCR (qPCR) and without directly evaluating protein levels. In this respect, studies have shown that qPCR measured mRNA expression closely mirrors the levels of GR, GILZ, FKBP5, and FKBP4 proteins (Goecke et al., 2007; Shi et al., 2003; Giraudier et al., 2002; Ward

et al., 1999). Moreover, analysing the transcription of GC related genes can add valuable contribution to our knowledge since chromatin accessibility of the regulatory regions (enhancers and promoters) is important for GC-inducible genes such as *GILZ*. Therefore, this approach represents a composite measure that accounts for GC signalling on different levels and this choice of mRNA targets can provide a good approximation of the GC signalling in T cell populations investigated. Nevertheless, it would be desirable that studies in the future assess protein levels of GC signalling pathway members as well as their functional sensitivity.

Finally, the correlative nature of our study prevents any inference of causality and warrants caution with interpretation. Therefore, it is still up to discover what could be the functional role of CD4+CCR7low TCM cells in MS-associated depression. Also, the small sample size of depressed MS patients in the COGEMS study alone (13) unfortunately did not allow further analysis and comparison of stress processing in MSD versus MS and HCs in this cohort. Future studies should expand in that direction.

4.5 Implications for practice and/or future research

In order to investigate the role of CD4+CCR7low TCM cell frequencies in MS-associated depression and its mechanism of action future studies in animal models are necessary. Tracking these cell subsets in the animal model of MS, the experimental autoimmune encephalomyelitis (EAE), before the onset of MS could give us insight into their role in mediating “depression-like” symptoms. Moreover, using immunophenotyping in clinical trials of depression treatment or DMTs in MS may offer an opportunity to investigate the relationship between depressive symptoms and immune correlates. For a long time, depression was perceived as a uniform disease, however, studies are demonstrating that MDD is not a monolith (Lynall et al., 2020; Felger and Miller, 2020) and that different subtypes exist, among them the inflammatory MDD. From the point of view of immunology MS-associated depression could represent is a more homogeneous group of patients than MDD without any comorbid disease and studying immune correlates of depression in patients with an underlying inflammatory condition could be a good approach to investigate the inflammatory subtype of MDD. Understanding the mechanisms underlying the

comorbidity of MS and MDD is also important to reduce the burden of diseases via prevention and early interventions.

Moreover, studying the interaction between different biological systems, e.g. peripheral and central stress processing is necessary for understanding multifaceted and interlinked aspects of stress, MS and MDD. It is especially important to investigate stress processing in depressed MS patients as well as non-depressed ones. The insula is here a specifically interesting object of studying, acting as a linking point between the brain and the immune system. It is of great importance to investigate if and under which conditions brain-immune interplay may become an endless loop, how long inflammatory memories are stable and how they can be altered. In years to come, new research may also be necessary to address if stress and anxiety can also lead to the reactivation of the insula.

5. Conclusions

Findings from this PhD thesis identified the immune cellular correlate of multiple sclerosis-associated depression linked to core affective symptoms and neuroinflammation. These cells were not associated with stress signalling markers, however, central and peripheral stress processing showed a differential association in multiple sclerosis patients in comparison to healthy controls. Further studies are needed to investigate the function of the described cells as well as to examine neuro-immune interactions in the context of stress. These findings can be relevant for understanding the biology that underlies comorbidity of inflammatory and affective disorders, shed a light on inflammatory subtypes of depression, as well as provide new avenues for the prevention and treatment of MS and MDD.

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Statutory Declaration

“I, Jelena Brasanac, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “Neuro-immune correlates of stress-related symptoms in multiple sclerosis” (“Identifizierung von neuroimmunologischen Korrelaten stress-bedingter Symptome bei Multipler Sklerose”), independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

Jelena Brasanac contributed the following to the below listed publications:

Publication 1: **Brasanac*, J**., Ramien*, C., Gamradt, S., Taenzer, A., Glau, L., Ritter, K., Patas, K., Agorastos, A., Wiedemann, K., Demiraley, C., Fischer, F., Otter, C., Bellmann-Strobl, J., Friese, M. A., Tolosa, E., Paul, F., Heesen, C., Weygandt, M., Gold, S. M., Immune signature of multiple sclerosis-associated depression, *Brain Behavior Immunity*, 2022 https://doi.org/10.1016/j.bbi.2021.11.022

Contribution:

- COGEMS study sample processing – isolation of peripheral blood mononuclear cells (PBMCs) by gradient centrifugation and cryopreservation
- Immunophenotyping COGEMS - preparation, staining and acquisition of all COGEMS samples using a multiparametric flow cytometer
- MRI acquisition of COGEMS participants
- Analysis of all immunophenotyping data (from all cohorts: COGEMS, DENIM, re-analysis of Patas et al., 2018 and Hasselmann et al., 2018) using the CITRUS algorithm and creation of Figure 1A, B, C, D, E; Figure 3A, B, C, D
- Statistical analysis in R and creation of figures 2A, B, E; 3E
- Conceptual planning of the manuscript together with the co-author and supervisor, interpretation of the data, independent drafting and revision of the manuscript, drafting the answer to the reviewers' comments and preparing the final version of the paper.

Publication 2: **Brasanac, J**., Hetzer, S., Asseyer, S., Kuchling, J., Bellmann-Strobl, J., Ritter, K., Gamradt, S., Scheel, M., Haynes, J-D., Brandt, A. U., Paul, F., Gold, S. M., Weygandt, M., Central stress processing, T-cell responsivity to stress hormones and disease severity in multiple sclerosis, *Brain Communications*, 2022 https://doi.org/10.1093/braincomms/fcac086

Contribution:

- COGEMS study sample processing – isolation of peripheral blood mononuclear cells (PBMCs) by gradient centrifugation and cryopreservation
- MRI acquisition of COGEMS participants
- Purification of CD4+ and CD8+ T cells from PBMCs using magnetic beads and quality control using flow cytometry
- Isolation of RNA from purified CD4+ and CD8+ T cells
- Synthesis of complementary DNA
- Quantitative analysis of gene expression using qPCR that was used in interaction analyses and creation of figure 4

- Conceptually planning the manuscript together with supervisors; independent drafting of the manuscript, revision of the manuscript, creating graphical abstract, drafting the answer to the reviewers, editing and finalizing figures, and preparing the final version of the paper.

_____

Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

Excerpt from Journal Summary List

Publication 1

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE,SSCI
Selected Categories: **"NEUROSCIENCES"** Selected Category Scheme: WoS
Gesamtanzahl: 273 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS NEUROSCIENCE	49,897	34.870	0.048890
2	NATURE NEUROSCIENCE	73,709	24.884	0.128020
3	TRENDS IN COGNITIVE SCIENCES	33,482	20.229	0.036270
4	NEURON	111,115	17.173	0.175220
5	ACTA NEUROPATHOLOGICA	28,031	17.088	0.036970
6	MOLECULAR PSYCHIATRY	28,622	15.992	0.046220
7	Molecular Neurodegeneration	6,772	14.195	0.011650
8	TRENDS IN NEUROSCIENCES	22,858	13.837	0.019470
9	Nature Human Behaviour	5,549	13.663	0.023120
10	BRAIN	64,627	13.501	0.061550
11	BIOLOGICAL PSYCHIATRY	50,155	13.382	0.045540
12	JOURNAL OF PINEAL RESEARCH	12,492	13.007	0.008170
13	BEHAVIORAL AND BRAIN SCIENCES	11,610	12.579	0.007760
14	Annual Review of Neuroscience	14,699	12.449	0.010490
15	PROGRESS IN NEUROBIOLOGY	15,161	11.685	0.010300
16	SLEEP MEDICINE REVIEWS	11,218	11.609	0.014840
17	ANNALS OF NEUROLOGY	43,728	10.422	0.039960
18	NEUROSCIENCE AND BIOBEHAVIORAL REVIEWS	36,525	8.989	0.048970
19	Brain Stimulation	9,206	8.955	0.015960
20	npj Parkinsons Disease	1,093	8.651	0.003040
21	FRONTIERS IN NEUROENDOCRINOLOGY	5,338	8.606	0.005050

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	Neurology-Neuroimmunology & Neuroinflammation	3,863	8.485	0.008390
23	Journal of Neuroinflammation	19,657	8.322	0.027070
24	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	4,791	8.090	0.004640
25	NEURAL NETWORKS	18,837	8.050	0.019420
26	Translational Neurodegeneration	1,759	8.014	0.003160
27	NEUROPSYCHOPHARMACOLOGY	30,856	7.853	0.034600
28	Acta Neuropathologica Communications	6,580	7.801	0.016320
29	Fluids and Barriers of the CNS	1,956	7.662	0.002170
30	Neurotherapeutics	6,764	7.620	0.009400
31	NEUROSCIENTIST	5,949	7.519	0.005010
32	Molecular Autism	3,579	7.509	0.007400
33	GLIA	17,858	7.452	0.016000
34	NEUROPSYCHOLOGY REVIEW	3,941	7.444	0.003460
35	Current Neuropharmacology	6,080	7.363	0.007730
36	JOURNAL OF HEADACHE AND PAIN	5,400	7.277	0.008140
37	BRAIN BEHAVIOR AND IMMUNITY	24,161	7.217	0.026930
38	Alzheimers Research & Therapy	5,593	6.982	0.011680
39	PAIN	45,325	6.961	0.031030
40	Translational Stroke Research	3,377	6.829	0.003920
41	BIPOLAR DISORDERS	6,185	6.744	0.007510
42	CURRENT OPINION IN NEUROBIOLOGY	17,009	6.627	0.025180
43	NEUROIMAGE	119,618	6.556	0.105820

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Selected JCR Year: 2020; Selected Categories: "NEUROSCIENCES"

With rank 37 out of 273, *Brain, Behavior and Immunity* is in the top 13.5% of neuroscience journals ranked by impact.

Printing copy(s) of the publication(s)

Publication 1

Brasanac*, J., Ramien*, C., Gamradt, S., Taenzer, A., Glau, L., Ritter, K., Patas, K., Agorastos, A., Wiedemann, K., Demiraley, C., Fischer, F., Otter, C., Bellmann-Strobl, J., Friese, M. A., Tolosa, E., Paul, F., Heesen, C., Weygandt, M., Gold, S. M., Immune signature of multiple sclerosis-associated depression, *Brain Behavior Immunity*, 2022 https://doi.org/10.1016/j.bbi.2021.11.022

Publication 2

Brasanac, J., Hetzer, S., Asseyer, S., Kuchling, J., Bellmann-Strobl, J., Ritter, K., Gamradt, S., Scheel, M., Haynes, J-D., Brandt, A. U., Paul, F., Gold, S. M., Weygandt, M., Central stress processing, T-cell responsivity to stress hormones and disease severity in multiple sclerosis, *Brain Communications*, 2022
https://doi.org/10.1093/braincomms/fcac086

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my dissertation for reasons of data protection.

Publication list

1. **Brasanac, J**., Ramien, C., Gamradt, S., Taenzer, A., Glau, L., Ritter, K., Patas, K., Agorastos, A., Wiedemann, K., Demiralay, C., Fischer, F., Otte, C., Bellmann-Strobl, J., Friese, M. A., Tolosa, E., Paul, F., Heesen, C., Weygandt, M., & Gold, S. M. (2022). Immune signature of multiple sclerosis-associated depression. *Brain, behavior, and immunity*, *100*, 174–182. https://doi.org/10.1016/j.bbi.2021.11.022

   Impact Factor (2022): 19.23

2. **Brasanac, J**., Hetzer, S., Asseyer, S., Kuchling, J., Bellmann-Strobl, J., Ritter, K., Gamradt, S., Scheel, M., Haynes, J. D., Brandt, A. U., Paul, F., Gold, S. M., & Weygandt, M. (2022). Central stress processing, T-cell responsivity to stress hormones and disease severity in multiple sclerosis. *Brain communications*, *4*(2), fcac086. https://doi.org/10.1093/braincomms/fcac086

3. **Brasanac, J**., Gamradt, S., Otte, C., Milaneschi, Y., Monzel, A. S., Picard, M., & Gold, S. M. (2022). Cellular specificity of mitochondrial and immunometabolic features in major depression. *Molecular psychiatry*, *27*(5), 2370–2371. https://doi.org/10.1038/s41380-022-01473-2

   Impact Factor (2022): 15.992

4. Meyer-Arndt, L., Kuchling, J., **Brasanac, J**., Hermann, A., Asseyer, S., Bellmann-Strobl, J., Paul, F., Gold, S. M., & Weygandt, M. (2022). Prefrontal-amygdala emotion regulation and depression in multiple sclerosis. *Brain Communications*, fcac152. https://doi.org/10.1093/braincomms/fcac152

5. Basso, L., Boecking, B., Neff, P., Brueggemann, P., El-Ahmad, L., **Brasanac, J.**, Rose, M., Gold, S. M., & Mazurek, B. (2022). Negative associations of stress and anxiety levels with cytotoxic and regulatory natural killer cell. *Frontiers in Psychology*. https://doi.org/10.3389/fpsyg.2022.871822

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6. Gamradt, S., Hasselmann, H., Taenzer, A., **Brasanac, J**., Stiglbauer, V., Sattler, A., Sajitz-Hermstein, M., Kierszniowska, S., Ramien, C., Nowacki, J., Mascarell-Maricic, L., Wingenfeld, K., Piber, D., Ströhle, A., Kotsch, K., Paul, F., Otte, C., & Gold, S. M. (2021). Reduced mitochondrial respiration in T cells of patients with major depressive disorder. *iScience*, *24*(11), 103312. https://doi.org/10.1016/j.isci.2021.103312

Impact Factor (2021): 5.74

7. Stiglbauer, V., Gamradt, S., Scherzer, M., **Brasanac, J**., Otte, C., Rose, M., Hofmann, T., Hinkelmann, K., & Gold, S. M. (2021). Immunological substrates of depressive symptoms in patients with severe obesity: An exploratory study. *Cell biochemistry and function*, *39*(3), 423–431. https://doi.org/10.1002/cbf.3608

Impact Factor (2021): 3.685

8. Weygandt, M., Behrens, J., **Brasanac, J**., Söder, E., Meyer-Arndt, L., Wakonig, K., Ritter, K., Brandt, A. U., Bellmann-Strobl, J., Gold, S. M., Haynes, J. D., & Paul, F. (2019). Neural mechanisms of perceptual decision-making and their link to neuropsychiatric symptoms in multiple sclerosis. *Multiple sclerosis and related disorders*, *33*, 139–145. https://doi.org/10.1016/j.msard.2019.05.025

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