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DISSERTATION

Different patterns of white matter and immunological alterations in the various phases of bipolar disorder

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CHAPTER I

Bipolar disorder: history and clinical features

Bipolar disorder (BD) is a prevalent recurrent and chronic mental disease (affecting 1-2% of the general population), associated with high rates of non-recovery, psychiatric and medical comorbidity, and progressive disability [1]. Clinically, BD is characterized by the occurrence of active phases of illness, mania and depression, alternated to asymptomatic periods of euthymia [1, 2].

The history of bipolar disorder

The origin of the concept of BD has its roots in ancient times, with mania and melancholia as two of the earliest described human diseases, and it is still very controversial in the modern nosology of psychiatric disorders [3].

Basing on the theory of Alcmaeon of Crotona (5th century BC) who thought that the origin of diseases was the disturbed interaction of body humors with the brain, Hippocrates (460-337 BC) was the first who systematically described mania (a state of severe anger or excitation, resulting from an excess of "yellow bile") and melancholia (a state of severe sadness, resulting from an excess of "black bile") as biologically defined illnesses [3, 4]. Then, Aretaeus of Cappadocia (1st century AD) explicitly described an intimate link between mania and melancholia, believing that they had the same etiology due to brain dysfunction, and thus giving the first conception of bipolarity [3, 4]. Specifically, in his view, mania was the phenomenological counterpart of melancholia, and represented a worsening of melancholia [3, 4].

For over 2000 years, mania was considered the main and most common form of mental illness, as described by leading clinicians such as Philippe Pinel (1801) [5]. Moreover, Wilhelm Griesinger (1845), one of the most important founders of German scientific psychiatry, considered excitatory phenomena of mania as causative of some depressive states, further suggesting a core role of mania and also a link between the two psychopathological conditions [3, 5]. Although during the 17th and 18th century several Authors described the recurrent longitudinal association

of mania and depression, only in the mid-nineteenth century the concept of a new and distinct psychiatric disorder, which was characterized by a continuous cycle of depression and mania, emerged with the description of Jean-Pierre Falret's *folie circulaire* and Jules Baillarger's *folie à double forme* [3, 4].

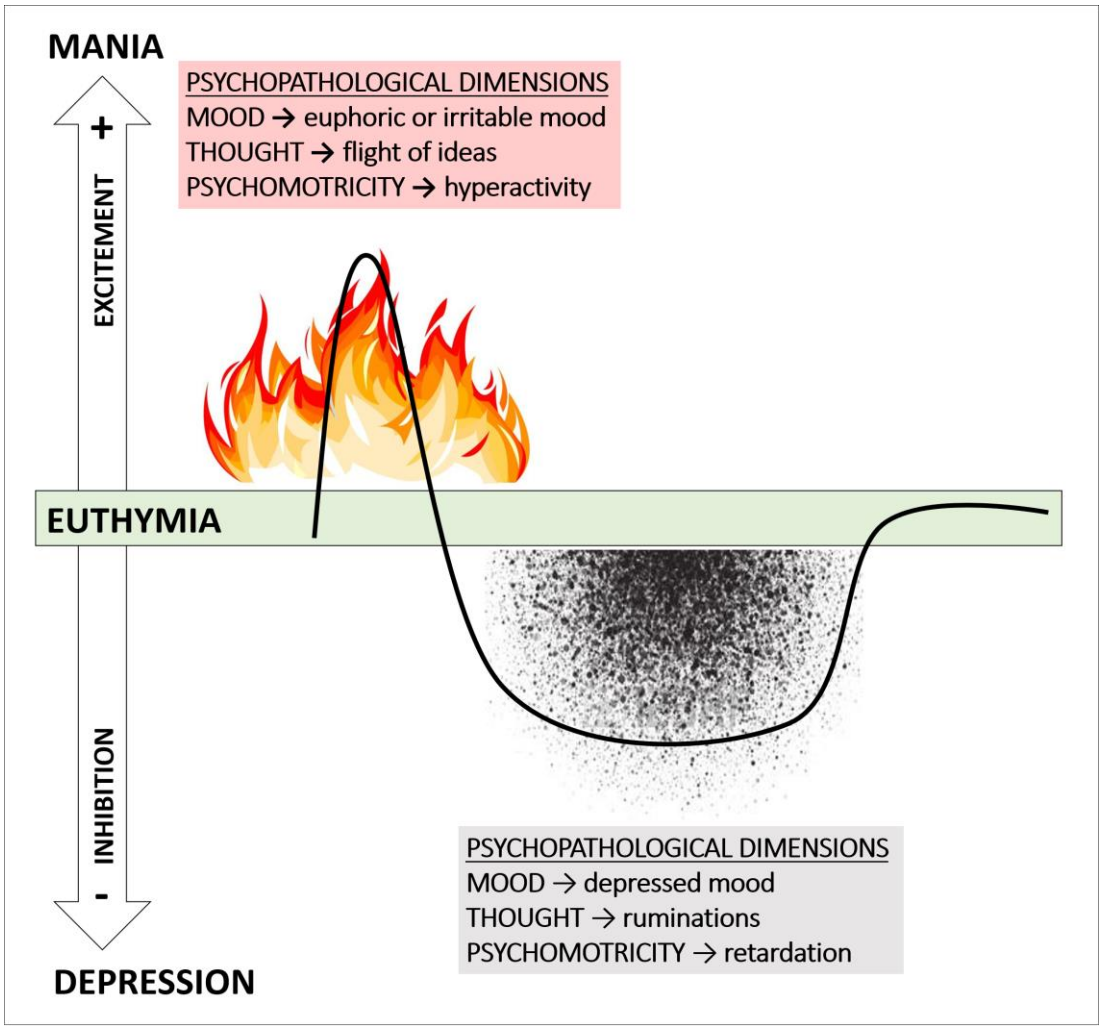
Finally, in 1890s Emil Kraepelin conceptualized the manic-depressive insanity, as a full spectrum of dysfunctions which included single episodes of mania or depression as well as cyclic recurrent manic and depressive episodes, giving a fundamental contribution in the modern psychiatric nosology [2-4]. The manic-depressive insanity, usually associated with psychotic features, was then rephrased as manic-depressive illness, to include the majority of subjects without psychosis [2, 6]. In particular, the Kraepelinian manic-depressive illness included a heterogeneous cluster of chronic-recurrent disorders characterized by intermittent or prolonged affective instability, resulting in states of excitement or inhibition of the main psychopathological dimensions, such as mood, thought and psychomotricity (activity/level of energy/volition) [2]. Mania was characterized by the excitement of such psychopathological dimensions which manifests in manic symptoms like euphoric or irritable mood, flight of ideas and hyperactivity [2]. On the other hand, depression was characterized by the inhibition of the same dimensions, resulting in depressive symptoms including depressed mood, ruminations and psychomotor retardation [2]. Furthermore, between the two extreme poles of mania and depression, Kraepelin also described, for the first time, different mixed states, as different combinations of inhibition or excitement of the various psychopathological dimensions [2]. Importantly, in accordance with the previous view, Kraepelin had broad criteria for mania, since both pure mania and many categories of mixed states were basically conditions of excitement [2, 5].

The Diagnostic and Statistical Manual of Mental Disorder (DSM)-I (1952) was the first attempt to categorize and standardize mental illness, and classified manic-depression basing on the Kraepelinian concepts [4]. With the advent of DSM-II (1968), manic-depressive illness was classified under "affective disorders", focalizing especially on mood alteration and giving less relevance to the other psychopathological dimensions (equally important for Kraepelin) [4]. Over the same period, basing on the work of Carl Wernicke (1900) in opposition to Kraepelin's view, Karl Kleist and Karl Leonhard (1950s) divided the manic-depressive insanity in bipolar recurrent psychosis and unipolar recurrent psychosis, describing mania and

depression as separate diseases and thus breaking the tight link between them [3, 4, 7]. This become the formal distinction of BD from major depressive disorder (MDD), which then was officially incorporated in the DSM-III (1980) [6, 8]. Importantly, MDD included both the unipolar depressive illness (as characterized by severe recurrent depressive episodes) and "neurotic depression" (which was mild/moderate, chronic, not episodic condition with predominance of anxious symptoms) [6]. This broadened the concept of depression, emphasizing its role as central in affective disorders [5, 6]. The subsequent editions of DSM (i.e., DSM-IV and DSM-5) maintained this approach moving further and further from Kraepelin's original characterization of manic-depressive illness [1, 4, 9]. Therefore, contemporary psychiatry considers depression and mania as different entities, and uses a broad definition of depression and a narrow definition of mania, describing only partially the complexity of BD [5].

Recently, various Authors challenged the current approach on BD by referring to some core concepts of the Kraepelinian view [4, 5, 10]. In particular, Hagop Akiskal (1980s and 1990s) has conceptualized the "bipolar spectrum", proposing an expansion of the diagnostic criteria for bipolar illness to better capture its complex clinical presentation and encompass its broader manifestations [10]. On the other hand, Athanasios Koukopoulos (2000s) proposed the "primacy of mania" hypothesis, in opposition to the current view, where depression is seen as more prominent, common, and problematic, while mania appears uncommon and treatment-responsive [5]. According to his view, mania is considered in a broader form, as a wide range of excitatory processes, occurring always before depression [5]. Mania is described as the *fire* of BD, seen as the core of the pathophysiology of the illness, while depression as its *ashes*, a consequence of the manic excitatory process [5].

Considering the classical clinical descriptions and the more recent contribution by various Authors, a current debate is still open on the psychopathology of BD, which needs to be further defined to guide diagnosis and therapy, as well as neurobiological research.



Clinical features of bipolar disorder. Bipolar disorder in accordance with the Kraepelinian view and primacy of mania hypothesis by Koukopoulos A.

Neurobiological research on bipolar disorder

As described, BD is characterized by a complex clinical presentation and manic, depressive, and euthymic episodes show very different psychopathological patterns and a still unclear relationship between each other. Thus, considering this clinical framework, such various clinical phases and different symptomatology of BD may be underpinned by distinct and/or common patterns of neurobiological alterations. Therefore, the investigation of the various phases of BD separately (instead of BD overall, regardless of the phases of illness), may help to better understand and clarify the specific neurobiological correlates and the pathophysiology of this disorder, which, to date, remain still unclear.

CHAPTER II

Different patterns of white matter alterations in the various phases of bipolar disorder

Introduction and background

In recent years, a growing number of studies have investigated neurobiological abnormalities in BD and, in particular, magnetic resonance imaging (MRI) has become a relevant non-invasive tool to explore *in-vivo* the pathophysiology of the disorder [11]. In this context, diffusion tensor imaging (DTI) - an MRI technique that measures the random movement of water molecules in the brain providing information about white matter (WM) microstructure and tracts [12] - has assumed a central role. Interestingly, WM changes resulted to be one of the most consistent alterations in BD. In particular, several whole brain studies have demonstrated widespread DTI alterations with all major classes of tracts implicated, involving prefrontal regions as well as projection, associative and commissural fiber tracts [13-16]. Among them, the midline regions, such as the corpus callosum and cingulum (a core component of the limbic system which plays a central role in the pathophysiology of BD [17]), were found to be the most constantly altered tracts in BD, as also confirmed in tractography studies (e.g., [13, 14, 18-21]). These works mostly reported a decrease in fractional anisotropy (FA) as well as an increase in mean diffusivity (MD) and radial diffusivity (RD) and, in few cases, changes in axial diffusivity (AD), reflecting microstructure abnormalities of WM in BD [13, 14]. These previous DTI studies, using both whole brain and tractographic approach on specific tracts such as the cingulum, investigated BD as a whole (regardless of the phase of illness) or patients in euthymic/remitted or depressed phases (e.g. [13-16, 18-38], and none of them included and directly compared all the various phases of BD.

Since BD presents a cyclic pattern with dramatic changes in clinical states across the different phases, showing acute phases characterized by full blown and opposite psychopathological states (mania and depression), as well as subclinical states

similar to healthy (euthymia), the investigation of state-dependent brain changes assumes particular relevance in this illness (see also Chapter I). Accordingly, some functional and metabolic data suggest state-dependent changes across the different phases of BD (e.g., [17, 39-43]). Beyond functional alterations, recent evidences in DTI studies suggest that dynamic changes also occur in WM microstructure, both in healthy after learning-induced plasticity [44-47] and in depressed patients when compared to those in remission [48, 49].

Therefore, considering the previous evidences along with the clinical framework of BD, we have conducted two studies aimed to investigate whole brain WM microstructure as well as structural connectivity of the cingulum bundle, considering and directly comparing the various phases of BD - i.e., mania, depression and euthymia [50, 51].

Diffusion tensor imaging (DTI) is a magnetic resonance imaging (MRI) technique that measures the random movement of water molecules in the brain, providing information about white matter (WM) microstructure *in vivo* [12, 16].

Distinct DTI parameters can be calculated to characterize water molecules diffusion in brain WM and, indirectly, fiber tract microstructure. Fractional anisotropy (FA) is a normalized measure of anisotropic diffusion, ranging from 0 (equal diffusion in all directions) to 1 (highly directional diffusion), and is affected by fiber coherence, myelin, extracellular diffusion and axonal density [16]. Mean diffusivity (MD) measures the average diffusion across the x, y, and z directions. Additionally, axial diffusivity (AD) measures the diffusion along fiber, while radial diffusivity (RD) describes the diffusion perpendicular to axons and is thought to be particularly sensitive to demyelination [12, 52].

Different approaches can be used for DTI data analyses, including tract-based spatial statistics (TBSS), and tractography [16]. In particular, TBSS is a whole-brain voxel-wise analysis that provide a comprehensive measure of WM alterations [53]. On the other hand, DTI tractography allows reconstruction of WM tracts and evaluation of their structural connectivity [54].

Methods

Sample and clinical assessment

The studies were conducted on 61 type I BD patients (21 in manic, 20 in depressive, and 20 in euthymic phases) and 42 healthy controls (HC). See **Table 1** for a detailed description of the sample. Subjects were recruited from the Psychiatric Clinic of Genoa (San Martino Polyclinic Hospital and Department of Neuroscience at the University of Genoa, Italy). The Ethical Committee of San Martino Polyclinic Hospital approved the studies, and written informed consent was obtained from all the participants.

Each participant was evaluated with standardized structured and/or semi-structured clinical instruments to obtain information on clinical and diagnostic features, course of illness, family history, and actual and past pharmacotherapy. The instruments were: the Mini International Neuropsychiatric Interview (MINI) [55]; the Structured Clinical Interview for DSM Axis-I Disorders/Patient edition (SCID-I/P) [56]; the Structured Clinical Interview for DSM Axis II Personality Disorders (SCID-II) [57]; the Structured Interview for Mood Disorder – Revised (SIMD-R) [58]; the Hamilton Depression Scale (HAM-D) with 17 items [59]; the Young Mania Rating Scale (YMRS) [60]. Physiologic, medical and psychopathologic history was also investigated, and physical and psychiatric examinations were conducted.

Inclusion criteria were as follows: diagnosis of BD type I, during manic or major depressive episodes, or euthymia, according to the DSM criteria [1] assessed by the SCID-I/P; score of 17-items HAM-D≥18 for depressed patients; score of YMRS≥13 for manic patients; scores of HAM-D<8 and YMRS<8 for euthymic patients; age between 18 and 60; ability to provide written informed consent. Exclusion criteria were as follows: diagnosis of schizophrenia, mental retardation, dementia, or other cognitive disorders; history of severe or decompensated somatic diseases, neurological diseases (e.g., stroke, cerebral vascular malformations, or epilepsy), previous head injury with loss of consciousness (for 5 or more minutes); current alcohol and substance abuse (during the preceding 3 months), history of alcohol or substance dependence, abuse of synthetic and/or new drugs; pregnancy and lactation; left-handedness; inability to undergo an MRI examination (claustrophobia, metal implants, etc.); previous treatment with electroconvulsive therapy, chemotherapy or brain radiotherapy. Healthy participants did not meet the DSM criteria for psychiatric

disorders, either at the time of study participation or in the past; they had a HAM-D score <8 and a YMRS score <8; they also met the same exclusion criteria indicated for patients.

Table 1. Subject demographic and clinical information

	BD				**HC**	**ANOVA** F (p)
	TOT	MANIC	DEPRESSED	EUTHYMIC		
Sample Size *n* (%)	61 (100%)	21 (34.4%)	20 (32.8%)	20 (32.8%)	42 (100%)	-
Age *mean* (SD)	44.6 (11.1)	45.6 (11.8)	44.9 (10.9)	43.1 (11)	44.3 (12.7)	0.258 (0.773)
Female *n* (%)	43 (70.5%)	18 (85.7%)	13 (65%)	12 (60%)	27 (64.3%)	1.321 (0.272)
HAM-D *mean* (SD)	-	6.9 (5.3)	21.5 (4)	3.6 (2.8)	1.0 (1.4)	103.460 (0.000)
YMRS *mean* (SD)	-	18.8 (5.6)	4.2 (2.8)	3.9 (2.7)	0.5 (1.0)	93.146 (0.000)
Age of Onset *mean* (SD)	25.1 (10.8)	25.4 (12.1)	25.3 (9.8)	24.6 (10.9)	-	0.009 (0.991)
Duration of Illness *mean* (SD)	19.6 (11.6)	20.9 (14.6)	19.5 (10.8)	18.2 (9)	-	0.257 (0.774)
Number of previous total episodes *mean* (SD)	8.6 (8.3)	10.0 (9.5)	8.8 (9.3)	6.9 (5.8)	-	0.713 (0.494)
Number of previous manic episodes *mean* (SD)	4.7 (4.8)	6.1 (5.6)	4.1 (4.9)	3.8 (3.9)	-	1.463 (0.240)
Number of previous depressive episodes *mean* (SD)	3.5 (4.3)	3.6 (4.8)	4.2 (4.6)	2.7 (3.2)	-	0.632 (0.535)
Mood Stabilizers *n* (%)	52 (85.2%)	16 (76.1%)	18 (90%)	18 (90%)	-	0.478 (0.622)
Antidepressants *n* (%)	22 (36.1%)	2 (9.5%)	11 (55%)	9 (45%)	-	5.835 (0.005)
Antipsychotics *n* (%)	35 (57.4%)	14 (66.7%)	12 (60%)	9 (45%)	-	1.009 (0.371)
Benzodiazepines *n* (%)	21 (33.4%)	6 (28.6%)	7 (35%)	8 (40%)	-	0.287 (0.752)
Unmedicated *n* (%)	2 (3.3%)	1 (4.8%)	0 (0%)	1 (5%)	-	0.488 (0.616)

Demographic and clinical information of the samples. In the last column the ANOVAs of comparisons between BD subgroups are shown.
Abbreviations: BD, bipolar disorder; HC, healthy controls; HAM-D, Hamilton Depression Scale; YMRS, Young Mania Rating Scale.

MRI data acquisition

Images were acquired using a 1.5-T GE scanner with a standard head coil. Foam pads were used to reduce head motion and scanner noise. DTI was acquired with a pure axial single-shot EPI sequence (diffusion sensitizing gradients along 60 non-collinear directions, b=1000 s/mm^2, 5 b=0 acquisitions, TR/TE=13750/93 ms, image matrix=128x128, FOV=24 cm, NEX=1, 55 contiguous axial 2.5 mm-thick slices). In addition, two anatomical sequences were acquired for all participants: a) sagittal three-dimensional (3D) T1-weighted SPGR (TR/TE=11.5/5 ms, IR=500 ms, flip angle=8 degree, FOV=25.6 cm, isotropic 1 mm^3 voxel-size); b) fluid-attenuated inversion recovery (FLAIR) (TR/TE/TI=8000/120/2000 ms, FOV=24 cm, matrix 256x192, 5 mm-thick contiguous slices). T1-weighted and FLAIR images from all participants were reviewed by a board-certified neuroradiologist, and none of them showed structural visible lesions.

DTI data preprocessing

Diffusion data were preprocessed and analyzed using tools from the Oxford University Centre for FMRIB software library (FSL 5.0, http:// www.fmrib.ox.ac.uk/fsl/) [61]. First, the b0 image of each subject was skull-stripped using the brain extraction tool. Since head motion can affect the DTI data and may be phase specific - manic patients could move more than depressed patients - we checked this issue in various ways. Each participant's motion was assessed by means of translation/rotation, and an exclusion criterion (translation>3 mm and rotation>3° in each direction) was set. All participants of the studies showed head motion of less than 1 mm. Furthermore, head motion parameter was compared between groups and revealed no significant differences among them (F=0.909; p=0.440). Then, the data were corrected for subject motion and eddy-current induced geometrical distortions, and the diffusion sensitizing gradients ("bvecs") were rotated to correct for motion. Subsequently, using the FMRIB's Diffusion Toolbox (FDT), the diffusion tensor (DT) was estimated in each voxel using a linear regression and FA, MD, RD and AD maps were derived.

TBSS study

In the first study, we aimed to investigate WM microstructure alterations in the different phases of BD, using a whole brain approach.

TBSS analysis was used to perform a voxel-wise analysis of the whole brain WM DT MRI measures (http://www.fmrib.ox.ac.uk/fsl/tbss/index.html) [53, 61]. Briefly, the individual FA images were non-linearly registered to the FMRIB58_FA standard space, provided within FSL, and averaged to create a mean FA image. The resulting mean FA image was then thinned to create a WM tract 'skeleton', which was thresholded at a FA=0.2 to include only WM voxels. Each subject's aligned FA data was then projected onto this skeleton and the resulting alignment-invariant representation of the central trajectory of WM pathways was used for voxel-wise statistical analysis. Similar processes were applied to non-FA data - MD, RD and AD maps - by using the individual registration and projection vectors obtained in the FA non-linear registration and skeletonization stages.

Voxel-wise differences in FA, MD, RD and AD values between BD patients and controls were tested using a permutation-based inference for non-parametric statistical thresholding ('randomize' program within FSL) [62] and two-sample t-test. The number of permutations was set to 5,000. Age and gender were entered into this analysis as confound regressors to ensure that any observed effect of group on FA, MD, RD and AD was independent of age- and gender-related changes. A p value <0.01, corrected for family-wise error (FWE) using the threshold-free cluster enhancement (TFCE) option in the 'randomize' tool [63], was set for between-groups comparison. The WM tracts were identified according to the JHU White Matter Tractography Atlas included in FSL [64-66]. Those voxels of the skeleton that resulted significantly different between patients and controls were transformed back to the native space, and the values of DT-MRI measures averaged within each cluster at tracts and skeleton level were extracted to perform the analysis of subgroup comparisons and correlation analyses.

The mean FA value of all tracts was entered into an ANOVA followed by post-hoc Games-Howell test to detect differences between the various subgroups - i.e., depressed, manic, euthymic patients, and HC. Subsequently, the FA value of each tract was entered into an ANOVA followed by post-hoc Games-Howell test to explore differences between the various subgroups at the single tracts level. The same procedure was performed for the MD, RD and AD values. All results were thresholded at a corrected p value of 0.01.

Then, the voxel-wise differences in FA, MD, RD and AD values between the various subgroups of patients and HC were also tested using 'randomize' program within

FSL and two-sample t-test, with age and gender as coregressors. A p value <0.01, corrected for FWE using the TFCE, was set for between-groups comparison**.** We also performed the same analyses at a p value <0.05, in order to explore the differences between subgroups with a less strict threshold. Finally, we extracted the total cluster size from each significant contrast, with the purpose of evaluate the overall alterations load in each subgroup.

Correlation analysis was performed to investigate the relationship between DTI findings and clinical data (i.e., YMRS and HAM-D scores).

Statistical analyses were performed in SPSS version 19.

Tractography study

In our second study, we investigated potential alterations in the structural connectivity (SC) of cingulum bundle in the various phases of BD, by using probabilistic tractography.

Voxel-wise probability density functions of the principal diffusion direction were estimated using Markov Chain Monte Carlo sampling with FSL's BEDPOSTX [67]. These were then used to estimate the connectivity between each voxel and the seed voxels using FSL's PROBTRACKX tool [67], in which 5,000 sample streamlines were taken for each seed voxel with a 0.2 curvature threshold, 0.5 mm step length, and 2,000 steps per sample.

Seed regions, waypoints, termination and exclusion masks were defined on the MNI152 2 mm T1 brain template. Perigenual anterior cingulate cortex (PACC) was used as seed region of interest (ROI) for the reconstruction of the cingulum bundle. The supragenual anterior cingulate cortex (SACC) and middle cingulate cortex (MCC) were used as waypoints, while the posterior cingulate cortex (PCC) as termination mask, basing on the JHU White Matter Tractography Atlas [64-66]. This procedure was separately performed on the left and right hemispheres. An exclusion mask was drawn on the brain midline to eliminate pathways crossing to the other hemisphere. Only streamlines that passed through the seed region, waypoints and termination mask were retained. These paths were thresholded at 1% to reduce the likelihood of including extraneous tracts. All data were visually inspected to confirm successful tracing in each individual and to detect possible artifacts. Individual FA maps were registered to the MNI152 1 mm T1 brain template, as implemented in FSL. Then, the resulting cingulum tracts were registered to the diffusion space and

used to extract mean FA of the left and right cingulum. The overall mean FA of the cingulum bundles was calculated by averaging left and right cingulum FA values, as a measure of tract integrity [68]. The same procedure was applied to obtain the mean MD, mean RD and mean AD of the cingulum.

Then, in order to investigate the alterations in specific portions of the cingulum, we obtained separate masks for the anterior part (from PACC to SACC), middle part (from SACC to MCC) and posterior part (from MCC to PCC), and calculated left, right and overall mean FA, MD, RD and AD values of each portion of the cingulum.

All the calculated DTI parameters were entered into statistical analyses. The mean FA, MD, RD and AD values of the cingulum were entered into a 4 (DTI parameters) x 4 (subgroups) mixed between-within subjects ANOVA followed by post-hoc Games-Howell test, in order to detect differences in the cingulum SC in the various subgroups. Then, each mean cingulum DTI value found to be altered was entered into an ANOVA followed by post-hoc Games-Howell test, in order to control which DTI parameter is specifically altered. Finally, the same statistical analyses were performed for each portion of the cingulum separately, in order to explore which part is mainly affected. All results were thresholded at a corrected p value of 0.05.

Correlation analyses were performed between clinical data (i.e., YMRS and HAM-D total scores) and the altered DTI parameters of cingulum SC.

Statistical analyses were performed in SPSS version 19.

Results

TBSS study - Patterns of microstructural white matter abnormalities in the various phases of bipolar disorder

Firstly, a widespread decrease in FA values and increase in MD and RD values was found in BD compared to controls in clusters belonging to various tracts, including the bilateral anterior thalamic radiation (ATR L/ATR R), cingulate gyrus (CG L/CG R), corticospinal tract (CST L/CST R), forceps major (Fmaj), forceps minor (Fmin), inferior fronto-occipital fasciculus (IFOF L/IFOF R), inferior longitudinal fasciculus (ILF L/ILF R), superior longitudinal fasciculus (SLF L/SLF R) and uncinate fasciculus (UF L/UF R), at $p<0.01$ TFCE corrected (**Figure 1**). No significant differences in AD values were found between groups.

Secondly, we carried out comparisons between the subgroups – i.e., depressed, manic and euthymic patients, and HC. Firstly, the ANOVA showed a significant difference between subgroups: in the mean FA of all tracts ($F=10.332$; $p=0.000$), with decreased values in depressed ($p=0.002$) and manic ($p=0.000$) patients compared to HC; in the mean MD ($F=9.437$; $p=0.000$), with only depressed patients showing increased value compared to HC ($p=0.001$); and in the mean RD ($F=9.715$; $p=0.000$), with increased values in depressed ($p=0.002$) and manic ($p=0.001$) patients compared to HC. Then, at the single tract level, the ANOVA showed a significant difference between subgroups in FA, MD and RD values in all the investigated tracts, at a corrected p value <0.01 (**Table 2**). Specifically, among subgroups, manic and depressed patients showed altered DTI parameters in the WM tracts, when compared to HC, at a corrected p value <0.01, while no significant differences were found in euthymic patients. Lowering the threshold at a $p<0.05$, a decrease in FA values mainly in Fmin, as well as a more widespread increase in RD values with the largest cluster in Fmin, was found in euthymic patients when compared to HC.

Then, the whole-brain comparisons at a corrected p value <0.01 confirmed: a diffuse decrease in FA values and a diffuse increase in MD and RD values in WM belonging to various tracts in the depressed subgroup when compared to HC (**Figure 2a**); a decrease in FA values and an increase in RD values but no differences in MD values in the manic subgroup when compared to HC (**Figure 2b**); no differences in FA, MD and RD values in the euthymic subgroup when compared to HC (but a localized decrease in FA and an increase in RD values was found at a $p<0.05$).

With regard to the clinical correlations, HAM-D and YMRS total scores did not show any significant correlation with FA, MD and RD values.

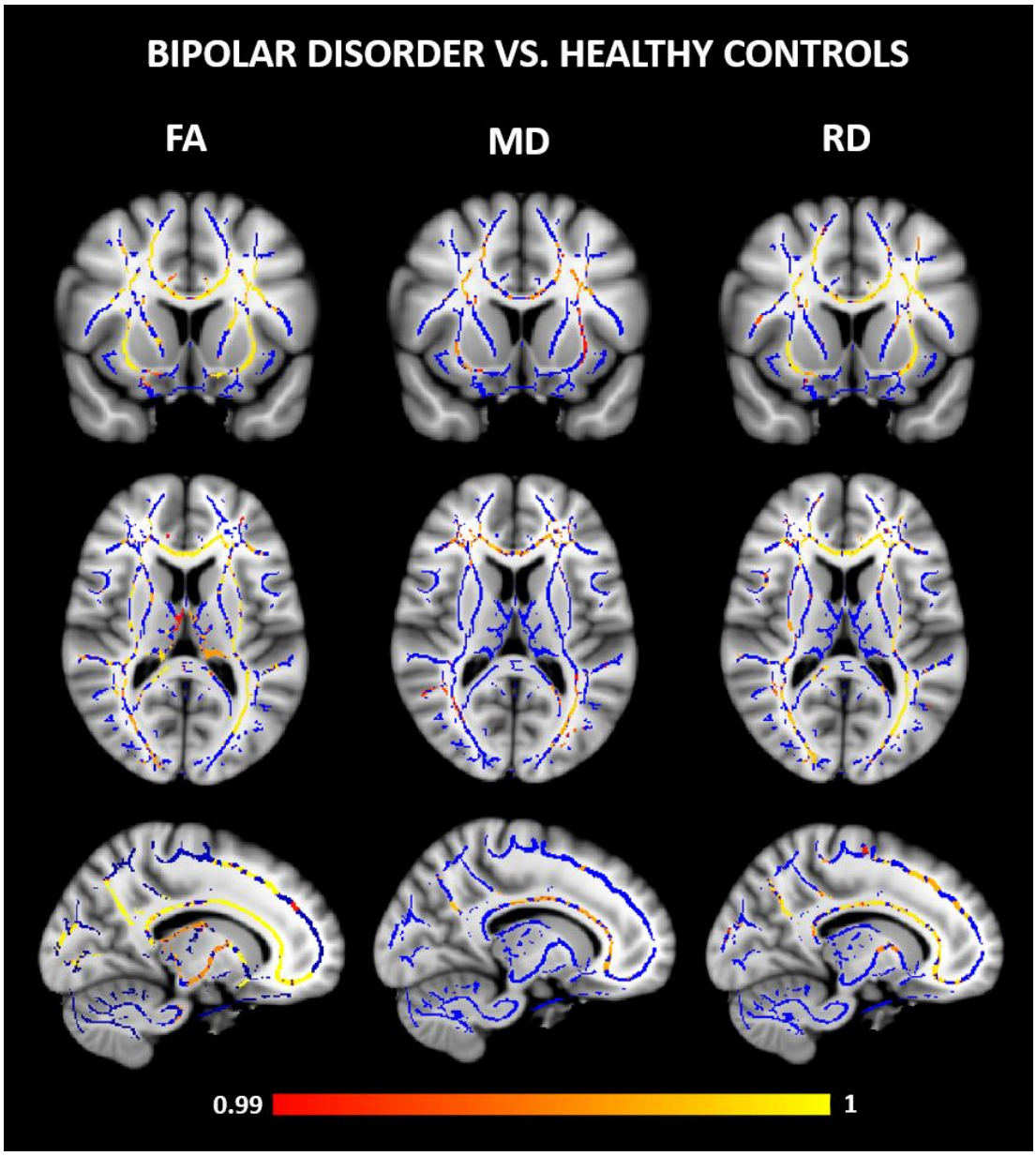


Figure 1. DTI parameters in BD vs HC: FA, MD and RD values
Results obtained from between-groups comparison showing in *red-yellow* the clusters of voxels with significantly decreased fractional anisotropy values (FA column), increased mean diffusivity values (MD column) and increased radial diffusivity values (RD column) in BD patients when compared with HC ($p<0.01$, TFCE corrected). For display purposes the statistically significant clusters are displayed as 1-p values. The white matter skeleton, thresholded at FA>0.2, is represented in *blue*. Group differences are mapped onto standard T1 Montreal Neurological Institute (MNI) template. Images are in radiological convention.
Abbreviations: DTI, diffusion tensor imaging; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; BD, bipolar disorder; HC, healthy controls.

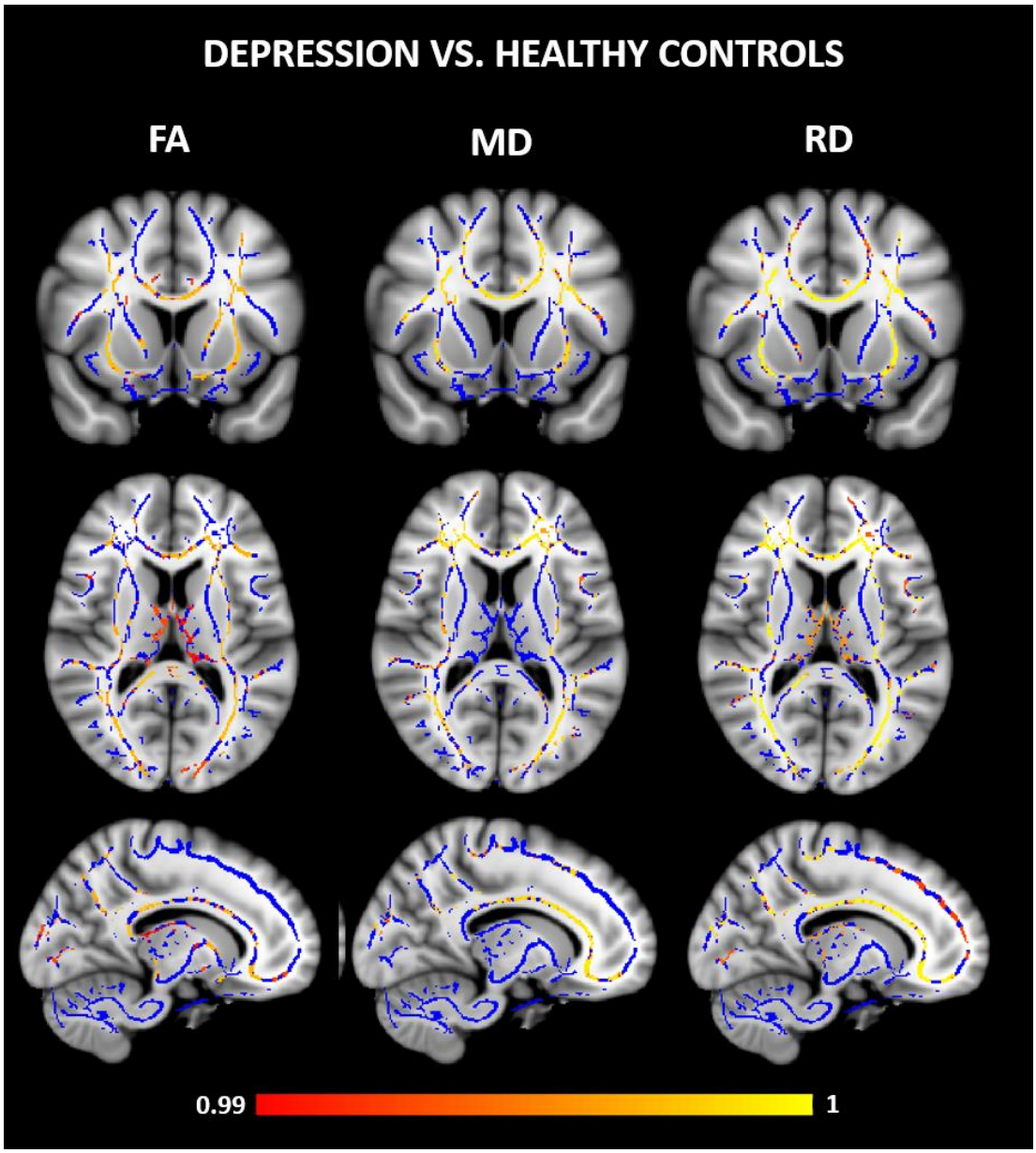


Figure 2a. DTI parameters in D vs HC: FA, MD and RD values
Results obtained from between-groups comparison showing in *red-yellow* the clusters of voxels with significantly decreased fractional anisotropy values (FA column), increased mean diffusivity values (MD column) and increased radial diffusivity values (RD column) in BD patients in depressive phase when compared with HC ($p<0.01$, TFCE corrected). For display purposes the statistically significant clusters are displayed as 1-p values. The white matter skeleton, thresholded at FA>0.2, is represented in *blue*. Group differences are mapped onto standard T1 Montreal Neurological Institute (MNI) template. Images are in radiological convention.
Abbreviations: DTI, diffusion tensor imaging; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; D, depressed patients; HC, healthy controls.

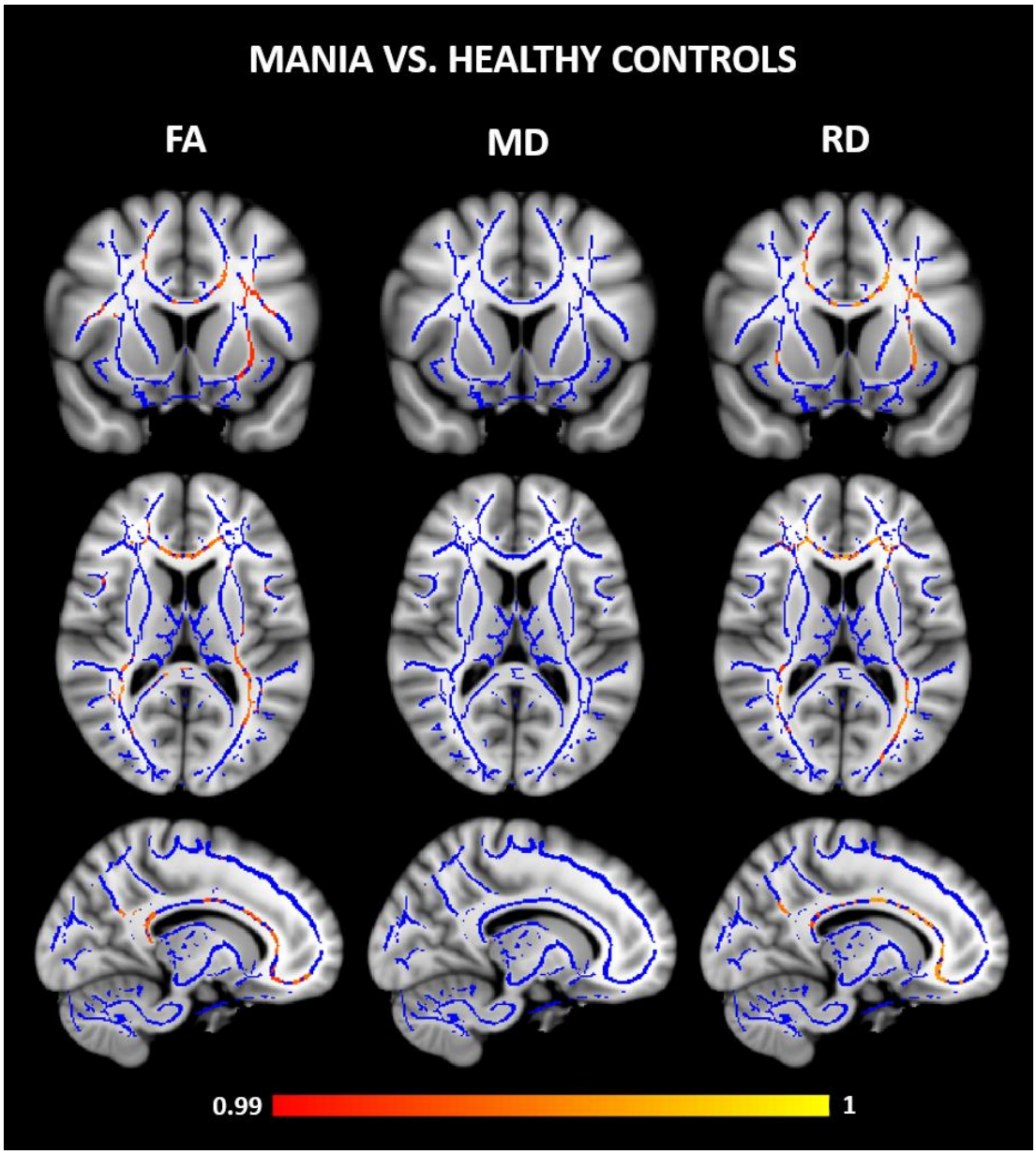


Figure 2b. DTI parameters in M vs HC: FA, MD and RD values
Results obtained from between-groups comparison showing in *red-yellow* the clusters of voxels with significantly decreased fractional anisotropy values (FA column), increased mean diffusivity values (MD column) and increased radial diffusivity values (RD column) in BD patients in manic phase when compared with HC ($p<0.01$, TFCE corrected). For display purposes the statistically significant clusters are displayed as 1-p values. The white matter skeleton, thresholded at $FA>0.2$, is represented in *blue*. Group differences are mapped onto standard T1 Montreal Neurological Institute (MNI) template. Images are in radiological convention.
Abbreviations: DTI, diffusion tensor imaging; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; M, manic patients; HC, healthy controls.

Table 2. FA, MD and RD differences between the various subgroups

	FA			MD			RD		
	ANOVA	**M vs. HC**	**D vs. HC**	**ANOVA**	**M vs. HC**	**D vs. HC**	**ANOVA**	**M vs. HC**	**D vs. HC**
WM Tracts	F (p)	p	p	F (p)	p	p	F (p)	p	p
ATR L	8.869 (0.000)	0.006	0.001	5.647 (0.001)		0.007	5.984 (0.001)		0.008
ATR R	8.309 (0.000)		0.001	4.913 (0.003)		0.004	5.447 (0.002)		0.005
CG L	5.105 (0.003)	0.008		6.810 (0.000)		0.005	7.603 (0.000)	0.005	0.003
CG R	6.229 (0.001)	0.005					6.018 (0.001)		0.005
CST L	5.735 (0.001)	0.000		7.658 (0.000)		0.004	6.352 (0.001)	0.001	
CST R	5.064 (0.003)	0.001		6.144 (0.001)		0.005	5.359 (0.002)	0.003	
Fmaj	5.739 (0.001)		0.008	6.810 (0.000)		0.004	6.714 (0.000)		0.007
Fmin	4.742 (0.004)	0.006		6.400 (0.001)		0.004	5.976 (0.001)		
IFOF L	8.801 (0.000)	0.002	0.003	9.710 (0.000)	0.007	0.001	9.422 (0.000)	0.004	0.002
IFOF R	8.562 (0.000)	0.000	0.004	7.055 (0.000)		0.003	7.880 (0.000)	0.008	0.004
ILF L	8.136 (0.000)	0.002	0.003	1.178 (0.000)	0.002	0.001	9.608 (0.000)	0.002	0.002
ILF R	8.253 (0.000)	0.009	0.002	5.781 (0.001)		0.006	7.891 (0.000)		0.002
SLF L	7.530 (0.000)	0.000		7.674 (0.000)		0.004	8.691 (0.000)	0.000	
SLF R	7.215 (0.000)	0.000		6.650 (0.000)		0.003	7.414 (0.000)	0.001	
UF L	8.250 (0.000)	0.004	0.001	10.676 (0.000)		0.000	9.941 (0.000)	0.002	0.001
UF R	7.943 (0.000)	0.004	0.002	8.070 (0.000)		0.005	7.734 (0.000)	0.007	0.003

ANOVA and Games-Howell post hoc test of FA, MD and RD values of each tract to detect differences between the various subgroups - i.e., depressed, manic and euthymic patients, and HC. All results were thresholded at a corrected p value of 0.01.

Abbreviations: FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; WM, white matter; M, manic patients; D, depressed patients; HC, healthy controls; ATR L, anterior thalamic radiation left; ATR R, anterior thalamic radiation right; CG L, cingulate gyrus left; CST L, corticospinal tract left; CST R, corticospinal tract right; Fmaj, forceps major; Fmin, forceps minor; IFOF L, inferior fronto-occipital fasciculus left; IFOF R, inferior fronto-occipital fasciculus right; ILF L, inferior longitudinal fasciculus left; ILF R, inferior longitudinal fasciculus right; SLF L, superior longitudinal fasciculus left; SLF R, superior longitudinal fasciculus right; UF L, uncinate fasciculus left; UF R, uncinate fasciculus right.

Tractography study - Abnormal structural connectivity of the cingulum in mania

Firstly, the 4 (DTI parameters) x 4 (subgroups) ANOVA and post-hoc analysis of the cingulum SC showed a significant main effect between the various subgroups (F=2.790; p=0.045) with manic patients showing decreased values when compared to HC (p=0.036) (interaction between DTI parameters and subgroups: F=1.511; p=0.145). Then, the ANOVAs for each DTI parameter of the whole cingulum showed a significant difference between subgroups in mean FA (F=2.829; p=0.042), MD (F=2.958; p=0.036) and RD (F=3.581; p=0.017), but not in AD (F=0.584; p=0.651). Among subgroups, the post-hoc analysis showed decreased FA (p=0.034) as well as increased MD (p=0.035) and RD (p=0.012) in manic patients when compared to HC (**Figure 3** and **Figure 4**). Furthermore, we performed explorative analyses on the SC of the left and right cingulum, separately. A significant difference between subgroups was found in the right cingulum bundle in FA (F=3.205; p=0.027), MD (F=2.890; p=0.039) and RD values (F=3.557; p=0.017). Specifically, among subgroups, manic patients showed increased MD (p=0.044) and RD values (p=0.022) as well as marginally decreased FA values (p=0.055), when compared to HC; and decreased FA values (p=0.032) when compared to euthymic patients. By contrast, no significant differences were found in the SC of the left cingulum.

Secondly, the 3 (altered DTI parameters: FA, MD, RD) x 4 (subgroups) ANOVA and post-hoc analysis of the anterior part of the cingulum SC showed a significant main effect between the various subgroups (F=2.780; p=0.045) with manic patients showing decreased values when compared to HC (p=0.012) (interaction between DTI parameters and subgroups: F=2.981; p=0.008). Then, the ANOVAs for each DTI parameter of the anterior part of the cingulum showed a significant difference between subgroups in mean FA (F=2.783; p=0.045), MD (F=3.838; p=0.012) and RD (F=3.918; p=0.011). Among subgroups, the post-hoc analysis showed decreased FA (p=0.011) as well as increased MD (p=0.039) and RD (p=0.011) in manic patients when compared to HC (**Figure 5**). In contrast, no significant differences were found in the DTI parameters in the middle part (main effect: F=2.156 p=0.098; interaction between DTI parameters and subgroups: F=1.306 p=0.256) and in the posterior part of the cingulum (main effect: F=1.010 p=0.392; interaction between DTI parameters and subgroups: F=1.009 p=0.420).

Finally, with regard to clinical correlations, neither HAM-D or YMRS total scores significantly correlated with the DTI parameters of the cingulum.

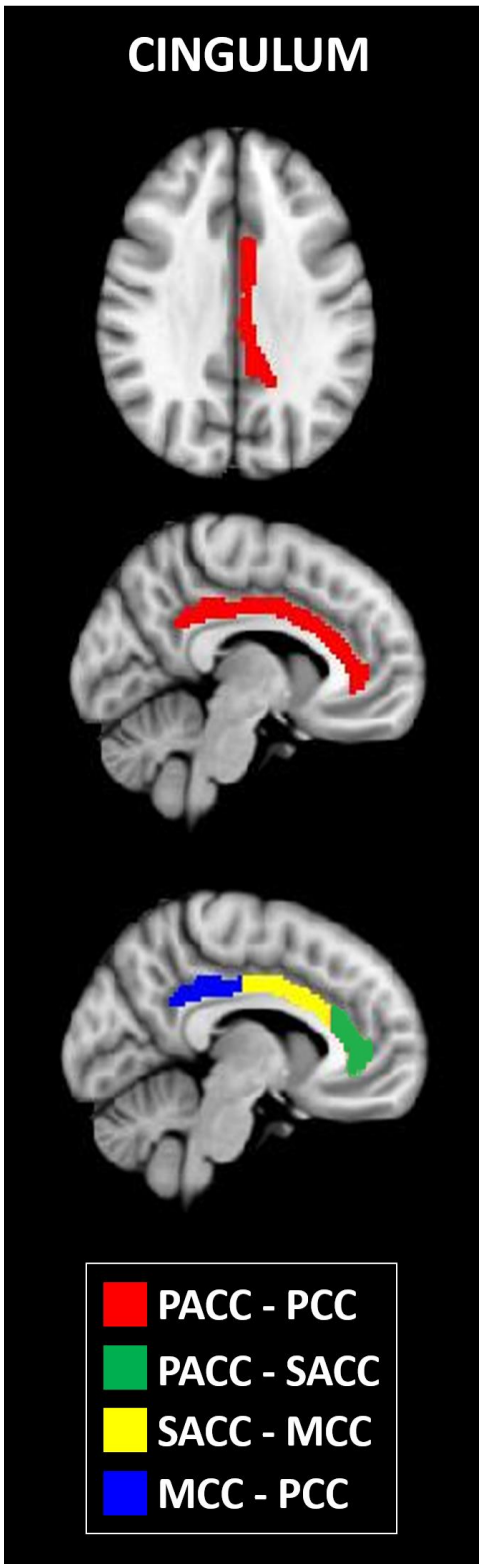


Figure 3. Probabilistic tractography reconstruction of the cingulum
Probabilistic tractography reconstruction from one individual (left side shown). The cingulum bundle is shown in the upper part of the figure. The various portions of the cingulum (in green: the anterior part from PACC to SACC; in yellow: the middle part from SACC to MCC; in blue: the posterior part from MCC to PCC) are shown in the lower part of the figure.
Abbreviations: PACC, perigenual anterior cingulate cortex; SACC, supragenual anterior cingulate cortex; MCC, middle cingulate cortex; PCC, posterior cingulate cortex.

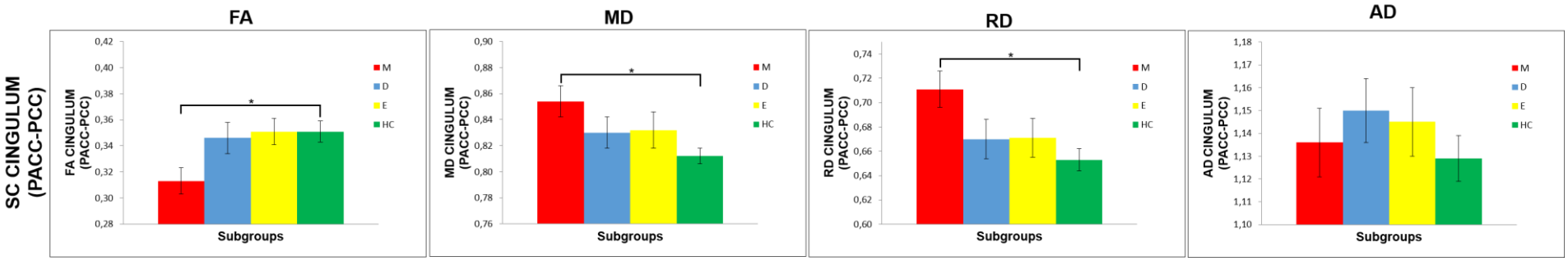


Figure 4. Differences in structural connectivity of the cingulum in mania, depression, euthymia and controls

ANOVA with Games-Howell post hoc test of the mean FA, mean MD, mean RD and mean AD values of the cingulum (from PACC to PCC) between the various subgroups. MD, RD and AD values are multiplied per 10^3.

Corrected $p<0.05$ *

Abbreviations: SC, structural connectivity; PACC; perigenual anterior cingulate cortex; PCC, posterior cingulate cortex; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; AD, axial diffusivity; M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

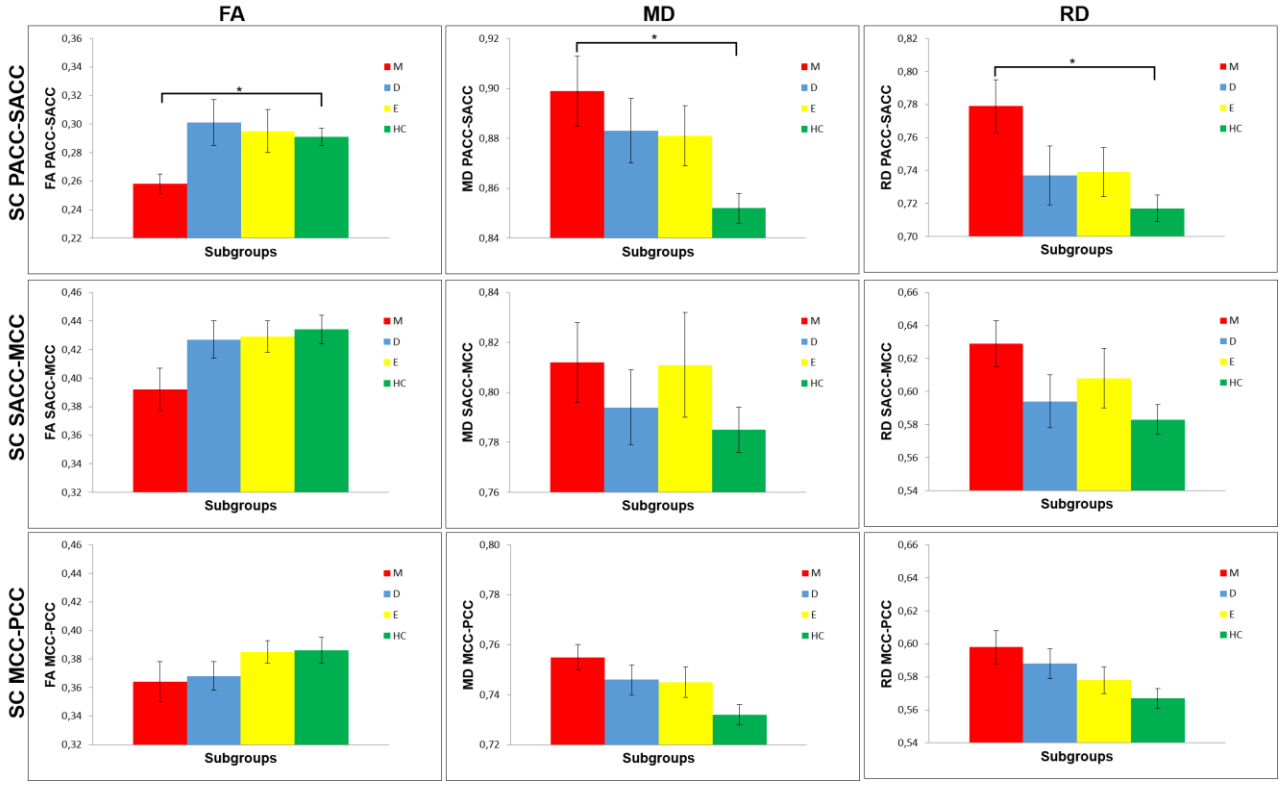


Figure 5. Differences in structural connectivity of the various portions of the cingulum in mania, depression, euthymia and controls
ANOVA with Games-Howell post hoc test of the mean FA values, mean MD values and mean RD values of the various portions of the cingulum (the anterior part from PACC to SACC, the middle part from SACC to MCC, the posterior part from MCC to PCC) between the various subgroups. MD and RD values are multiplied per 10^3.
Corrected $p<0.05$ *
Abbreviations: SC, structural connectivity; PACC, perigenual anterior cingulate cortex; SACC, supragenual anterior cingulate cortex; MCC, middle cingulate cortex; PCC, posterior cingulate cortex; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

Limitations

The main limitation of the two studies is medication confound, since almost all the patients in our sample were undergoing pharmacotherapy. Thus, a psychotropic medication load was generated for each patient, reflecting the number and dosage of the various medications, as a measure of drug therapy [69]. This was done by converting antipsychotics into chlorpromazine dose equivalents [70], mood stabilizers into lithium dose equivalents [70], antidepressants into imipramine dose equivalents [70], and benzodiazepines into diazepam dose equivalents [71]. The codes 0, 1, 2 or 3 were then used, respectively, for: no medication, and dose equivalents below, equal or above the mean effective daily dose [72]. A composite measure of medication load was generated by summing all the individual medication codes for each category for each individual BD patient [49]. Then, we examined the potential impact of psychotropic medication load on our findings, by correlating the resulting pharmacological loads with DTI parameters. In both our studies, total medication load, as well as medication loads for the different classes of drugs, did not show any significant correlation with DTI parameters (Bonferroni-corrected $p>0.05$), with the only exception of a significant correlation between medication load and RD value in Fmaj ($\rho=0.381$; $p=0.003$) in the TBSS work. Nevertheless, we cannot exclude some influence of treatment on our findings, since patients in different phases of illness are exposed to different medications classes (e.g., antipsychotics are more common among manic patients while antidepressant are more used in the depressive phase), or subgroup differences could be affected in a non-linear way and relationships between these factors may present a degree of complexity that is not captured by linear correlations. However, taken together, these results suggest that DTI changes, even if potentially associated with pharmacotherapy, are not the mere consequence of drug treatment in our sample [69].

Age and illness duration may represent additional confounding factors. Our sample consisted of patients at different age and stages of the disease. However, the mean age did not show any significant difference between the various subgroups, as well as the mean illness duration. Furthermore, no significant correlations of DTI parameters with age and illness duration were found in both studies (Bonferroni-

corrected p>0.05). Thus, these results suggest the absence of major effects of these clinical factors on WM alterations in our sample.

Finally, our studies suffer of the typical limits of cross-sectional studies. An ideal study on differences between the various phases of illness would be conducted longitudinally in the same individual going through manic, depressive and euthymic phases. However, such longitudinal studies are extremely difficult to implement, and could be affected by some similar confounders (e.g., ethically it is not possible to keep participants with the same treatment in the different phases). Therefore, the present findings should be regarded as preliminary, but informative and hypothesis generating for longitudinal studies.

Discussion

The main findings of our work are the following. In our TBSS study, we found a widespread alteration in WM microstructure (decrease in FA and increase in MD and RD parameters) in BD, showing distinct patterns of changes in the different phases of illness. In particular, such WM abnormalities were larger in the active phases of illness (i.e., depression and mania) with respect to euthymia. With regard to our tractography study, we detected a reduction of the cingulum SC (especially its anterior part) in mania.

Our TBSS study confirmed a widespread WM alteration in BD, as reported in previous studies [13, 14] and, furthermore, showed for the first time a different pattern of WM changes in the various phases of illness. Importantly, the subgroup comparisons showed that the diffuse WM abnormalities were prevalent in the active phases of illness (depression and mania) with respect to euthymia (**Figure 6**). Moreover, in our sample, depressed, manic and euthymic patients showed a relatively different spatial pattern of WM abnormalities, with a widespread distribution of alterations in the depressive phase; a more constant involvement of midline structures, such as Fmin and CG, as well as some lateral tracts, such as SLF and CST, in the manic phase; and more localized alterations in the midline tract Fmin in the euthymic phase.

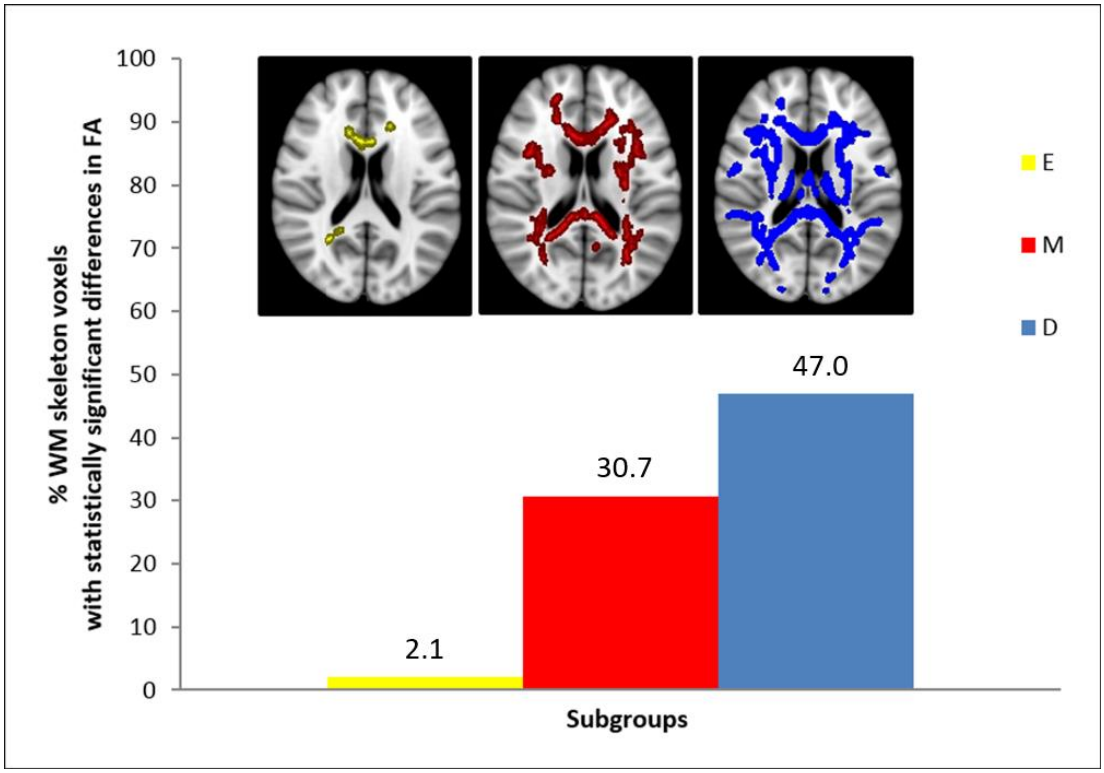


Figure 6. WM alterations in the euthymic, manic and depressed phases of bipolar disorder

Summary figure that shows the percentage and distribution of WM alterations in the different phases of BD. The histograms represent the percentage of the total number of WM skeleton voxels in all the tracts that show statistically significant reduction in FA values, while the brains represent the distribution of the same voxels in euthymia (yellow), mania (red) and depression (blue), with respect to controls (at a corrected $p<0.05$). A gradient of increasing WM abnormalities from the euthymic (low degree and localized WM alterations mainly in the midline structures) to the manic (more diffuse WM alterations affecting both midline and lateral structures) and, finally, to the depressive phase (high degree and widespread WM alterations), is shown in the figure.

Abbreviations: BD, bipolar disorder; WM, white matter; FA, fractional anisotropy; E; euthymic phase; M; manic phase; D, depressive phase.

Interestingly, in our tractography study, we confirmed and further extended both our TBSS findings in mania and previous data on cingulum alteration in BD (without regard of the phase of illness) [13, 14, 18-21], by showing reduced SC in the cingulum (especially in its anterior part) in mania specifically, as distinguished from depressed and euthymic patients, when compared to HC.

Together, our findings suggest that acute phases of illness may be associated with acute state-dependent microstructural WM changes. This raises the intriguing question of which hypothetical pathophysiologic mechanisms mediate such dynamic alterations of WM during the bipolar phases.

At the cellular level, the FA reduction is indicative of a generic loss of WM integrity or directionality; however, a concomitant increase in RD suggests a more specific alteration in oligodendroglial and myelin microstructure [16]. These findings are potentially consistent with the results from postmortem studies in BD patients that showed reductions in glia cell density and abnormalities of gene expression related to the perineuronal and myelinating oligodendroglia [73].

However, it is still unclear if these alterations are chronic and stable or acute and partially transitory, as suggested by the observation of FA reduction along with disruption of integrity of the nerve-sheath in healthy subjects who experienced acute stress, and by a preclinical study on acutely stressed mouse brain [74, 75]. Since the prolonged activation of the stress system, which could trigger the active phases of BD [76-78], induces an increase in pro-inflammatory factors [79], and since increased levels of these factors have been associated with a loss of WM integrity [80], we speculated that the diffuse WM microstructural abnormalities might reflect a stress-related inflammatory damage.

In conclusion, for the first time, our results showed widespread WM alterations, occurring significantly in the active phases of BD rather than in euthymia, associated with decreased SC in midline regions in mania specifically. Accordingly, a state-dependent and dynamic WM change seems to occur during the active phases of BD. However, the pathogenic mechanisms underlying this pattern of WM microstructural abnormalities are unclear.

CHAPTER III

Immunological alterations and their correlation with white matter abnormalities in the various phases of bipolar disorder

Introduction and background

As described in Chapter II, WM abnormalities have been consistently detected in BD, occurring significantly in the active phases rather than in euthymia, and in mania especially [50, 51].

Independently, recent evidences suggest that BD is associated with distinct immunological abnormalities, concerning both cytokines and immune cells, in the peripheral circulation and central nervous system [81-83]. Data on cytokines alterations in BD, although heterogeneous, mainly show increased level/activity of pro-inflammatory cytokines, e.g., interleukin (IL)-6, especially in the active phases [81, 84]. Moreover, these findings are further supported by evidences of increased levels in other inflammation markers, such as C-reactive protein (CRP), in BD and in mania especially [85, 86]. Recently, circulating frequencies of T cells and their subpopulations, which play a key role in cell-mediated immune responses, have been found to be substantially altered in BD patients [87-100]. These studies were conducted on either euthymic patients [87, 88, 91, 92, 95] or BD patients overall (regardless of the phase of illness) [89, 93, 94, 97, 98], while studies addressing specifically depression [99, 100] or mania [90] are rather sparse. Thus, studies that comprehensively investigate and compare the immunological abnormalities in each different phase of BD are still lacking.

It is conceivable that WM microstructural and immunological alterations, both detected in BD, could be pathogenically related. Indeed, a previous study showed that increased levels of pro-inflammatory factors are associated with loss of WM integrity in healthy subjects [80]. More recently, two studies demonstrated a correlation between immune alterations (concerning cytokines such as tumor necrosis factor (TNF)α, interferon (IFN)γ, IL-8, IL-10, or T cells, i.e., Th17) and WM changes in the depressive phase of BD [99, 101]. These findings corroborate the

hypothesis of a possible relationship between WM and immunological alterations in BD prompting a systematic analysis on this potential relationship across all the different phases of BD, and in mania in particular.

Therefore, we have performed a study [102] aimed to: (I) characterize WM abnormalities in each single phase of BD (replicating our previous finding in an independent sample); (II) characterize the immunological alterations in each single phase of BD; (III) investigate correlations between these two potential biomarkers of the disease.

We hypothesized that: (I) WM alterations are mainly detected in the active phases of illness, and in mania especially, on the basis of our previous studies [50, 51]; (II) immunological abnormalities can be detected in the manic phase, coherently with the WM changes; (III) a correlation between WM and immunological alterations can be detected especially in the manic phase, accordingly to a potential immune-related damage of WM in BD.

Methods

Subjects and clinical assessment

In the present study, we have recruited an independent sample with respect to our previous work [50, 51]. Subjects were recruited from the Psychiatric Clinic of Genoa (San Martino Polyclinic Hospital and Department of Neuroscience at the University of Genoa, Italy). The Ethical Committee of San Martino Polyclinic Hospital approved the study, and written informed consent was obtained from all the participants. The study was conducted on 60 BD type I patients - 20 in manic, 20 in depressive and 20 in euthymic phases - and 20 HC (see **Table 1** for a detailed description of the sample).

Each participant was evaluated with the same clinical instruments as in our previous studies (i.e., MINI, SCID-I/P, SCID-II, SIMD-R, HAM-D, YMRS); physical and psychiatric examinations were also conducted.

As in our previous work, inclusion criteria included diagnosis of BD type I, during manic or major depressive episodes, or euthymia (score of 17-items HAM-D≥18 for

depressed patients; score of YMRS≥13 for manic patients; scores of HAM-D<8 and YMRS<8 for euthymic patients), age between 18 and 60, ability to provide written informed consent. Exclusion criteria were the same as in our previous studies (including other major psychiatric, neurological or somatic disorders, with a particular attention on immune-inflammatory or infectious illnesses, as well as inability to undergo an MRI examination). HC did not meet the DSM criteria for psychiatric disorders, had a HAM-D score <8 and a YMRS score <8, and met the same exclusion criteria indicated for patients.

For each participant, MRI data and blood sample were collected.

Table 1. Subject demographic and clinical information

	BD				HC
	BD TOT	MANIC BD	DEPRESSED BD	EUTHYMIC BD	
Sample Size *n* (%)	60 (100%)	20 (33.3%)	20 (33.3%)	20 (33.3%)	20 (100%)
Age *mean* (SD)	47.0 (8.9)	47.2 (7.9)	45.7 (8.8)	48.2 (10.1)	41.3 (14.1)
Female *n* (%)	36 (60%)	13 (65%)	11 (55%)	12 (60%)	9 (45.0%)
Tobacco Smokers *n* (%)	39 (65%)	14 (70%)	14 (70%)	11 (55%)	8 (40%)
BMI Kg/m^2 *mean* (SD)	24.9 (4.8)	24.7 (4.7)	24.8 (5.8)	25.3 (3.9)	22.9 (2.3)
Duration of Illness *mean* (SD)	16.3 (12.7)	16.0 (12.0)	14.2 (12.0)	18.8 (14.1)	-
Number of Episodes *n* (%)	5.6 (3.7)	5.5 (4.2)	5.6 (3.8)	5.6 (3.1)	-
Inpatients *n* (%)	36 (60%)	14 (70%)	20 (100%)	2 (10%)	-
HAM-D *mean* (SD)	-	7.0 (6.0)	21.1 (4.1)	1.5 (2.6)	-
YMRS *mean* (SD)	-	19.4 (5.4)	3.4 (2.7)	2.0 (2.6)	-
Psychotic Features *n* (%)	6 (10%)	4 (20%)	2 (10%)	0 (0%)	-
Mood Stabilizers *n* (%)	47 (78.3%)	17 (85%)	15 (75%)	15 (75%)	-
Antidepressants *n* (%)	30 (50%)	4 (20%)	14 (70%)	12 (60%)	-
Antipsychotics *n* (%)	41 (68.3%)	15 (75%)	16 (80%)	10 (50%)	-
Benzodiazepines *n* (%)	41 (68.3%)	15 (75%)	18 (90%)	8 (40%)	-
Unmedicated *n* (%)	1 (1.7%)	0 (0%)	0 (0%)	1 (5%)	-

Abbreviations: BD, bipolar disorder; HC, healthy controls; BMI, body mass index; HAM-D, Hamilton Depression Scale; YMRS, Young Mania Rating Scale.

DTI analyses

The MRI acquisition protocol, as well as DTI preprocessing and TBSS analysis that were here applied, were the same used in our previous studies, deeply described in Chapter II and briefly summarized here below.

MRI data acquisition, DTI processing and TBSS analysis

Images were acquired using a 1.5-T GE scanner. DTI was acquired with a pure axial single-shot EPI sequence (diffusion sensitizing gradients along 60 non-collinear directions, b=1000 s/mm^2, 5 b=0 acquisitions, TR/TE=13750/93 ms, image matrix=128x128, FOV=24 cm, NEX=1, 55 contiguous axial 2.5 mm-thick slices). In addition, the two anatomical sequences were also acquired for all participants (i.e., 3D T1-weighted SPGR and FLAIR), which were reviewed by a board-certified neuroradiologist, and none of them showed structural visible lesions.

Diffusion data were preprocessed and analyzed using FSL. A standard preprocessing was performed, including brain extraction, head motion and eddy-current corrections. Moreover, head motion of all participants was less than 2 mm, and showed no significant differences among groups (χ^2=3.540; p=0.316). Subsequently, DT was estimated in each voxel using a linear regression and FA, RD and AD maps were derived.

TBSS analysis was used to perform a voxel-wise analysis of the whole-brain WM DT MRI measures. Briefly, FA images were non-linearly registered to standard space, averaged to create a mean FA image which was "skeletonized" with a threshold of FA=0.2, and then projected onto this resulting skeleton. Similar processes were applied to non-FA data – i.e., RD and AD maps. Then, TBSS data were entered in between-groups comparisons in order to investigate differences among manic, depressed and euthymic patients, and HC.

Comparison between groups of DTI parameters

Firstly, voxel-wise differences in FA values between groups were tested using a permutation-based inference for non-parametric statistical thresholding ("randomize" program) and ANOVA. The number of permutations was set to 5,000. Age and gender were entered into this analysis as confound regressors to ensure that any observed effect of group on FA was independent of age- and gender-related changes. A p value <0.05, corrected for FWE using the TFCE option in the randomize tool,

was set for between-groups comparisons. After detecting significant differences in the ANOVA, the randomize function and two-sample t-tests, with age and gender as coregressors, were performed in order to reveal the voxel-wise differences in FA values between the various groups (i.e., mania, depression, euthymia, and HC). A p value <0.05, corrected for FWE using the TFCE and for multiple comparisons using Bonferroni (Bonf) correction (6 comparisons, $p_{TFCE\text{-}Bonf}<0.008$), was set for between-groups comparisons. The significant results in the t-tests were then masked with the significant clusters resulting from the correspondent ANOVA, to detect the t-tests differences only in those areas that were found to be significantly different in the ANOVA. Subsequently, differences in RD and AD values between groups were investigated separately using the same methodology, in order to better characterize the potential FA alterations – i.e., randomize function and ANOVAs with a TFCE and Bonferroni corrected $p<0.05$ (2 ANOVAs, $p_{TFCE\text{-}Bonf}<0.025$); t-tests with TFCE and Bonferroni corrected $p<0.05$ (6 comparisons, $p_{TFCE\text{-}Bonf}<0.008$); masking of t-test differences with the significant clusters resulting from the correspondent ANOVA.
Secondly, the clusters showing combined alterations across different DTI parameters in the previous voxel-wise ANOVAs were identified according to the JHU-ICBM-DTI-81 White-Matter Labels atlas included in FSL. The values of DT-MRI measures were averaged and extracted from these significant clusters of each altered tract (with a threshold of voxels number >200) and entered into ANOVAs, with age and gender as covariates (a Bonferroni-corrected $p<0.05$ was set for ANOVAs), followed by Bonferroni-corrected post-hoc t-tests, and then into correlation analyses (see below).

Immunological analyses

Immunofluorescence analysis by flow cytometry of T cell subpopulations

Firstly, analysis of cell expression of membrane antigens was performed by immunofluorescence and flow cytometry, in order to achieve data relative to frequencies of circulating T cell subpopulations: total CD4+, CD4+CD28+CD45RA+ (CD4+ naïve), CD4+CD28+CD45RA- (CD4+ central memory), CD4+CD28-CD45RA- (CD4+ effector memory), CD4+CD28-CD45RA+ (CD4+ terminal effector memory); total CD8+, CD8+CD28+CD45RA+ (CD8+ naïve), CD8+CD28+CD45RA- (CD8+ central memory), CD8+CD28-CD45RA- (CD8+ effector memory) and CD8+CD28-CD45RA+ (CD8+ terminal effector memory) [103, 104]. In particular, 100 μl of fresh blood sample was incubated with

specific fluorochrome-conjugated monoclonal Antibodies (mAbs) at 4 °C for 30 minutes in the dark and red cells were lysed with 2 ml of BD FACS Lysing solution (Beckton Dickinson (BD) Biosciences, CA, USA) and resuspended in 200 µl of the same solution. The following mAbs were used: allophycocyanin (APC)-cyanin 7 (Cy7) conjugated anti-CD4 (Clone RPA-T4), phycoerythrin (PE)-conjugated anti-CD28 (Clone CD28.2), fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA (Clone HI100), Brilliant Violet (BV) 421-conjugated anti-CD8 (Clone RPA-T8), and Horizon V500-conjugated anti-CD3 (Clone UCHT1) [(BD)]. The following gate strategy was used: identification of CD3+ T cells using CD3 versus SSC plot, control of lymphocyte scatter in FSC-A versus SSC-A plot, selection of singlets using FSC-A versus FSC-H, definition of CD4+ and CD8+ populations in CD4 versus CD8 plot. To identify antigen exposure history and differentiation status, CD45RA versus CD28 plot was used on CD4+ and CD8+ populations. Samples were analyzed on a FACS Canto II flow cytometer (BD) by the FACS DIVA version 6.0 software (BD).

Secondly, the cytokine profile of peripheral CD4+ and CD8+ T lymphocytes, in terms of IFNγ, IL-17A, IL-4, and IL-10 production, was analyzed by intracellular staining and flow cytometry analyses, as already described [105-107]. In particular, peripheral blood mononuclear cells (PBMCs) were purified from the venous heparinized blood samples by centrifugation on Ficoll-Hypaque gradient (Biochrom AG, Berlin, Germany) for 30 min at 2000 rpm. Then, $1x10^6$ PBMCs, re-suspended in culture X-VIVO10 medium (Lonza, Basel, CH), were stimulated with phorbol-12-myristate-13-acetate (PMA 50 ng/ml, Sigma, St. Louis, MO, USA) and Ionomycin (2 µg/ml, Sigma) for 5 hours at 37 °C. Brefeldin A (BFA 10 µg/ml, Sigma) was added to the cells for the last 3 hours of incubation. After activation, the samples were stained with fluorochrome-conjugated mAbs specific for surface markers BV421-conjugated anti-CD3 (BD) PerCP-Cy5.5 anti-CD8 (e-Biosciences, MA, USA) and viability dye Violet Aqua/Dead Fixable Dead Cell stain (Life Technologies - Thermo Fisher Scientific, MA, USA), before fixing and permeabilizing the lymphocytes with the Cytofix/Cytoperm kit (BD) following the manufacturer's instructions. The cells were washed in Perm-Wash buffer (BD) and incubated with anti-cytokine fluorochrome-conjugated mAbs: PE-Cy7 conjugated anti-IFNγ (Clone B27) [(BD)], FITC-conjugated anti-IL-4 (Clone 8D4-8) [(BD)], APC-conjugated anti-IL-17A (Clone N49-653) [(BD)] and PE-conjugated anti-IL-10 (Clone JES3-19F1) [(BD)]. Thereafter, the samples were washed in Perm-Wash buffer, fixed with FACS Lysing

solution (BD) and stored at 4 °C until the acquisition. The following gate strategy was used to measure CD3+CD8- (i.e., CD3+CD4+ T cells) and CD3+CD8+ cytokine-producing T cells: identification of live cells using viability dye versus SSC dot plot, gating of CD3+ T cells using CD3 versus SSC plot, control of lymphocyte scatter in FSC-A versus SSC-A plot, selection of singlets using FSC-A versus FSC-H, definition of CD3+CD8- and CD3+CD8+ populations in CD3 versus CD8 plot. The samples were analyzed using a FACS Canto II flow cytometer (BD) by the FACS DIVA version 6.0 software (BD).

All immunological analyses concerning T cell subpopulations are expressed as frequency referred to the total CD3+ population. FACS analyses were performed by two independent researchers expert in flow cytometry and the mean of the two measures were considered. Laboratory analyses were performed blind to clinical information.

Analysis of cytokine concentrations

Plasma levels of IL-6 cytokine were analyzed by ELISA using a commercially available Human IL-6 ELISA Kit High Sensitivity (ABCAM, Cambridge Science Park, UK) according to the manufacturer's instructions. CV% was 4.4%, LLOQ was 0.8 pg/ml, range of sensitivity of the kit was 1.56 pg/ml - 50 pg/ml.

Plasma concentrations of IFNγ, IL-10, IL-4, IL-17A, IL-1β, TNFα, and IL-6 cytokines were measured by a bead-based immunoassay (BDTM Cytometric Bead Array, BD) [108], according to the manufacturer's instructions [84] using the FACS Canto II cytometer (BD) equipped with the FACS DIVA version 6.0 software (BD). Plasma cytokine concentration was expressed in picograms per milliliter by FlowCytomix Pro Software (BD). LLOQs was 0.274 pg/ml for each cytokine and range of sensitivity was 0.274 pg/ml - 200 pg/ml.

Comparison between groups of immunological parameters

Firstly, the frequency of circulating CD4+ T cells was entered into an ANOVA between the various groups followed by Bonferroni-corrected post-hoc tests (corrected $p<0.05$). After detecting significant differences, the frequencies of CD4+ T cell subpopulations - i.e., CD4+CD28+CD45RA+, CD4+CD28+CD45RA-, CD4+CD28-CD45RA-, and CD4+CD28-CD45RA+ T cells - were entered into between-groups comparisons (4 comparisons, $p_{Bonf}<0.012$), followed by Bonferroni-

corrected post-hoc tests. Subsequently, the frequencies of circulating cytokine-producing CD4+ T cells – i.e., CD4+IFNγ+, CD4+IL4+, CD4+IL17+, and CD4+IL10+ T cells - were entered into between-groups comparisons (4 comparisons, $p_{Bonf}<0.012$), followed by Bonferroni-corrected post-hoc tests.

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Finally, the plasma concentration of IL-6 cytokine (as assessed by both ELISA and CBA techniques), as well as TNFα, IL-1β, IFNγ, IL-4, IL-17A and IL-10 cytokines (as assessed by CBA technique), was entered into between-groups comparisons followed by Bonferroni-corrected post-hoc tests. Correlations between cytokines level and T cells frequency were also explored.

All the ANOVAs and t-test were controlled for age and gender, which were used as covariates, and a p value <0.05 corrected for multiple comparisons using Bonferroni correction was set for all comparisons.

Relationship between DTI, immunological and clinical parameters

The DTI parameters that showed significant differences among groups were entered into whole brain voxel-wise regression analyses with data relative to the frequency of those T cell subsets that showed significant differences among groups (with age and gender as covariates), in the whole cohort of subjects. A TFCE and Bonferroni-corrected $p<0.05$ was set.

Then, the significant DTI and immunological alterations - i.e., the DTI parameters extracted from the significant clusters of each altered tract and the peripheral frequencies of those T cell subsets that showed significant differences between groups - were entered into a partial correlation analysis (with age and gender as covariates), to further confirm their relationship in the whole sample.

The DTI parameters extracted from the significant clusters of each altered tract in a specific group were entered into partial correlation analyses with data relative to those T cell subsets that showed significant alterations in the same group (with age and gender as covariates). The same correlation analyses were carried on also in the groups that did not show such DTI and immunological alterations, in order to control the specificity of significant DTI-immune correlations.
Potential clinical correlations of DTI and immunological alterations were explored. The significant DTI and immunological data were entered into Spearman correlation analysis with clinical parameters – i.e., YMRS total score and HAM-D total score – in the BD sample.

Data were tested for normal distribution using the Kolmogorov–Smirnov test. For those data that did not reach normal distribution, a cube-root transformation was adopted to make distribution less skewed. Parametric tests were performed for normally distributed data, while non-parametric tests were used for data that were still skewed after cube-root transformation.
Statistical analyses were performed in SPSS version19.

Results

DTI alterations in BD patients

The TBSS analysis was performed in order to detect significant changes in the DTI parameters in the different phases of BD (in an independent sample with respect to our previous work).
A widespread voxel-wise significant reduction in FA values in the manic phase and, to a lesser extent, in the depressive phase was found with respect to HC; no significant changes were detected in the euthymic phase. Furthermore, a significant RD increase was found in the manic phase only, with respect to HC, while no significant differences in the depressive and euthymic phases were detected; AD values did not show alterations in any group (**Figure 1a and Figure 1b**).
The clusters showing combined FA-RD alterations were mainly located in the body of corpus callosum (BCC), genu of corpus callosum (GCC), bilateral anterior corona radiata (ACR R and ACR L), left superior corona radiata (SCR L) and left posterior

corona radiata (PCR L). Interestingly, a significant decrease in FA and increase in RD values in the BCC and SCR L were exclusively found in the manic phase, while the other tracts showed DTI changes both in manic and depressive phases (**Figure 1c, Figure 1d, Table 2a** and **Table 2b**).

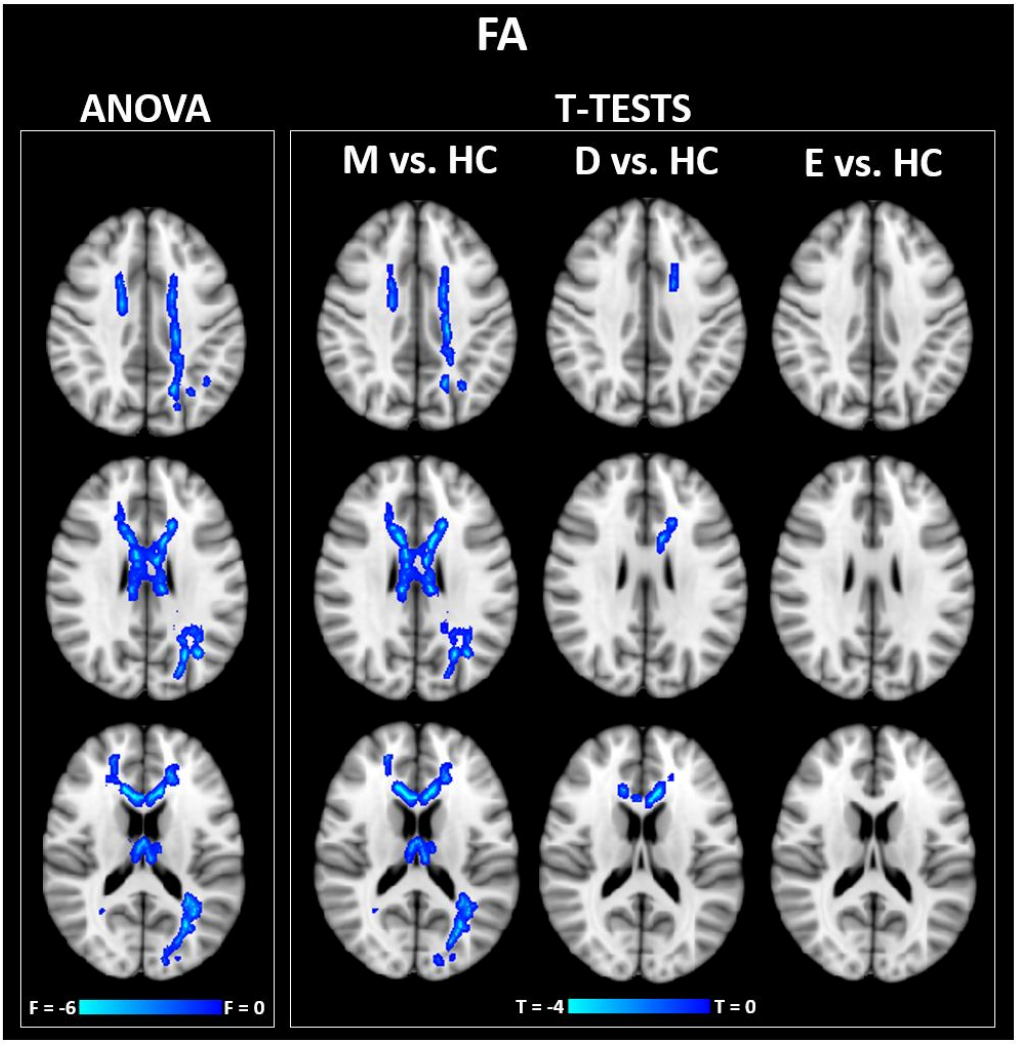


Figure 1a. WM alterations in the various phases of BD

Results from between-groups comparison (ANOVA and t-tests, with age and gender as covariates) showing clusters with significantly decreased FA values (*blue-light blue*), in BD patients in the different phases of illness (i.e., mania, depression and euthymia) compared with HC. The f-map of FA values (ANOVA) is thresholded at a TFCE corrected $p<0.05$. The t-maps of FA values (t-tests) are thresholded at a TFCE and Bonferroni corrected $p<0.05$, and masked with the significant clusters resulting from the correspondent ANOVA. Group differences are mapped onto standard T1 Montreal Neurological Institute (MNI) template at z=37, z=27 and z=17. The color bar represents voxel-wise f-values and t-values. The significant clusters have been modified using the fill function of FSL software for visual purpose.

Abbreviations: WM, white matter; TBSS, tract-based spatial statistics; DTI, diffusion tensor imaging; FA, fractional anisotropy; BD, bipolar disorder; M, mania; D, depression; E, euthymia; HC, healthy controls.

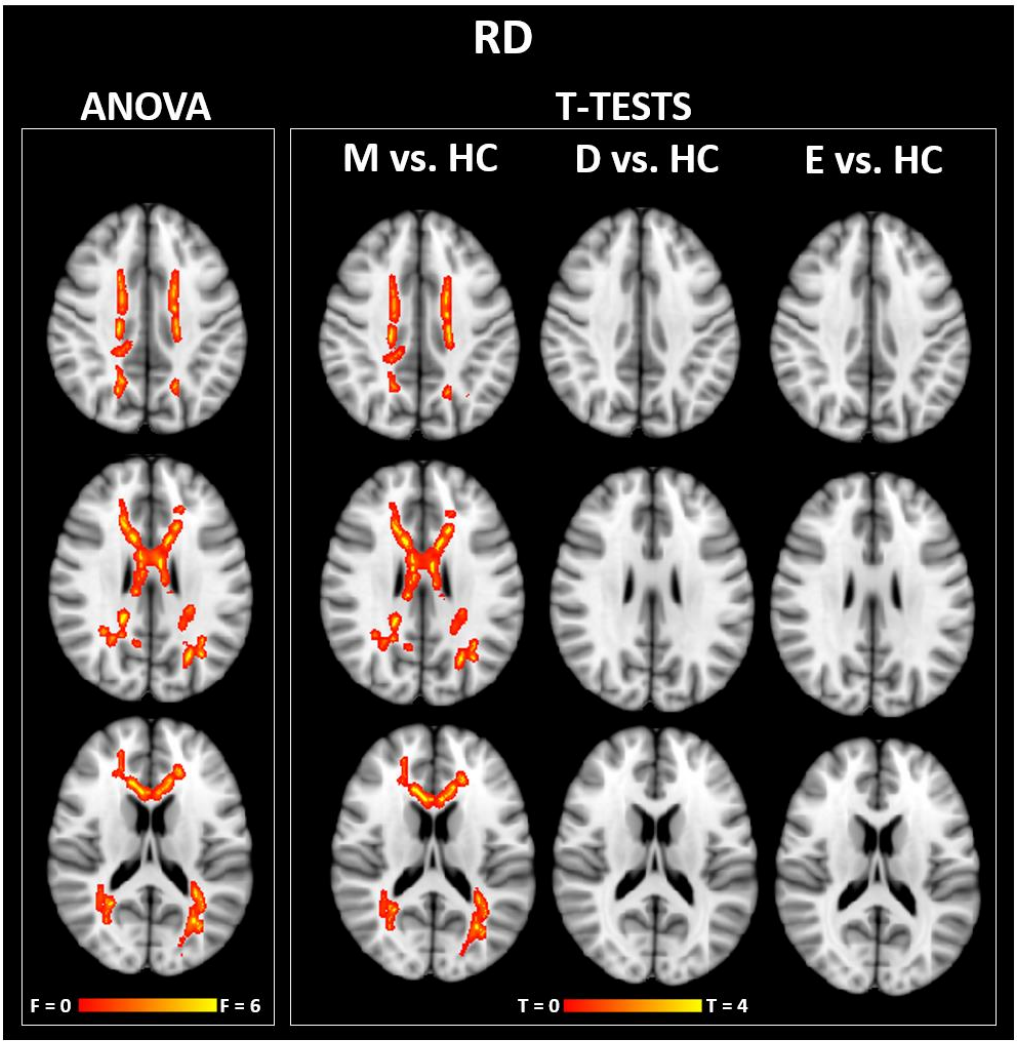


Figure 1b. WM alterations in the various phases of BD
Results from between-groups comparison (ANOVA and t-tests, with age and gender as covariates) showing clusters with significantly increased RD values (*red-yellow*), in BD patients in the different phases of illness (i.e., mania, depression and euthymia) compared with HC. The f-map of RD values (ANOVA) is thresholded at a TFCE and Bonferroni corrected $p<0.05$. The t-maps of RD values (t-tests) are thresholded at a TFCE and Bonferroni corrected $p<0.05$, and masked with the significant clusters resulting from the correspondent ANOVA. Group differences are mapped onto standard T1 Montreal Neurological Institute (MNI) template at z=37, z=27 and z=17. The color bar represents voxel-wise f-values and t-values. The significant clusters have been modified using the fill function of FSL software for visual purpose.
Abbreviations: WM, white matter; TBSS, tract-based spatial statistics; DTI, diffusion tensor imaging; RD, radial diffusivity; BD, bipolar disorder; M, mania; D, depression; E, euthymia; HC, healthy controls.

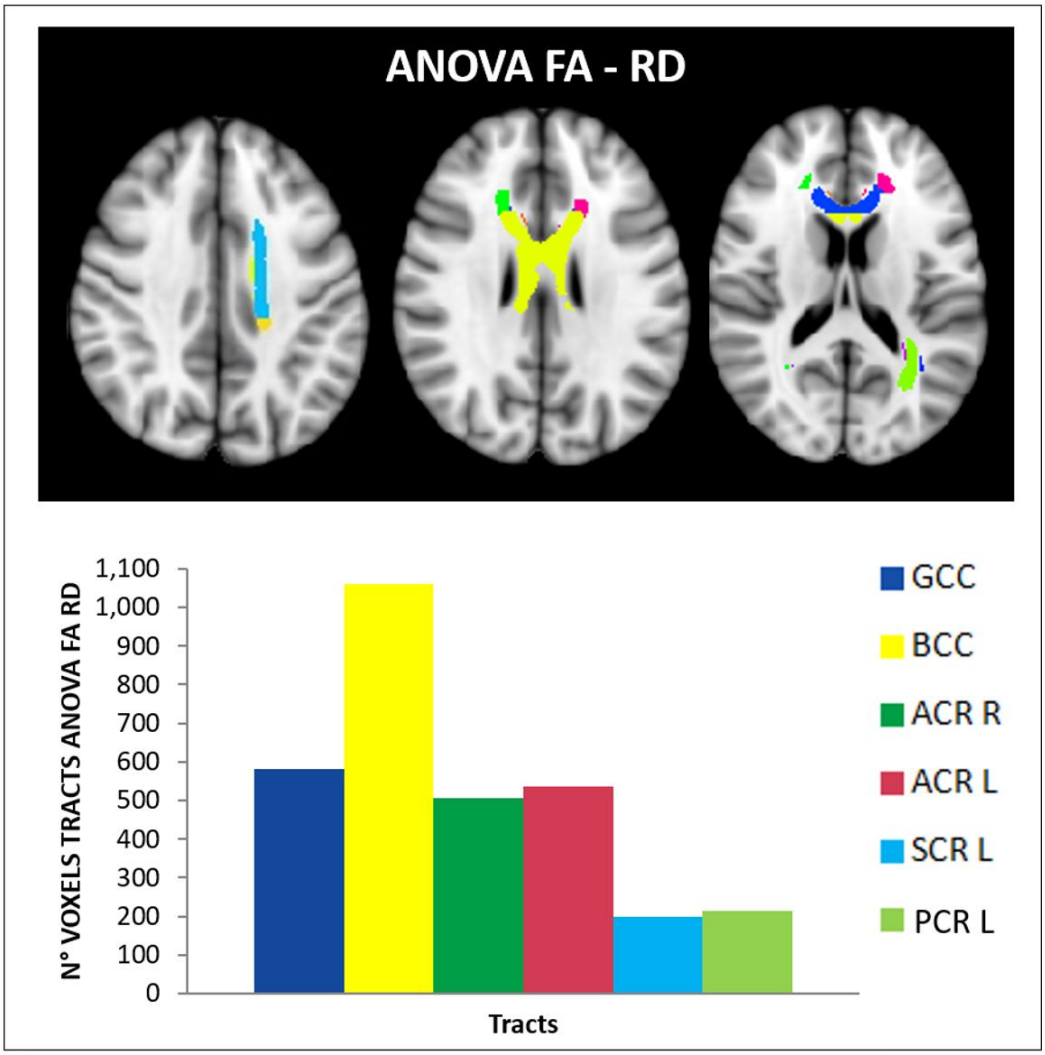


Figure 1c. WM alterations in the various phases of BD

Upper part: The main altered WM tracts showing overlapping FA-RD alterations (in the ANOVAs, see Figure 1a and 1b) in BD patients. The resulting WM alterations are differently colored according to the JHU-ICBM-DTI-81 White-Matter Labels atlas and mapped onto standard T1 Montreal Neurological Institute (MNI) template at z=37, z=27 and z=17.

Lower part: The cluster size of altered voxels is displayed for each altered tract (number of altered voxels > 200).

Abbreviations: BD, bipolar disorder; WM, white matter; FA, fractional anisotropy; RD, radial diffusivity; CGC, genu of corpus callosum; BCC, body of corpus callosum; ACR R, anterior corona radiata right; ACR L, anterior corona radiata left; SCR L, superior corona radiata left; PCR L, posterior corona radiata left.

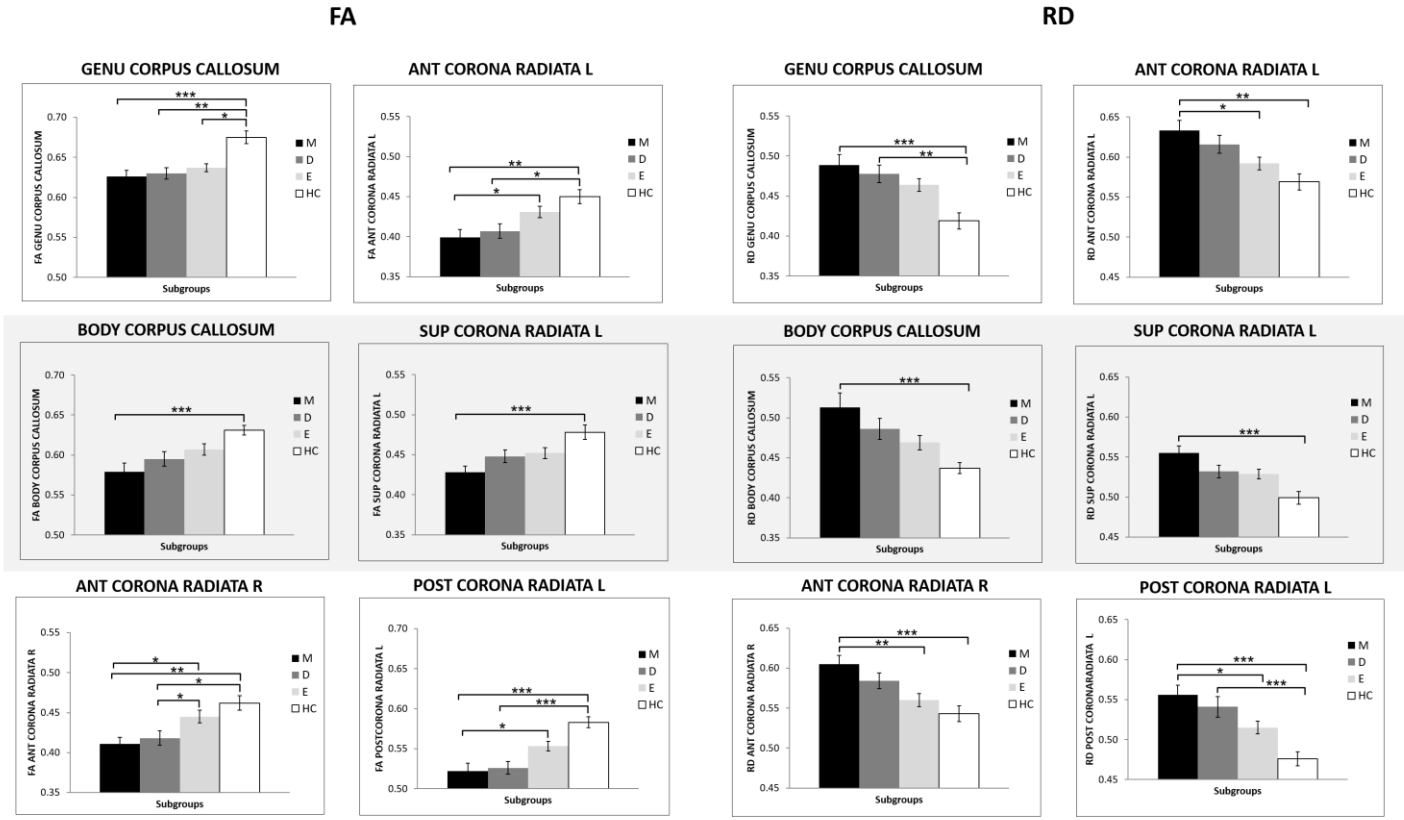


Figure 1d. WM alterations in the various phases of BD

Between-groups comparison (ANOVAs, with age and gender as covariates, followed by Bonferroni-corrected post-hoc tests) of FA and RD values extracted from each altered tract (see Figure 1c).

Corrected $p<0.05$*; $p<0.01$**; $p<0.001$***

Abbreviations: WM, white matter; FA, fractional anisotropy; RD, radial diffusivity; BD, bipolar disorder; M, mania; D, depression; E, euthymia; HC, healthy controls.

Table 2a. Comparisons between groups of the DTI parameters of the altered WM tracts

WM TRACTS	ANOVA	M vs. HC	D vs. HC	E vs. HC	M vs. D	M vs. E	D vs. E
	F (p)	*(p)*	*(p)*	*(p)*	*(p)*	*(p)*	*(p)*
FA GCC	**6.530 (0.001)**	**M<HC (0.001)**	**D<HC (0.002)**	**E<HC (0.036)**			
FA BCC	**5.792 (0.001)**	**M<HC (0.001)**					
FA ACR R	**6.826 (0.000)**	**M<HC (0.004)**	**D<HC (0.011)**			**M<E (0.015)**	**D<E (0.049)**
FA ACR L	**6.108 (0.001)**	**M<HC (0.005)**	**D<HC (0.021)**			**M<E (0.027)**	
FA SCR L	**5.111 (0.003)**	**M<HC (0.002)**					
FA PCR L	**11.219 (0.000)**	**M<HC (0.000)**	**D<HC (0.000)**			**M<E (0.022)**	
RD GCC	**6.167 (0.001)**	**M>HC (0.001)**	**D>HC (0.006)**				
RD BCC	**5.755 (0.001)**	**M>HC (0.001)**					
RD ACR R	**6.765 (0.000)**	**M>HC (0.001)**				**M>E (0.005)**	
RD ACR L	**5.787 (0.001)**	**M>HC (0.003)**				**M>E (0.025)**	
RD SCR L	**6.882 (0.000)**	**M>HC (0.000)**					
RD PCR L	**9.734 (0.000)**	**M>HC (0.000)**	**D>HC (0.001)**			**M>E (0.023)**	

ANOVAs and Bonferroni-corrected post hoc tests (with age and gender as covariates) showing differences in the DTI parameters of altered WM tracts between the various groups - i.e., manic, depressed, euthymic patients, and HC. The significant results are highlighted in bold.

Abbreviations: DTI, diffusion tensor imaging; FA, fractional anisotropy; RD, radial diffusivity; WM, white matter; GCC, genu corpus callosum; BCC, body corpus callosum; ACR R, anterior corona radiata right; ACR L, anterior corona radiata left; SCR L, superior corona radiata left; PCR L, posterior corona radiata left; M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

Table 2b. Mean values of the DTI parameters of the altered WM tracts

WM TRACTS	**M**	**D**	**E**	**HC**
	mean (SD)	*mean (SD)*	*mean (SD)*	*mean (SD)*
FA GCC	0.626 (0.039)	0.630 (0.035)	0.637 (0.026)	0.675 (0.036)
FA BCC	0.579 (0.051)	0.595 (0.043)	0.607 (0.031)	0.631 (0.030)
FA ACR R	0.411 (0.037)	0.418 (0.040)	0.445 (0.038)	0.462 (0.040)
FA ACR L	0.399 (0.045)	0.407 (0.042)	0.431 (0.033)	0.450 (0.041)
FA SCR L	0.428 (0.037)	0.444 (0.039)	0.452 (0.034)	0.478 (0.042)
FA PCR L	0.522 (0.046)	0.526 (0.040)	0.553 (0.027)	0.583 (0.033)
RD GCC	0.489 (0.060)	0.478 (0.053)	0.464 (0.037)	0.419 (0.046)
RD BCC	0.513 (0.081)	0.486 (0.061)	0.469 (0.042)	0.437 (0.035)
RD ACR R	0.605 (0.050)	0.584 (0.045)	0.560 (0.036)	0.543 (0.045)
RD ACR L	0.633 (0.058)	0.616 (0.051)	0.592 (0.036)	0.569 (0.048)
RD SCR L	0.555 (0.044)	0.532 (0.037)	0.529 (0.027)	0.499 (0.036)
RD PCR L	0.556 (0.055)	0.541 (0.058)	0.515 (0.036)	0.476 (0.041)

Mean and standard deviation of the extracted FA and RD values from the significantly altered WM tracts in BD groups and HC.
Abbreviations: DTI, diffusion tensor imaging; FA, fractional anisotropy; RD, radial diffusivity; WM, white matter; GCC, genu corpus callosum; BCC, body corpus callosum; ACR R, anterior corona radiata right; ACR L, anterior corona radiata left; SCR L, superior corona radiata left; PCR L, posterior corona radiata left; M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

Immunological alterations in BD patients

Phenotypic analyses were performed in order to measure the frequencies of different circulating T cell subpopulations in the various phases of BD.

The frequency of total CD4+ T cells showed a significant increase in the manic phase with respect to HC. Analysis of CD4+ T cell subpopulations revealed that such increase was dependent on the expansion of CD4+CD28+ T cell subsets (that include both naïve and central memory CD4+ T cells) (**Figure 2a**, **Table 3a** and **Table 3b**).

The frequency of total CD8+ T cells showed a significant decrease in the manic phase with respect to HC. Interestingly, this reduction was dependent on contraction of CD8+CD28- T cell subsets, including both effector memory (CD8+CD28-CD45RA-) T cells and terminal effector memory (CD8+CD28-CD45RA+) T lymphocytes, and was associated with significant reduction of CD8+IFNγ+ T cells (**Figure 2b**, **Table 3a** and **Table 3b**).

Finally, the levels of cytokine concentrations in the plasma of BD patients and HC showed a significant increase of IL-6 exclusively in the manic phase, while no significant changes in plasma levels of the other tested cytokines were found (**Table 4a** and **Table 4b**). Furthermore, IL-6 levels showed a significant inverse correlation with CD8+CD28- T cells frequency (**Table 5**).

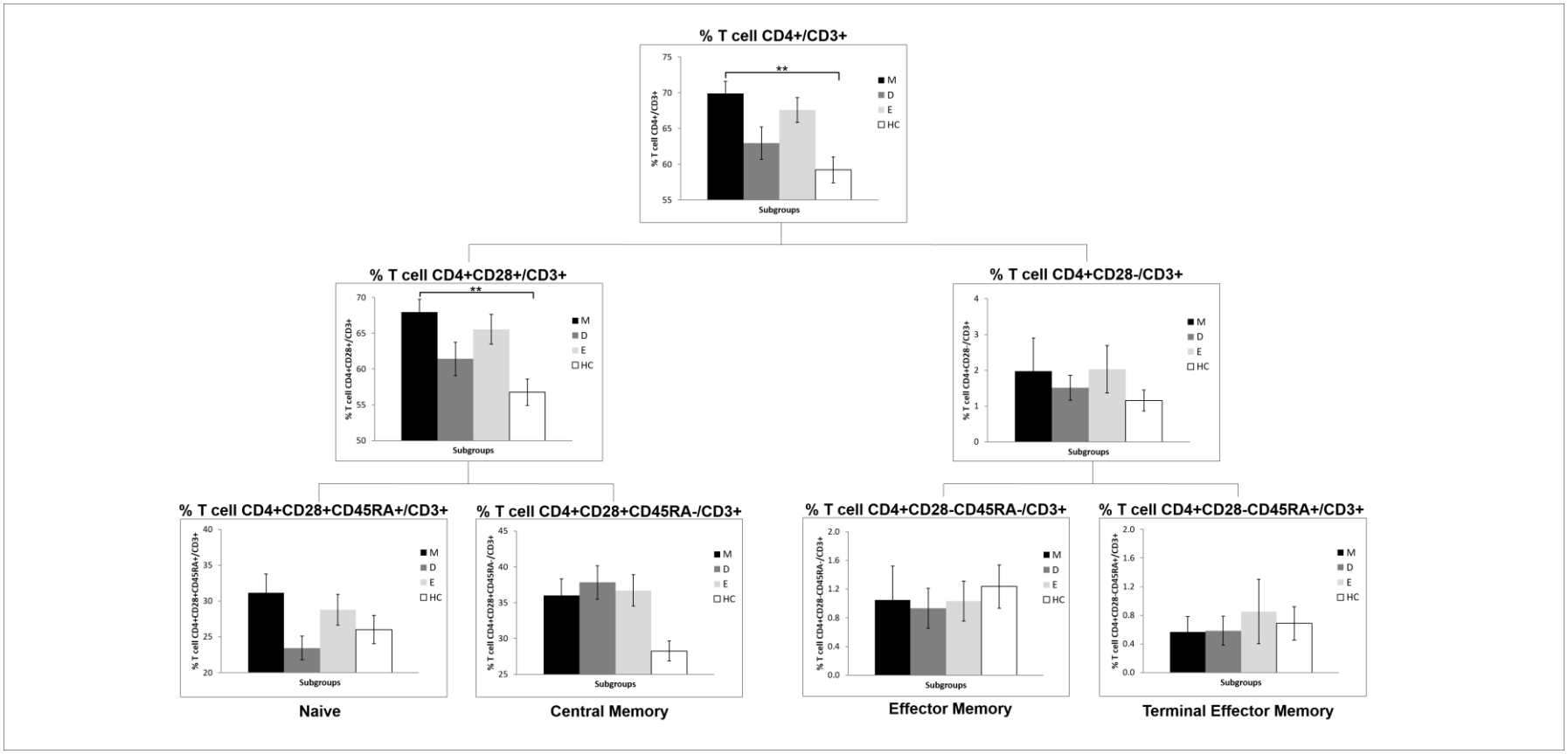


Figure 2a. Alterations of circulating T cell subsets in the various phases of BD

Comparative analyses (ANOVAs, with age and gender as covariates, followed by Bonferroni-corrected post-hoc tests) on different CD4+ T cell subsets (total CD4+ T cells as well as naïve CD4+CD28+CD45RA+, central memory CD4+CD28+CD45RA-, effector memory CD4+CD28-CD45RA- and terminal effector memory CD4+CD28-CD45RA+ T cell subsets) among BD patients in the various phases of illness and HC.

Corrected $p<0.05$*; $p<0.01$**; $p<0.001$***

Abbreviations: BD, bipolar disorder; M, mania; D, depression; E, euthymia; HC, healthy controls.

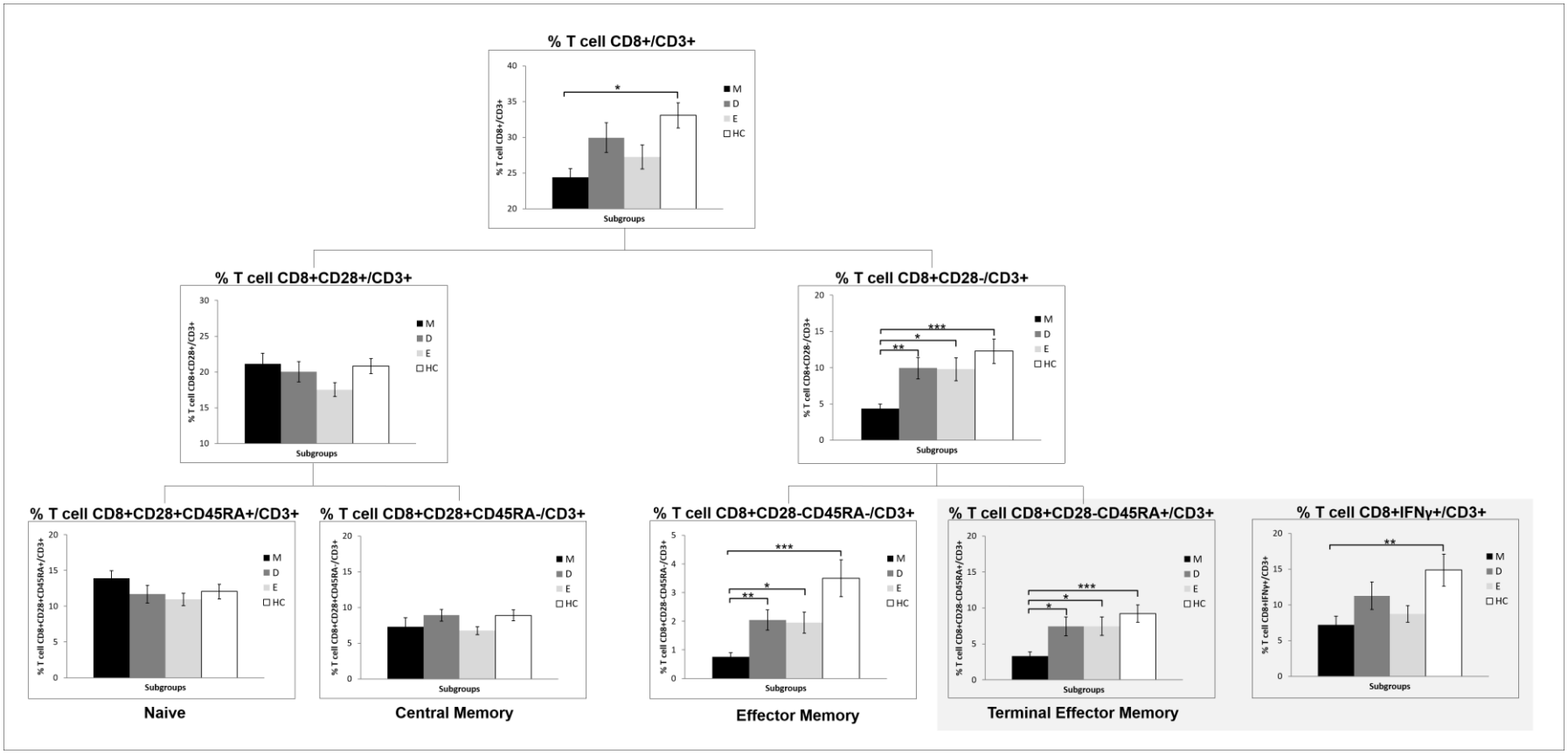


Figure 2b. Alterations of circulating T cell subsets in the various phases of BD

Comparative analyses (ANOVAs, with age and gender as covariates, followed by Bonferroni-corrected post-hoc tests) on different CD8+ T cell subsets (total CD8+ T cells, naïve CD8+CD28+CD45RA+, central memory CD8+CD28+CD45RA-, effector memory CD8+CD28-CD45RA- and terminal effector memory CD8+CD28-CD45RA+ T cell subsets, as well as CD8+IFNγ+ T cells) among BD patients in the various phases of illness and HC.

Corrected $p<0.05$*; $p<0.01$**; $p<0.001$***

Abbreviations: BD, bipolar disorder; M, mania; D, depression; E, euthymia; HC, healthy controls.

Table 3a. Comparisons between groups of T cells

T CELLS	**Kruskal-Wallis Test**	**ANOVA**	**M vs. HC**	**D vs. HC**	**E vs. HC**	**M vs.D**	**M vs. E**	**D vs. E**
	χ^2 (p)	*F (p)*	*(p)*	*(p)*	*(p)*	*(p)*	*(p)*	*(p)*
CD3+		1.587 (0.200)						
CD4+		**4.850 (0.004)**	**M>HC (0.005)**					
CD4+CD28+		**4.629 (0.005)**	**M>HC (0.005)**					
CD4+CD28+CD45RA+		2.115 (0.106)						
CD4+CD28+CD45RA-		3.597 (0.017)		D>HC (0.019)				
CD4+CD28-	2.989 (0.393)							
CD4+CD28-CD45RA-	3.840 (0.279)							
CD4+CD28-CD45RA+	0.908 (0.824)							
CD4+IFNγ+		1.080 (0.363)						
CD4+IL17+		0.305 (0.822)						
CD4+IL10+	1.553 (0.670)							
CD4+IL4+	3.152 (0.369)							

CD8+		**3.069 (0.033)**	**M<HC (0.026)**		
CD8+CD28+		1.601 (0.196)			
CD8+CD28+CD45RA+		3.370 (0.023)	M>HC (0.026)		
CD8+CD28+CD45RA-		2.165 (0.099)			
CD8+CD28-		**7.869 (0.000)**	**M<HC (0.000)**	**M<D (0.007)**	**M<E (0.014)**
CD8+CD28-CD45RA-		**9.858 (0.000)**	**M<HC (0.000)**	**M<D (0.008)**	**M<E (0.015)**
CD8+CD28-CD45RA+		**6.641 (0.000)**	**M<HC (0.000)**	**M<D (0.030)**	**M<E (0.027)**
CD8+IFNγ+		**4.702 (0.005)**	**M<HC (0.004)**		
CD8+IL17+	1.402 (0.705)				
CD8+IL10+	0.418 (0.937)				
CD8+IL4+	6.296 (0.098)				

ANOVAs and Bonferroni-corrected post hoc tests (with age and gender as covariates) for normally distributed data, or Kruskal-Wallis test for non-normally distributed data, showing differences in T cells frequencies between the various groups, i.e., manic, depressed, euthymic patients, and HC. The p values of the ANOVAs are Bonferroni-corrected. The significant results are highlighted in bold.
Abbreviations: M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

Table 3b. Mean values of T cells

T CELLS	**M** *mean (SD)*	**D** *mean (SD)*	**E** *mean (SD)*	**HC** *mean (SD)*
CD3+	23.495 (7.672)	23.355 (8.261)	29.310 (10.360)	23.740 (11.515)
CD4+	69.935 (7.419)	62.950 (10.082)	67.605 (7.756)	59.215 (8.124)
CD4+CD28+	67.953 (8.111)	61.433 (10.427)	65.570 (9.336)	56.750 (8.203)
CD4+CD28+CD45RA+	31.172 (11.744)	23.439 (7.462)	28.794 (9.604)	26.008 (8.812)
CD4+CD28+CD45RA-	35.996 (10.482)	37.841 (10.432)	36.704 (9.739)	28.254 (6.147)
CD4+CD28-	1.981 (4.098)	1.516 (1.558)	2.030 (2.964)	1.155 (1.331)
CD4+CD28-CD45RA-	1.046 (2.118)	0.933 (1.246)	1.034 (1.240)	1.235 (1.362)
CD4+CD28-CD45RA+	0.568 (0.967)	0.585 (0.910)	0.853 (2.017)	0.686 (1.047)
CD4+IFNγ+	7.769 (6.254)	8.707 (3.933)	9.464 (5.227)	9.085 (4.845)
CD4+IL17+	0.453 (0.367)	0.409 (0.210)	0.503 (0.432)	0.370 (0.173)
CD4+IL10+	0.418 (1.083)	0.187 (0.194)	0.183 (0.163)	0.227 (0.154)
CD4+IL4+	0.307 (0.666)	0.535 (1.152)	0.301 (0.556)	0.512 (0.911)

CD8+	24.436 (5.070)	29.950 (9.265)	27.260 (7.490)	33.070 (7.859)
CD8+CD28+	21.114 (6.699)	20.016 (6.324)	17.538 (4.268)	20.820 (4.776)
CD8+CD28+CD45RA+	13.920 (4.564)	11.679 (5.446)	10.955 (3.923)	12.047 (4.545)
CD8+CD28+CD45RA-	7.303 (5.592)	8.915 (3.591)	6.766 (2.511)	8.894 (3.402)
CD8+CD28-	4.345 (2.802)	9.931 (6.636)	9.791 (7.060)	12.266 (7.598)
CD8+CD28-CD45RA-	0.761 (0.608)	2.045 (1.575)	1.953 (1.647)	3.507 (2.882)
CD8+CD28-CD45RA+	3.294 (2.770)	7.426 (5.808)	7.457 (5.776)	9.224 (5.317)
CD8+IFNγ+	7.199 (5.418)	11.258 (8.583)	8.738 (5.084)	14.860 (9.947)
CD8+IL17+	0.030 (0.029)	0.042 (0.044)	0.036 (0.049)	0.047 (0.052)
CD8+IL10+	0.921 (3.766)	0.080 (0.077)	0.250 (0.477)	0.149 (0.199)
CD8+IL4+	0.291 (0.834)	0.617 (1.255)	0.129 (0.214)	0.244 (0.402)

Mean and standard deviation of the peripheral frequencies of total CD3+, CD4+ and CD8+ T cells, and their subpopulations.
Abbreviations: M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

Table 4a. Comparisons between groups of plasma cytokines

CYTOKINES	**Kruskal-Wallis Test**	**M vs. HC**	**D vs. HC**	**E vs. HC**	**M vs. D**	**M vs. E**	**D vs. E**
	χ^2 *(p)*	*U (p)*	*U (p)*	*U (p)*	*U (p)*	*U (p)*	*U (p)*
ELISA							
IL-6	**17.767 (0.000)**	**M>HC 79 (0.002)**				**M>E 66 (0.000)**	
CBA							
IL-6	5.694 (0.127)	M>HC 123.5 (0.024)					
TNFα	2.651 (0.449)						
IL-1b	0.592 (0.898)						
IFNγ	2.026 (0.567)						
IL-4	0.003 (1.000)						
IL-17	6.080 (0.108)						
IL-10	2.453 (0.484)						

Kruskal-Wallis tests and Bonferroni-corrected Mann-Whitney tests showing differences in cytokines levels between the various groups, i.e., manic, depressed, euthymic patients, and HC. The significant results are highlighted in bold.
Abbreviations: IL, interleukin; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma; M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

Table 4b. Mean values of plasma cytokines

CYTOKINES	**M** *mean (SD)*	**D** *mean (SD)*	**E** *mean (SD)*	**HC** *mean (SD)*
ELISA				
IL-6	6.496 (7.589)	2.557 (3.166)	1.730 (4.805)	1.523 (1.011)
CBA				
IL-6	4.723 (8.295)	1.457 (2.971)	1.417 (2.512)	0.289 (0.726)
TNFα	0.036 (0.130)	0.094 (0.249)	1.724 (7.579)	0.721 (1.871)
IL-1b	0.132 (0.592)	0.084 (0.375)	2.397 (10.719)	0.429 (1.784)
IFNγ	<DL	<DL	0.316 (1.413)	0.449 (2.010)
IL-4	0.196 (0.876)	0.088 (0.393)	2.369 (10.596)	0.294 (1.314)
IL-17	0.765 (3.423)	<DL	3.059 (13.680)	3.109 (7.948)
IL-10	0.310 (0.896)	0.042 (0.129)	0.398 (1.550)	0.021 (0.093)

Mean and standard deviation of plasma levels of cytokines.
Abbreviations: IL, interleukin; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma; M, manic patients; D, depressed patients; E, euthymic patients; HC, healthy controls.

Table 5. Correlations between IL-6 and T cells

T CELLS	IL-6 ρ (p)
CD3+	-0.182 (0.108)
CD4+	0.122 (0.284)
CD4+CD28+	0.108 (0.343)
CD4+CD28+CD45RA+	-0.051 (0.657)
CD4+CD28+CD45RA-	0.211 (0.061)
CD4+CD28-	-0.141 (0.215)
CD4+CD28-CD45RA-	-0.121 (0.290)
CD4+CD28-CD45RA+	-0.127 (0.265)
CD4+IFNγ+	-0.204 (0.073)
CD4+IL17+	0.001 (0.993)
CD4+IL10+	-0.132 (0.248)
CD4+IL4+	-0.001 (0.993)
CD8+	-0.059 (0.604)
CD8+CD28+	**0.229 (0.043)**
CD8+CD28+CD45RA+	0.126 (0.270)
CD8+CD28+CD45RA-	0.101 (0.374)
CD8+CD28-	**-0.279 (0.013)**
CD8+CD28-CD45RA-	**-0.257 (0.022)**
CD8+CD28-CD45RA+	-0.205 (0.070)
CD8+IFNγ+	-0.182 (0.111)
CD8+IL17+	-0.080 (0.485)
CD8+IL10+	-0.120 (0.297)
CD8+IL4+	-0.050 (0.663)

Spearman correlation analysis (ρ) between IL-6 level and T cells frequency. The significant results are highlighted in bold.

Correlations between DTI and immunological alterations in BD patients

In order to verify the existence of statistical associations between DTI and immunological alterations, a whole brain voxel-wise regression analysis was performed between those DTI parameters and peripheral frequencies of CD8+ T cell subpopulations that were found to be altered in the previous analyses. Taking into consideration the whole cohort of subjects, a significant direct relationship between decreased FA values (especially in the corpus callosum and corona radiata) and reduced frequencies of circulating CD8+ terminal effector memory and CD8+IFNγ+ (but not CD8+ effector memory) T cells was observed. Coherently, an inverse relationship was found between increased RD values and reduced peripheral frequencies of both CD8+ terminal effector memory and CD8+IFNγ+ T cell subpopulations (**Figure 3 and Table 6**).

Then, the relationship between DTI and immunological changes was investigated in the manic phase in particular, since only mania was found to be characterized by combined and specific WM and immunological alterations. Consistently, a significant correlation of FA-RD abnormalities in the BCC and SCR L with reduced frequencies of circulating CD8+ terminal effector memory and CD8+IFNγ+ T cells (but not CD8+ effector memory T cells, coherently with the whole brain regression analysis) was observed in the manic phase. These DTI-immunological relationships were specific for the manic phase because not present in depression, euthymia or HC (**Table 7**).

With regard to clinical correlations, YMRS total score was found to be inversely correlated with peripheral frequencies of effector memory and terminal effector memory CD8+ T cells, and directly correlated with plasma levels of IL-6. No significant clinical correlations were found with DTI parameters (**Table 8**).

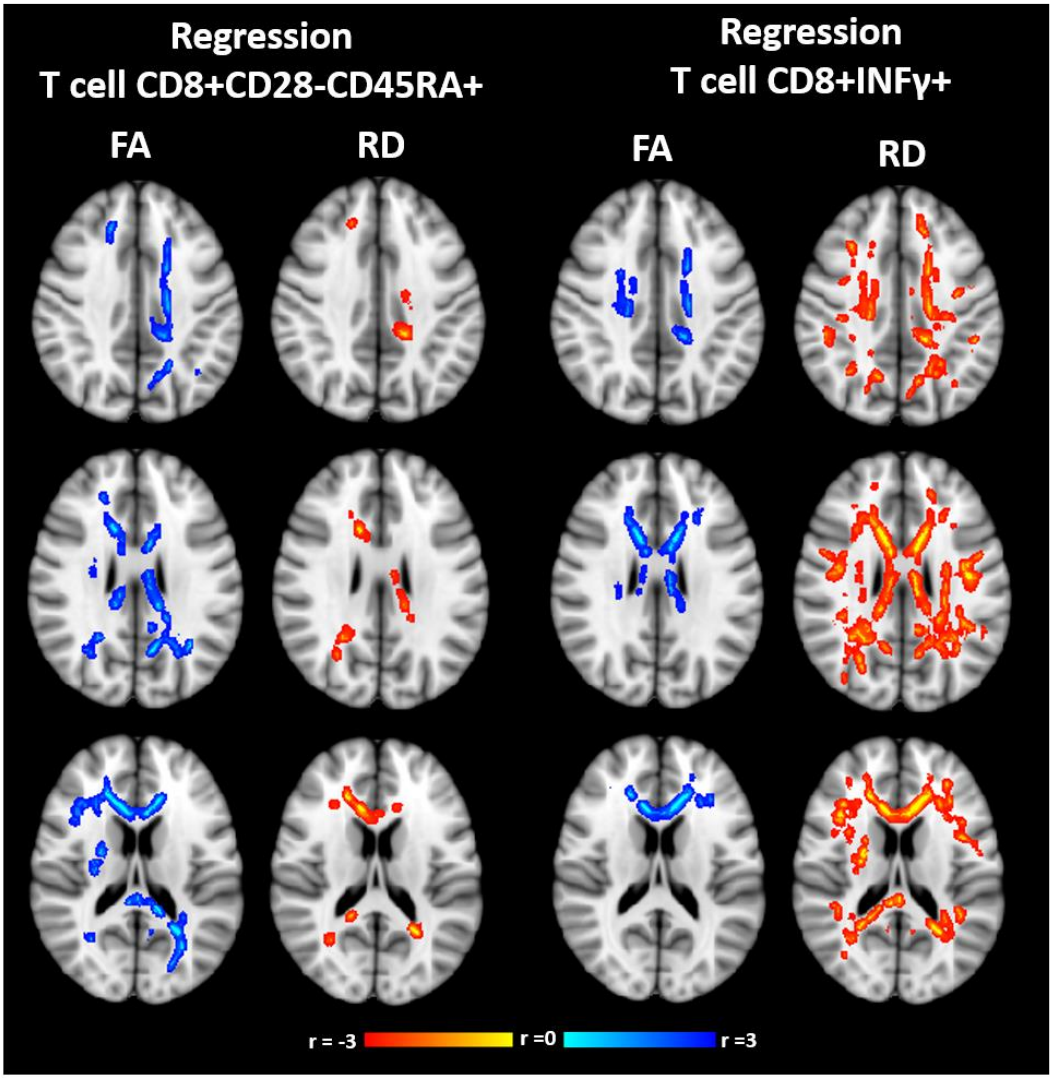


Figure 3. Correlations between WM and T cells alterations
Results from whole brain voxel-wise regression analyses between DTI and T cells alterations (with age and gender as covariates) showing clusters with significant correlations of FA and RD with terminal effector memory CD8+CD28-CD45RA+ and CD8+IFNγ+ T cells. The regression maps of FA and RD values are thresholded at a TFCE and Bonferroni-corrected $p<0.05$, and mapped onto standard T1 Montreal Neurological Institute (MNI) template at z=37, z=27 and z=17. The color bar represents voxel-wise r-values. Direct correlations between decreased FA and reduced frequencies in the circulation of the different T cell subsets are shown in *blue-light blue*, while inverse correlations between increased RD and reduced frequencies in the circulation of the different T cell subsets are shown in *red-yellow*. The significant clusters have been modified using the fill function of the FSL software for visual purpose.
Abbreviations: WM, white matter; DTI, diffusion tensor imaging; FA, fractional anisotropy; RD, radial diffusivity; BD, bipolar disorder; M, mania; D, depression; E, euthymia; HC, healthy controls.

Table 6. Correlation between DTI and immunological alterations

WM TRACTS	CD8+CD28-CD45RA+	CD8+IFNγ+
	r (p)	*r (p)*
FA GCC	0.354 (0.001)	0.396 (0.000)
FA BCC	0.367 (0.001)	0.369 (0.001)
FA ACR R	0.376 (0.001)	0.299 (0.008)
FA ACR L	0.288 (0.011)	0.298 (0.009)
FA SCR L	0.359 (0.001)	0.325 (0.004)
FA PCR L	0.298 (0.008)	0.265 (0.020)
RD GCC	-0.349 (0.002)	-0.390 (0.000)
RD BCC	-0.369 (0.001)	-0.357 (0.001)
RD ACR R	-0.354 (0.001)	-0.357 (0.001)
RD ACR L	-0.303 (0.007)	-0.305 (0.002)
RD SCR L	-0.335 (0.003)	-0.360 (0.001)
RD PCR L	-0.324 (0.004)	-0.305 (0.007)

Partial correlation analysis (r), with age and gender as covariates, of DTI parameters of WM tracts with peripheral frequencies of CD8+ T cell subpopulations - i.e., CD8+CD28-CD45RA+ and CD8+IFNγ+ T cells, in the whole sample.

Abbreviations: DTI, diffusion tensor imaging; WM, white matter; FA, fractional anisotropy; RD, radial diffusivity; GCC, genu corpus callosum; BCC, body corpus callosum; ACR R, anterior corona radiata right; ACR L, anterior corona radiata left; SCR L, superior corona radiata left; PCR L, posterior corona radiata left.

Table 7. Correlations between WM and T cells abnormalities in the various subgroups

	MANIA			
	FA *r (p)*		**RD** *r (p)*	
T CELLS	**BCC**	**SCR L**	**BCC**	**SCR L**
CD8+CD28-	**0.478 (0.045)**	0.404 (0.096)	**-0.504 (0.033)**	-0.396 (0.103)
CD8+CD28-CD45RA-	0.325 (0.189)	-0.227 (0.366)	-0.351 (0.153)	-0.142 (0.575)
CD8+CD28-CD45RA+	**0.485 (0.041)**	**0.487 (0.040)**	**-0.483 (0.042)**	-0.368 (0.133)
CD8+IFNγ+	**0.611 (0.007)**	**0.661 (0.003)**	**-0.582 (0.011)**	**-0.576 (0.012)**
	DEPRESSION			
	FA *r (p)*		**RD** *r (p)*	
T CELLS	**BCC**	**SCR L**	**BCC**	**SCR L**
CD8+CD28-	0.298 (0.230)	0.067 (0.792)	-0.345 (0.161)	-0.211 (0.400)
CD8+CD28-CD45RA-	0.191 (0.447)	0.146 (0.562)	-0.247 (0.323)	-0.293 (0.239)
CD8+CD28-CD45RA+	0.302 (0.223)	0.063 (0.804)	-0.340 (0.168)	-0.176 (0.484)
CD8+IFNγ+	0.126 (0.619)	0.017 (0.946)	-0.127 (0.614)	-0.086 (0.733)

	EUTHYMIA			
	FA *r (p)*		**RD** *r (p)*	
T CELLS	**BCC**	**SCR L**	**BCC**	**SCR L**
CD8+CD28-	0.085 (0.739)	0.312 (0.207)	-0.093 (0.713)	-0.042 (0.868)
CD8+CD28-CD45RA-	0.086 (0.733)	0.120 (0.635)	-0.115 (0.648)	0.126 (0.619)
CD8+CD28-CD45RA+	0.205 (0.415)	0.375 (0.125)	-0.207 (0.409)	-0.152 (0.546)
CD8+IFNγ+	0.007 (0.978)	0.351 (0.167)	0.017 (0.947)	-0.267 (0.301)
	HEALTHY CONTROLS			
	FA *r (p)*		**RD** *r (p)*	
T CELLS	**BCC**	**SCR L**	**BCC**	**SCR L**
CD8+CD28-	-0.102 (0.688)	0.067 (0.793)	0.100 (0.692)	0.001 (0.998)
CD8+CD28-CD45RA-	-0.108 (0.670)	-0.033 (0.897)	0.048 (0.849)	-0.029 (0.910)
CD8+CD28-CD45RA+	-0.031 (0.902)	0.337 (0.171)	0.075 (0.769)	-0.144 (0.569)
CD8+IFNγ+	0.382 (0.118)	0.184 (0.464)	-0.365 (0.136)	-0.171 (0.499)

Partial correlation analysis (r), with age and gender as covariates, of FA and RD values in the BCC and SCR L with peripheral frequencies of CD8+ T cell subpopulations - i.e., CD8+CD28-CD45RA+ and CD8+IFNγ+ T cells (as well as CD8+CD28-CD45RA- T cells, as control) - in the manic group (as well as in the other groups, i.e., depressed and euthymic patients and HC, as control). The significant results are highlighted in bold.
Abbreviations: WM, white matter; FA, fractional anisotropy; RD, radial diffusivity; BCC, body corpus callosum; SCR L, superior corona radiata left.

Table 8. Correlations of clinical parameters with WM and immunological alterations in BD

WM TRACTS	**YMRS** ρ *(p)*	**HAM-D** ρ *(p)*
FA GCC	-0.061 (0.642)	-0.046 (0.727)
FA BCC	-0.131 (0.318)	-0.025 (0.852)
FA ACR R	-0.204 (0.118)	-0.241 (0.063)
FA ACR L	-0.165 (0.208)	-0.199 (0.126)
FA SCR L	-0.050 (0.706)	0.008 (0.952)
FA PCR L	-0.220 (0.090)	-0.251 (0.053)
RD GCC	0.052 (0.695)	0.018 (0.892)
RD BCC	0.094 (0.476)	0.031 (0.815)
RD ACR R	0.278 (0.031)	0.164 (0.211)
RD ACR L	0.208 (0.111)	0.138 (0.295)
RD SCR L	0.156 (0.234)	-0.113 (0.390)
RD PCR L	0.253 (0.051)	0.158 (0.228)
IMMUNOLOGICAL PARAMETERS		
CD8+CD28-	**-0.284 (0.028)**	0.064 (0.628)
CD8+CD28-CD45RA-	**-0.326 (0.011)**	0.142 (0.279)
CD8+CD28-CD45RA+	**-0.290 (0.025)**	0.020 (0.877)
CD8+IFNγ+	-0.220 (0.094)	0.018 (0.891)
IL-6 (ELISA)	**0.420 (0.001)**	0.216 (0.101)

Spearman correlation analysis (ρ) of clinical parameters - i.e., YMRS and HAM-D - with altered DTI (FA and RD values of the altered WM tracts) and immunological (CD8+CD28-CD45RA+, CD8+IFNγ+ and CD8+CD28-CD45RA- T cells, and plasmatic IL-6) parameters, in the BD sample. The significant results are highlighted in bold.

Abbreviations: DTI, diffusion tensor imaging; WM, white matter; FA, fractional anisotropy; RD, radial diffusivity; GCC, genu corpus callosum; BCC, body corpus callosum; ACR R, anterior corona radiata right; ACR L, anterior corona radiata left; SCR L, superior corona radiata left; PCR L, posterior corona radiata left; BD, bipolar disorder; YMRS, Young mania rating scale; HAM-D, Hamilton depression scale.

Limitations

A number of limitations to the current study should be noted.

Body mass index (BMI) could affect our results, since previous reports have found that obesity partly explains elevated inflammatory markers in mood disorder [109]. However, the mean BMI in our BD and HC groups showed values within the normal range, and no significant difference between groups was detected. Moreover, BMI showed no significant correlation with the altered immunological (and DTI) data. All the differences between groups in the relevant DTI and immunological data, as well as their correlations, were still significant even when further controlled for BMI. Therefore, these control analyses suggest that BMI does not affect our DTI-immunological findings.

Another potential confounding factor is tobacco smoking, since a complex relationship between mood disorders, inflammation and smoking has been described [110]. The percentage of tobacco smokers was higher in BD groups with respect to controls, in accordance with the clinical evidence that tobacco smoking among psychiatric patients is more common than in the general population (e.g., [111]). However, no significant difference in the relevant DTI and immunological parameters was detected between tobacco smokers and non-smokers (with the exception of higher level of IL-6 in smokers; $U=487.5$, $p=0.007$). All the differences in DTI and T cells data were still significant even after controlling for smoking. With regard to IL-6, this parameter was significantly different between groups ($\chi^2=7.802$, $p=0.050$) and showed higher values in manic group with respect to HC ($U=18$, $p=0.014$), when considering the smokers subjects only. Finally, the correlation between the relevant DTI and immunological alterations still remained significant after controlling for smoking. Therefore, these control analyses suggest that tobacco smoking does not affect our DTI-immunological findings.

Illness duration may represent an additional confounding factor. Our sample consisted of patients at different stages of the disease. However, no significant difference was detected in illness duration and number of episodes lifetime between patients groups, suggesting a similar course of illness. Furthermore, illness duration did not show significant correlations with immunological parameters and alterations in the different WM tracts. The only exception was an inverse correlation between illness duration and FA in the GCC ($\rho=-0.262$, $p=0.043$). Interestingly, in our study,

the GCC was the only area that was found to be altered in both mania and depression as well as euthymia. Coherently, the GCC was found to be one of the most consistent altered tract in DTI studies on bipolar subjects, both on BD sample as a whole and in euthymic patients [13]. Thus, one can suggest that WM alterations can accumulate into this brain region specifically, with the increase of duration and number of active episodes of illness.

One of the main limitation of the study is medication confound, since almost all the patients in our sample were undergoing pharmacotherapy. The medication load (calculated as in our previous studies) was found to correlate with FA and RD values in various WM tracts in the whole BD cohort, with the relevant exception of the BCC, at uncorrected $p<0.05$ (only the correlation with the bilateral ACR survived after Bonferroni correction; $r=-0.421$, $p=0.001$). No significant correlation was detected with immunological data. Moreover, when manic patients were taken into consideration separately, no significant correlation between medication load and both DTI and immunological parameters was detected. Furthermore, when medication load was entered as additional covariate, the correlation analysis between WM and immunological alterations in the manic phase held or even increased (**Figure 4**). Taken together, these data suggest that WM and immunological alterations, and especially their relationship in the manic phase, even if potentially associated with pharmacotherapy, are not the mere consequence of drug treatment in our sample [69].

Finally, as our previous works, this study suffers from the typical limits of cross-sectional analyses and studies. Longitudinal studies are needed to confirm and complement our findings.

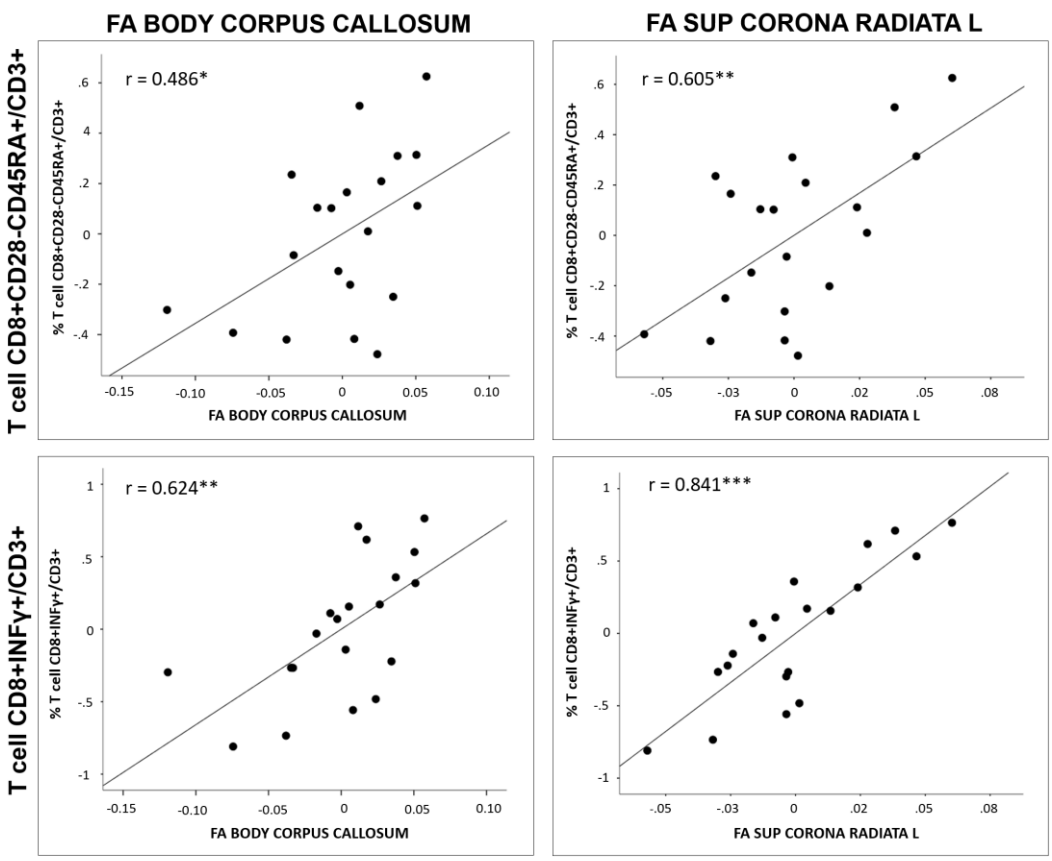


Figure 4. Correlations between WM and T cells changes corrected for medication load in mania

Partial correlations analysis, with age and gender as well as medication load as covariates, of FA values in the body of corpus callosum and left superior corona radiata with the frequencies of CD8+CD28-CD45RA+ and CD8+IFNγ+ T cells subpopulations in the circulation of BD patients in the manic phase.

$p<0.05$*; $p<0.01$**; $p<0.001$***

Abbreviations: WM, white matter; FA, fractional anisotropy; RD, radial diffusivity.

Discussion

Main findings

The main findings of our study are the following: (I) a widespread combined FA-RD alteration was found mainly in the manic phase, with relatively specific involvement of the BCC and SCR L; (II) peripheral immunological alterations were detected in the manic phase, mainly characterized by an increase in CD4+ T cells as well as a decrease in total CD8+ T cells and their subpopulations effector memory (CD8+CD28-CD45RA-), terminally differentiated effector memory (CD8+CD28-CD45RA+) and CD8+IFNγ+; (III) a statistical association between WM and immunological alterations was found in the whole cohort, and a correlation of FA-RD alterations in the BCC and SCR L with reduced CD8+ terminally differentiated effector memory and CD8+IFNγ+ T cells was detected in mania (**Figure 5**).

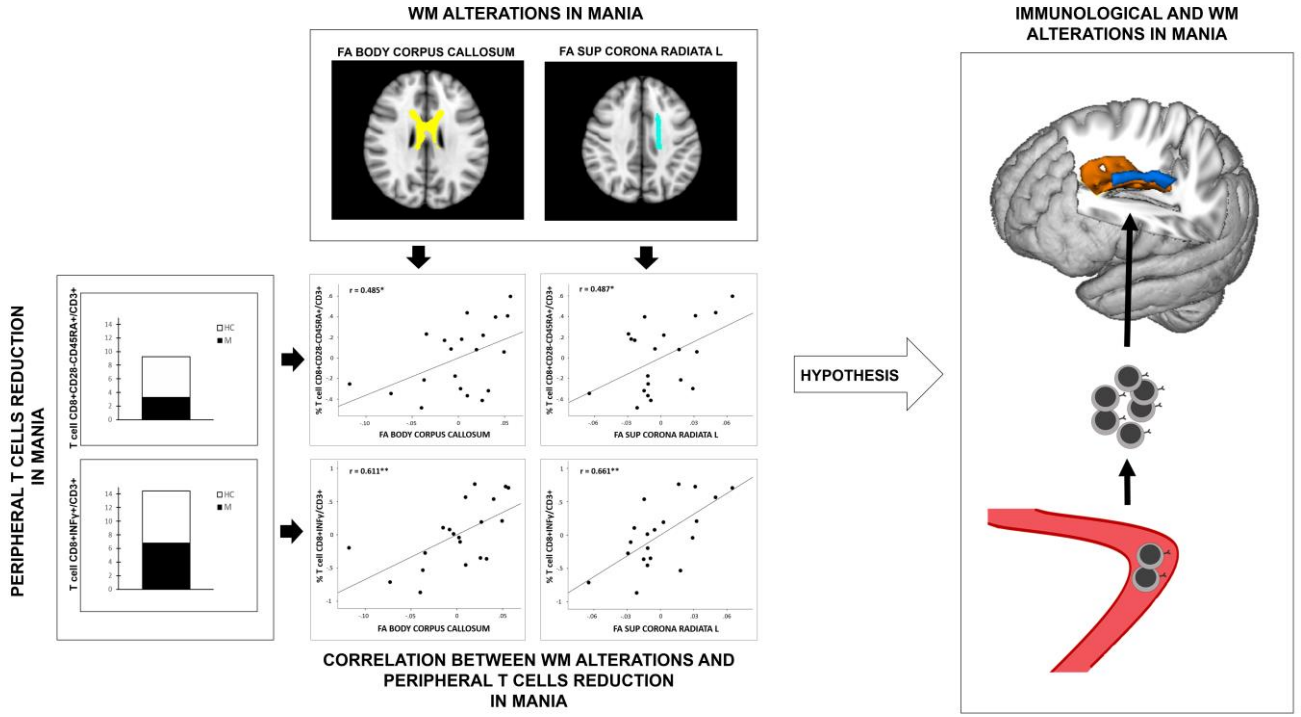


Figure 5. Schema of results and hypothesis on the relationship between WM and immunological alterations in mania

The most remarkable DTI and immunological alterations in mania consist in: (i) relatively specific WM alterations mainly located in the body of corpus callosum and left superior corona radiata (*upper panel)*; (ii) reduction in the frequency of circulating terminal effector memory CD8+CD28-CD45RA+ and CD8+IFNγ+ T cell subpopulations (*left panel*); (iii) significant partial correlation (with age and gender as covariates) between the cited WM and immunological alterations (*middle panel*).

Our hypothesis is that in mania CD8+CD28-CD45RA+ T cells (which are cells prone to migration in the peripheral tissues) and CD8+IFNγ+ T cells (which are the activated CD8+ T cells and include activated CD8+CD28-CD45RA+ T lymphocytes) leave the blood stream to migrate into the brain where they induce an immune-related WM damage *(right panel)*.

Abbreviations: WM, white matter; DTI, diffusion tensor imaging; FA, fractional anisotropy; M, mania; HC, healthy controls.

WM alterations in mania

In this study, widespread WM alterations were significantly detected in the active phases of illness, confirming our previous whole brain findings on an independent BD sample [50]. In the present work, altered WM tracts showed a progressively decreasing trend of FA-RD changes from mania (high alterations) to depression (moderate alterations) and to euthymia (low alterations). The prominence of WM abnormalities in mania, especially in midline structures such as BCC, is in accordance to the previous tractography study from our group, which showed significant structural disconnectivity in midline regions in mania only [51]. Taken together, these data further support the hypothesis of dynamic changes of WM abnormalities across the different phases of illness, showing a more consistent impairment in mania.

Immunological alterations in mania

Based on the hypothesis that immunological alterations may be pathogenically involved in BD, we analyzed the distribution of T cell subpopulations and cytokine concentrations in the peripheral blood of BD patients in the different phases of illness. Interestingly, the most significant immunological alterations were observed in mania, which was characterized by increased peripheral levels of CD4+ T cells and IL-6, as well as decreased frequency of CD8+ T cells, belonging specifically to the effector memory, terminal effector memory and IFNγ+ subpopulations. Trends of circulating T cell subset frequencies similar to those observed in manic phase were also detected in depressive and euthymic phases of BD, although here they did not reach statistically significant differences compared to those of controls. Although a previous work suggested an activation of cell-mediated immunity in bipolar mania [90], studies detailing the immunological alterations are lacking so far. Our findings might identify the immunological stigmata of mania in an increase of early activated (CD4+CD28+) T cells and a decrease of effector memory (CD8+CD28-CD45RA-) and terminal effector memory (CD8+CD28-CD45RA+) T cells, paralleled by reduction of CD8+IFNγ+ T cells (corresponding to functionally activated CD8+ T cells). Interestingly, the loss of the CD28 co-receptor, which is observed in a heterogeneous CD8+ T cell population, occurs after prolonged stimulation. The expression of the CD45RA molecule within these cells characterizes the shift from effector memory to terminal effector memory cells. This represents a progression to a

low proliferative/high release of cytokines profile that is associated to increasing expression of adhesion molecules and chemokine receptors, ultimately dictating their localization in peripheral tissues [112]. Hence, terminal effector memory CD8+ T cells are effectors cells prone to migration in the peripheral tissues [113]. Together, our data suggest the occurrence of an acute immune response in manic patients, as further corroborated by the increase of a cytokine such as IL-6, which is related to acute inflammation. Interestingly, in our sample we could also observe a correlation between the increased IL-6 serum levels and the decrease of CD8+CD28- T cells, hence the chronically stimulated and/or activated CD8+ T cells, suggesting that the two phenomena may be pathogenically related. Signs of immune activation in BD have been hypothetically related to autoimmune and infection processes, which interestingly were found to be more prevalent in patients with affective disorders; these data have made attractive the autoimmune and infectious hypotheses of BD, but clear proof supporting them needs still to be provided (e.g., [96, 114, 115]).

Relationship between WM and immunological alterations in mania

In order to investigate potential underpinnings for WM abnormalities in BD, we then investigated their relationship with immunological abnormalities, and a significant correlation between WM and immunological alterations was found in our sample. In this context, a previous study on the depressive phase of BD showed a significant correlation between WM alterations and Th17 cells levels, but no significant differences were detected when patients were compared to HC (coherently with our findings in depression) [99]. Our work extends previous results by showing that, notably, both the prominent WM alterations and the reduction of effectors CD8+ T cells were found to be strongly associated to the manic phase. In particular, reduced FA and increased RD in the BCC and SCR were found to significantly correlate with a reduction of circulating CD8+ terminal effector memory T cells (which are cells prone to migration in the peripheral tissues) and CD8+IFNγ+ T cells (which are the activated CD8+ T cells and include activated CD8+ terminal effector memory T lymphocytes), but not with CD8+ effector memory T cells, in the manic phase. A decrease in FA with concomitant increase in RD was suggested to reflect alterations in oligodendroglial and myelin microstructure of WM [16, 37], which, in turn, have been related to T cell-mediated pathogenesis in various diseases [116]. In the absence of an easily accessible tissue where confirmatory histological investigations could be

performed, it is remarkable that our study, combining sensitive radiological investigations with immunological analyses, had the possibility to specifically constrain correlative analyses between structural and immunological alterations. The high statistical level of correlation observed in these analyses might indicate a direct relationship between the two abnormalities, leading to the hypothesis of a reciprocal interdependency.

Collectively, these findings suggest an acute immune response in mania, sustained by early generated CD4+ T cell compartment (likely with T helper function), leading to activation of CD8+ T cell subpopulations that leave the circulation to migrate in peripheral tissues such as, hypothetically, the brain areas where WM damage occurs. This phenomenon is reminiscent of what observed in chronic inflammatory neurological diseases such as multiple sclerosis (MS). There, reduction of CD8+ T cells and increased CD4+/CD8+ ratio in peripheral circulation associated with accumulation of CD8+ T cells in acute and chronic inflammatory WM lesions are observed, suggesting that CD8+ T cells recognize components of the myelin sheath and could contribute to the WM damage as effector cells [117, 118]. However, studies are needed to formally demonstrate presence and localization of specific subsets of CD8+ T effector cells in post-mortem brains of BD subjects to prove their pathogenic potential in mania.

Conclusions

In summary, both the prominent WM and immunological alterations were found to be strongly associated to mania. For the first time, our data highlight immunological markers of mania highly correlating with structural alterations in WM tracts. They also support an innovative pathogenic mechanism that recognizes in a subpopulation of CD8+ effector T cells a major candidate to be responsible for the structural WM lesions underlying mania. Savitz J. (one of the leading researcher in this field) wrote a Commentary on our work pointing out that our findings narrow the gap on the pathophysiological basis of mania and its link with immune dysregulation which, to date, has been sparsely investigated and is poorly understood [119]. Finally, the Author, in accordance with our findings and hypothesis, also highlighted the possibility of a mechanistic overlap between BD and other inflammatory illnesses such as MS [119].

CHAPTER IV

Clinical and neurobiological evidences on the relationship between bipolar disorder and multiple sclerosis

Introduction and background

Considering previous data, as well as our findings, a deeper investigation of the relationship between BD and MS may be relevant for a better understanding of the still unknown pathogenethic processes behind both of them. But what do BD and MS have in common?

Epidemiology and clinical features of bipolar disorder and multiple sclerosis

Clinically, it is well known that MS may be associated with psychiatric comorbidities, and, to date, this relationship has been investigated mainly from an epidemiological point of view [120, 121]. In this context, besides depression and anxiety, which are the most studied mental health conditions in MS, BD has assumed a growing interest [120]. Although the association between BD and MS has been already described since the late '70s [122], the recent literature on this issue remains still sparse [120]. However, the frequency of BD and its impact on the life of individuals with MS is well underscored [120]. The overall prevalence of BD in MS range from 0 to 16% [120, 123]. Most recent studies suggest that BD is roughly twice as common in MS, both at the time of diagnosis (3.15% in MS, as compared to 1.69% in healthy subjects) [120] and in the MS population more broadly (4.7% in MS vs. 2.3% in healthy subjects) [120, 123]. Some studies described percentages further greater, finding that MS patients present up to 30 fold-increase in the incidence of BD [96, 121, 124]. On the other hand, replicated epidemiological studies have demonstrated that BD shows high rates of inflammatory medical comorbidities (up to 48%) [125]. In particular, BD has been associated with many autoimmune disorders, including MS [125, 126].

Moreover, BD and MS seem to show some similar features from a clinical point of view. Horrobin and Lieb (1981) hypothesized, for the first time, that the relapsing-

remitting nature of BD may be driven by the immune system, as seen in other relapsing-remitting inflammatory disorders, such as MS [127]. Indeed, the vast majority of patients with MS initially follow a relapsing-remitting course, defined by acute exacerbations from which they typically completely or incompletely recover, with periods of relative clinical stability in between [128]. This is reminiscent of what is observed in the clinical course of BD, where the occurrence of active phases of illness is alternated to periods of euthymia. Interestingly, a high percentage of BD occurring in MS patients present with manic episodes (single/recurrent) [129]. Furthermore, among psychiatric symptomatology occurring during MS, manic symptoms such as emotional instability and euphoria are quite common [130].

Collectively, epidemiological data as well as relapsing-remitting course and some symptomatologic presentations support the hypothesis of an association between MS and BD, especially in its manic phase. However, the mechanisms underlying these associations are unknown and, to date, have been poorly investigated. Family studies investigating the involvement of human leukocyte antigen (HLA) genes have demonstrated a common genetic susceptibility among patients with BD and MS [131-133]. Moreover, downregulation of the genes related to the processes of energy metabolism, mitochondrial function and oligodendrocytes activity, along with upregulation of the genes involved in immune response and inflammation, have been reported in various brain areas of both BD and MS [133, 134]. These data further support a common biological basis between these two illnesses, and one can thus hypothesize that this might reflect in similar patterns of brain alterations.

Microstructural white matter alterations in multiple sclerosis

MS, which is the prototype of inflammatory demyelinating diseases of the central nervous system, is characterized by multifocal WM demyelination, gliosis and neuroaxonal damage [135]. Widespread demyelinating WM lesions (WML) are the hallmark of the disease and are visible on standard T2-weighted MRI scans as hyperintensities [136]. However, the disease spreads beyond the MRI visible lesions [137] to the normal-appearing WM (NAWM) [138], where subtle microstructural alterations are already detectable in the early stages of MS when the MRI visible lesions are still few [139]. Previous DTI studies in MS have shown decreased FA along with increased MD and RD values [138, 140, 141] especially within the T2-visible WML, where such alterations were correlated to pathological findings such as

demyelination, axonal loss, inflammation and gliosis [142]. Although to a lesser degree, DTI parameters were reported consistently altered in NAWM of MS patients (reduced FA, augmented MD and RD) [22]; this was related to clinical severity and disease duration [143, 144], as well as with neuropathological signs (patchy edema, glial hyperplasia, demyelination, transection-induced axon degeneration, perivascular infiltration containing T lymphocytes) [145-147]. The corpus callosum and, in general, the periventricular WM regions are vulnerable sites for WM alteration and lesion development, especially in the early stages of MS [141, 148].
Although BD is not a primarily demyelinating disorder and does not typically show WML, it is characterized (in particular in the manic phase) by widespread microstructural WM abnormalities especially in the midline structures. Interestingly, this resembles some of the described DTI alterations in MS. However, investigations that compare such neurobiological alterations between BD and MS are still lacking.

Therefore, we conducted a study aimed to characterize DTI similarities or differences in the regional extent of WM pathological changes, between patients with mania and MS patients with early disease and small number of WML. We focused our analysis on midline structures such as the corpus callosum and cingulum, considering the consistent alteration found in these areas across both the diseases [149].

Methods

The bipolar mania (M-BD) group was composed by 23 patients, and was part of the samples used in our previous studies [50, 51, 102]. As described in the previous chapters, type I BD diagnosis was assessed with the SCID-I/P in accordance to DSM criteria and manic patients were included in the study when scoring ≥13 at YMRS and <8 at 17-item HAM-D. The MS group (part of a sample used in other work [150]) was composed of 23 patients with a recent diagnosis of relapsing-remitting MS (RR-MS) according to McDonald's (2010) criteria [151], with a minimum WML burden, and sex and age-matched to the M-BD group. Clinical diagnosis was also supported by neurophysiological and cerebrospinal fluid findings. MS patients were enrolled in the study at least one month after steroid administration or the last

relapse. Median Expanded Disability Status Scale (EDSS) score was 1.5 (range 0-2.5). For each patients' sample (i.e., M-BD and MS) a corresponding 23-subject-population of matched HC - HC(M-BD) and HC(MS) groups respectively - was included (**Table 1**). Exclusion criteria for all groups were: inability to provide written informed consent, diagnosis of other psychiatric, neurologic or cognitive affections, any major systemic or organ comorbidities, history of present or previous drug and/or alcohol addiction, left-handedness, pregnancy and lactation, the inability to undergo an MRI examination. Both studies on BD and MS were approved by the local Ethical Committee of San Martino Polyclinic Hospital, and all subjects provided written informed consent.

Table 1. Subject demographic and clinical information

	M-BD	**HC (M-BD)**	**MS**	**HC (MS)**
Sample Size *n*	23	23	23	23
Female *n* (%)	14 (60.8)	14 (60.8)	14 (60.8)	14 (60.8)
Age *mean* (SD)	42 (12)	44 (12)	40 (11)	30 (4.5)

Abbreviations: M-BD, mania - bipolar disorder; MS, multiple sclerosis; HC, healthy controls

All scans were acquired on the same 1.5-T GE scanner, using a standard brain T/R 8-channel phased-array coil. The DTI protocol was always oriented on the pure (scanner's) axial plane and shared these same identical parameters within all subjects: single shot echo planar imaging sequence, five b=0 acquisitions before the subsequent b=1000 s/mm2 diffusion weighted acquisitions, TR=14000ms, minimum TE, in-plane matrix 128x128, FOV=240mm, NEX=1, 56 contiguous slices, slice thickness=2.5mm, no gap. The total number of non-collinear directions of the diffusion sensitizing gradients was 30 for the MS sample and corresponding HC, while 60 for the M-BD sample and corresponding HC: this difference can be considered acceptable and uninfluential in the classic DTI metrics analysis, according to previous work [152-154]. In addition, two anatomical sequences were also acquired for all participants (i.e., 3D T1-weighted SPGR and FLAIR). These two last scans were both reviewed by a board-certified neuroradiologist. No occasional findings and/or neurological comorbidities in the whole cohort, as well as

significant WMLs in the psychiatric and HC groups, were found, so that none of the subjects was excluded from the study.

A standard preprocessing was run on the DTI series from all subjects, featuring eddy current geometric compensation, brain extraction and fitting of the DT, using tools from FSL [61, 155, 156]. DT parametric maps were generated for FA, MD and RD. The obtained maps were all non-linearly co-registered on the standard FMRIB58_FA template of FSL, via standard TBSS pre-processing [53], to achieve an overlap of the WM tracts between all subjects. An *a-priori* ROI analysis was conducted. Specifically, masks of the whole corpus callosum (as well as its sub-portions of genu, body and splenium) and bilateral cingulum, as provided by the JHU ICBM-DTI-81 White-Matter Labels atlas in FSL [64, 65], were used. The masks were then skeletonised in order to exclude the peripheral voxels that could suffer from registration biases.

Finally, mean values of DTI parameters (i.e., FA, MD and RD) were extracted from each tract of all subjects. In order to improve normality for group-level comparison, the DTI metrics for each ROI were transformed to z-values within each patients' sample by using the corresponding HC group as reference, separately - i.e., the M-BD group was normalized onto the HC(M-BD) group while the MS group onto the HC(MS) group (after normalization, the two HC groups were then considered together as a single reference HC group). The z-scored DTI metric values (zFA, zMD and zRD) of the three groups (HC, M-BD and MS) were then entered into group-level analysis.

In the MS group, in addition to the tract analysis of the corpus callosum and cingulum, a segmentation of WML was performed on FLAIR images using the automated lesion prediction algorithm (LPA) from lesion segmentation toolbox (LST) [157, 158] of Statistical Parametric Mapping 12 (SPM12) [159]. A subsequent revision and manual correction was operated by a board certified neuroradiologist. Then, the same non-linear transformations used for the DT parametric maps were applied to obtain individual lesion mask registered to the FMRI58_FA space. Finally, each patient's binary lesion mask was inverted and multiplied for its DT parametric maps to assign a zero value to all sites where a lesion was outlined. The mean value of non-zero-voxels (with no visible lesions) from the tract ROIs in the MS group (MS nL) were thus considered deriving from NAWM only and entered in between-groups comparisons with the M-BD group. A lesion probability map [148] (voxel

values within the 0.0 – 1.0 range) relative to the MS group was also computed and standardized onto the FMRI58_FA space. The map was then used to quantify the FLAIR burden of impairment at ROI level, by calculating the number-of-voxel-weighted average of lesion probabilities within each tract.

A univariate ANOVA was performed for each tract (i.e., corpus callosum: whole tract, as well as genu, body and splenium; bilateral cingulum), in full factorial mode with type 3 sum of squares including intercept, assigning the z-scored mean DTI metric (i.e., zFA, zMD and zRD, separately) as dependent variable, the group as a fixed factor, age and sex as covariates. The marginal means were estimated by group, main effects were compared and Bonferroni adjustment for multiple comparisons was included. Significance level (alpha) was set to 0.05. Additionally, homogeneity tests were performed, together with descriptive statistics. Finally, in order to control for potential effects of WML in the MS group, the same statistical analysis was independently performed on the MS nL group.

Statistical analyses were performed by means of SPSS.

Results

A decrease in FA values and an increase in MD and RD values in the whole corpus callosum were found in the MS group (at a higher degree) as well as in the M-BD sample (at a lesser degree) when compared to HC, although statistically significant for MS only.

Considering the sub-regions of the corpus callosum separately, a different pattern of DTI alterations was observed. Consistent and highly significant changes in FA, MD and RD values were found in the posterior regions (body and especially splenium of corpus callosum) in MS. Coherently, an intense lesional burden, as assessed by the lesion probability map, was detected in the same areas in the MS sample. Conversely, a significant increase in MD and RD values was found in the anterior portion of corpus callosum (i.e., genu) in the M-BD groups, with respect to both MS and HC. Moreover, in M-BD a progressively decreasing trend of DTI alterations was detected from the genu (higher degree) to the body (moderate degree) and to the splenium (low degree) of the corpus callosum.

Although no significant differences in DTI parameters were found in the cingulum between groups, a relative decrease in FA and increase in RD values can be observed in the M-BD group, and to a lesser extent in the MS sample, with respect to HC.

All the above results are summarized in **Table 2a** and **Table 2b**.

Finally, after correcting for lesions, the MS nL group showed a modest but consistent reduction of DTI alterations when compared to the MS group in all the corpus callosum regions (but not in the cingulum). The lesion burden recorded in each tract is listed in **Table 3**.

Table 2a. Mean values of DTI parameters of the investigated WM tracts

WM Tracts	HC	M-BD	MS	MS nL
	mean (SE)	*mean (SE)*	*mean (SE)*	*mean (SE)*
FA CC	-0.062 (0.142)	-0.604 (0.201)	-0.755 (0.199)	-0.488 (0.194)
FA gCC	-0.092 (0.15)	-0.668 (0.212)	-0.418 (0.21)	-0.252 (0.209)
FA bCC	-0.047 (0.135)	-0.484 (0.191)	-0.265 (0.189)	-0.044 (0.187)
FA sCC	-0.024 (0.163)	-0.307 (0.23)	-1.817 (0.228)	-1.536 (0.209)
FA Cing	-0.044 (0.151)	-0.512 (0.214)	-0.308 (0.121)	-0.309 (0.212)
MD CC	0.041 (0.164)	0.411 (0.232)	1.379 (0.23)	1.229 (0.23)
MD gCC	0.064 (0.152)	0.716 (0.215)	-0.232 (0.213)	-0.331 (0.212)
MD bCC	0.03 (0.143)	0.357 (0.202)	0.844 (0.2)	0.771 (0.201)
MD sCC	0.008 (0.168)	-0.225 (0.237)	2.325 (0.235)	2.202 (0.228)
MD Cing	0.003 (0.152)	0.062 (0.216)	0.342 (0.214)	0.354 (0.214)
RD CC	0.061 (0.151)	0.618 (0.214)	0.981 (0.212)	0.748 (0.209)
RD gCC	0.086 (0.151)	0.76 (0.214)	0.095 (0.212)	-0.053 (0.21)
RD bCC	0.046 (0.139)	0.492 (0.197)	0.472 (0.195)	0.301 (0.194)
RD sCC	0.02 (0.164)	0.232 (0.232)	2.161 (0.23)	1.929 (0.215)
RD Cing	0.03 (0.143)	0.394 (0.203)	0.341 (0.201)	0.348 (0.201)

Mean and standard error of the extracted FA, MD, and RD values from the investigated WM tracts in M-BD, MS and HC.
Abbreviations: DTI, diffusion tensor imaging; WM, white matter; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; CC, corpus callosum; gCC, genu corpus callosum; bCC, body corpus callosum; sCC, splenium corpus callosum; Cing, bilateral cingulum; M-BD, mania – bipolar disorder; MS, multiple sclerosis; nL, no lesion; HC, healthy controls.

Table 2b. Comparisons between groups of the DTI parameters of the investigated WM tracts

WM Tracts	ANOVA	M-BD vs. HC	MS vs. HC	MS vs. M-BD	MS nL vs. HC	MS nL vs. M-BD
	F (p)	*(p)*	*(p)*	*(p)*	*(p)*	*(p)*
FA CC	**4.825 (0.010)**		**MS<HC (0.017)**			
FA gCC	2.550 (0.084)					
FA bCC	1.758 (0.178)					
FA sCC	**21.220 (0.000)**		**MS<HC (0.000)**	**MS<M-BD (0.000)**	**MS nL<HC (0.000)**	**MS nL<M-BD (0.000)**
FA Cing	1.650 (0.198)					
MD CC	**11.255 (0.000)**		**MS>HC (0.000)**	**MS>M-BD (0.011)**	**MS nL>HC (0.000)**	**MS nL>M-BD (0.043)**
MD gCC	**5.243 (0.007)**	**M-BD>HC (0.048)**		**MS<M-BD (0.007)**		**MS nL<M-BD (0.002)**
MD bCC	**5.450 (0.006)**		**MS>HC (0.004)**		**MS nL>HC (0.010)**	
MD sCC	**39.289 (0.000)**		**MS>HC (0.000)**	**MS>M-BD (0.000)**	**MS nL>HC (0.000)**	**MS nL>M-BD (0.000)**
MD Cing	0.884 (0.417)					
RD CC	**6.640 (0.002)**		**MS>HC (0.002)**		**MS nL>HC (0.027)**	
RD gCC	**3.667 (0.030)**	**M-BD>HC (0.038)**				**MS nL<M-BD (0.022)**
RD bCC	2.406 (0.96)					
RD sCC	**30.548 (0.000)**		**MS>HC (0.000)**	**MS>M-BD (0.000)**	**MS nL>HC (0.000)**	**MS nL>M-BD (0.000)**
RD Cing	1.375 (0.258)					

ANOVA results (controlled for age and gender) followed by Bonferroni-corrected post-hoc tests between groups for the corpus callosum (whole tract and sub-regions: genu, body, splenium) and bilateral cingulum.
Abbreviations: FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity; HC, healthy controls; M-BD, mania - bipolar disorder; MS, multiple sclerosis; nL, no lesion.

Table 3. Regional lesion probability for the MS sample

WM Tracts	Mean Probability
Whole corpus callosum	15.10%
Corpus callosum genu	7.40%
Corpus callosum body	17.00%
Corpus callosum splenium	13.80%
Bilateral cingulum	4.5 %

The regional value, expressed in percentage, was obtained from the average of the values of each voxel contained in the various tracts, where each voxel's value represents the WML relative frequency (i.e., the number of patients with a WML in that voxel divided for the total number of patients).
Abbreviations: MS, multiple sclerosis; ROI, region of interest; WML, white matter lesion.

Limitations

The main limitation of the study is the relatively small samples size, which could reduce the statistical power of the measurements, and the retrospective nature of the work. Another possible weakness of this study could be represented by the different number of DTI directions (30 vs. 60) in the scan protocols between the groups. Different values of FA have been previously described as the number of directions increases. However, this effect has been detected for a number of directions lower than 30, while after this value the FA reaches a plateau and the variance in its computation is comparable with the error of the measure [152-154]. Thus, even though the ideal case is to use a unique protocol of acquisition, it can be assumed that the difference in number of directions in our data does not affect the obtained results.

Discussion

This study, for the first time, investigated and directly compared brain WM alterations in M-BD and MS, the prototype of demyelinating inflammatory neurological disease, using DTI and focusing on the corpus callosum and cingulum as those tracts which are mainly altered in these conditions. We observed common changes in DTI parameters in the M-BD and MS groups, with the greatest abnormalities in the whole corpus callosum in MS. However, considering the corpus callosum sub-portions separately, we found an opposite anterior-posterior pattern of DTI alterations in the M-BD and MS groups. In particular, in the anterior region (i.e., genu) the M-BD group showed the highest level of WM alterations, whereas in the posterior region (i.e., splenium) the WM impairment was significantly greater in MS (**Figure 1**).

Specifically, the M-BD group showed a progressively decreasing trend of DTI alterations from the anterior to the posterior areas of the corpus callosum, reaching a statistically significant increase in MD and RD in the genu only. Coherently, a trend to reduction in FA and increase in RD (though not significant) was detected also in the cingulum in M-BD. This is in accordance with previous work on BD, in which the highest degree of DTI changes was detected in the anterior sections of the midline regions, especially in mania [51, 160].

By contrast, the DTI alterations were characterized by an inverse pattern in the MS group, showing a progressively increasing trend from the anterior to the posterior areas. Accordingly, the body and especially the genu appeared relatively spared, while the splenium was found to be highly compromised, showing significantly altered FA, MD and RD values, not only in MS but also in MS nL. Lesion correction was consistently associated to a milder alteration of DTI parameters in those regions where the lesion burden was higher (i.e., body and splenium of corpus callosum), while it was less or not relevant in those regions characterized by few or no FLAIR-detectable lesions (such as the cingulum). Thus, after lesion correction, the DTI values of MS nL tended to set between those of the MS and M-BD groups, but still remaining highly altered. These findings confirm that DTI alterations, especially in the more compromised regions such as the splenium, extend beyond the FLAIR-detectable lesions to the surrounding NAWM, independently from the lesion burden. This is also in line with some previous studies, which show that the initial damage in the early stages of MS is mainly localized in the deep, periventricular and posterior WM [148, 161].

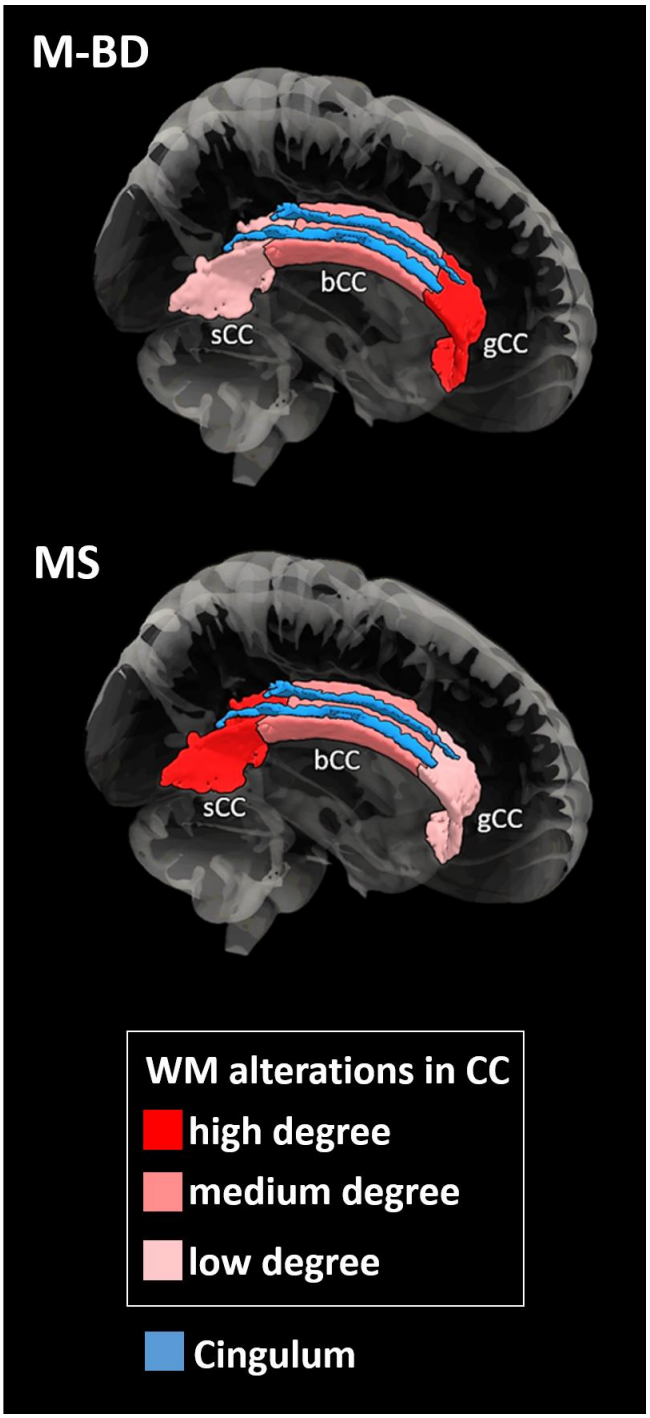


Figure 1. Schema of WM alterations in bipolar mania and multiple sclerosis
In red shades: WM DTI alterations in the corpus callosum with opposite spatial pattern in its sub-regions in M-BD and MS. In blue: cingulum.
Abbreviations: M-BD, mania - bipolar disorder; MS, multiple sclerosis; gCC, genu corpus callosum; bCC, body corpus callosum; sCC, splenium corpus callosum; WM, white matter; DTI, diffusion tensor imaging.

In conclusion, common WM changes were found in M-BD and MS, but the topographical pattern of alterations seem to vary between the two groups. Thus, it's tempting to speculate that M-BD and MS might share common pathophysiological features, with the DTI abnormalities reflecting the same phenomenon at different levels of severity or a similar manifestation of two different pathological processes. Future works, such as combined WM and immunological investigations, as well as histological and postmortem studies, are needed to shed light on this issue and clarify the still unknown pathogenethic processes behind both BD and MS.

CHAPTER V

Preliminary longitudinal data on white matter and immunological changes in the various phases of bipolar disorder

In order to better understand and characterize the apparently state-dependent WM and immunological changes in BD, our transversal (inter-subjects) findings need to be confirmed and extended in a longitudinal (intra-subject) perspective.

Therefore, we are conducting a longitudinal study, collecting both DTI and bio-humoral follow-up data of our sample and investigating WM and immunological alterations in BD patients across their different phases of illness.

In particular, basing on our previous transversal findings, we aimed to investigate: (I) DTI alterations (i.e., FA and RD parameters) in midline structures (i.e., corpus callosum) in BD patients passing from active phases to euthymia; (II) immunological alterations (i.e., CD4+ and CD8+ T cell subpopulations) in BD patients passing from active phases to euthymia.

DTI follow up study: preliminary data

Methods

Subjects who were part of the samples of our previous transversal studies [50, 51, 102] (baseline, T0) were re-evaluated after a minimum period of 6 months (T1). In particular, 20 manic and 21 depressed patients from the T0 sample were re-evaluated during the euthymic phase (T1). The evaluation at T1 comprised the same clinical scales and MRI protocol as at T0. Please see the Methods section of Chapter II e III for a complete description of clinical evaluation, inclusion and exclusion criteria, as well as MRI parameters acquisitions.

DTI analyses were performed by using FSL toolboxes with the same approach as in our previous studies. After a standard preprocessing of the diffusion data, the maps of FA and RD values were derived. TBSS analysis was used to perform a voxel-wise analysis of the whole-brain DTI measures, which were then entered in between-

groups comparisons (age and gender were entered as covariates, and a p value <0.05, corrected for FWE using the TFCE, was set). In particular, FA as well as RD maps were compared between manic or depressed groups at T0 and HC, in order to detect WM areas with significant alterations in the active phases of illness. Then, the resulting significantly altered areas were masked with the corpus callosum (from the JHU-ICBM-DTI-81 White-Matter Labels atlas included in FSL), in order to obtain a mask of the significant FA and RD alterations within the corpus callosum during the active phases of BD. These masks were applied to DTI maps of manic or depressed patients at T0 as well as the same patients in euthymia at T1, to extract the correspondent FA and RD values. Finally, these DTI parameters were compared using paired t-tests between T0 and T1 patients' groups (by means of SPSS), in order to detect potential longitudinal changes across the different phases of illness.

Results

The voxel-wise between-groups comparison showed a significant reduction of FA, especially in midline regions, in manic patients at T0 when compared to HC. Coherently, a significant increase in RD was detected again in manic patients at T0 when compared to HC. No significant changes were observed in FA or RD values in the depressed group at T0 when compared to HC. See **Figure 1A**.

FA and RD values were then extracted from these altered areas (within the corpus callosum) in manic subjects at T0 as well as in the same subjects during euthymia at T1. The paired t-test between patients' groups at T0 and T1 showed a significant increase in FA values ($t=2.141$; $p=0.045$) as well as a significant decrease in RD values ($t=2.117$; $p=0.048$) in BD patients passing from the manic phase (T0) to euthymia (T1). See **Figure 1B**.

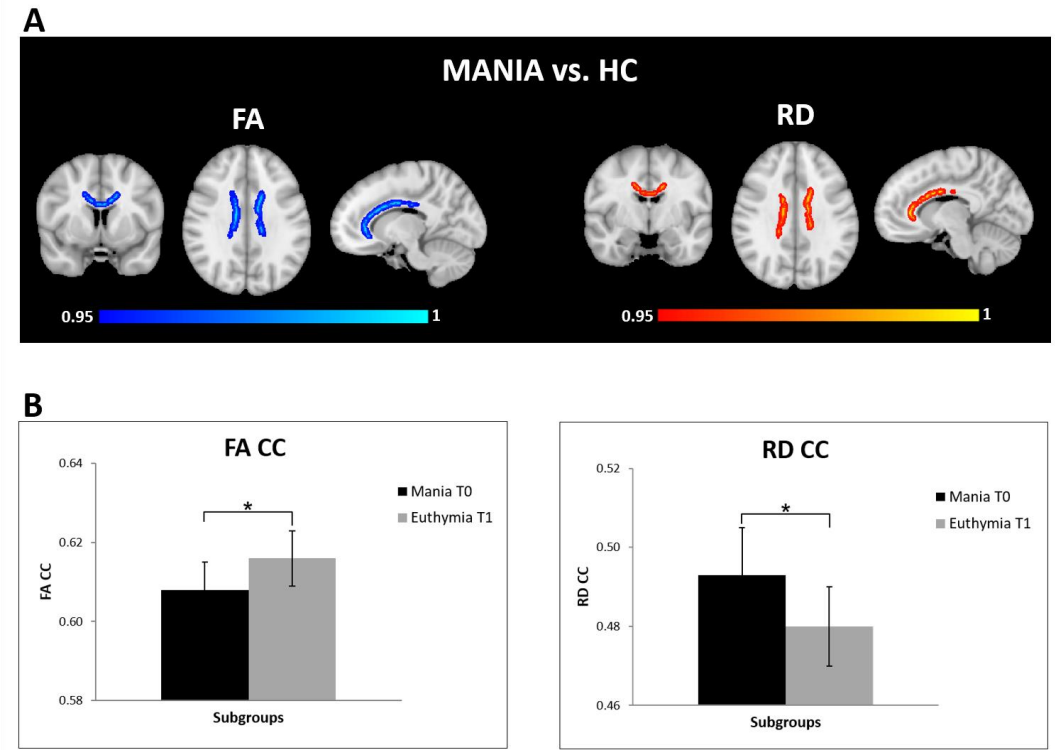


Figure 1. WM alterations of midline structures in mania and their changes in euthymia
A. Results from between-groups comparison (t-tests, with age and gender as covariates) showing clusters with significantly decreased FA values (*blue-light blue*) and significantly increased RD values (*red-yellow*) within the corpus callosum, in BD patients in manic phase when compared to HC ($p<0.05$, TFCE corrected). For display purposes the statistically significant clusters are displayed as 1-p values. Group differences are mapped onto standard T1 Montreal Neurological Institute (MNI) template. The significant clusters have been modified using the fill function of FSL software for visual purpose.
B. Between-groups comparison (paired t-test) of FA and RD values extracted from the altered cluster within the corpus callosum (see part A) in BD patients passing from mania (T0) to euthymia (T1); $p<0.05$*.
Abbreviations: WM, white matter; FA, fractional anisotropy; RD, radial diffusivity; CC, corpus callosum; BD, bipolar disorder; HC, healthy controls.

Immunological follow up study: preliminary data

Methods

In a subset of the previous group, blood samples were also collected at T1. In particular, 20 BD patients in active phases (9 in manic and 11 in depressive phases) from the T0 sample were re-evaluated during the euthymic phase (T1).

Immunological analyses were performed by using the same methodology as in our previous work (see Chapter III). Immunofluorescence analysis by flow cytometry was used to quantify circulating CD4+ and CD8+ T cell subpopulations (i.e., CD4+CD28+, CD4+CD28-, CD8+CD28+, and CD8+CD28- T cells). Finally, these immunological parameters were compared using Wilcoxon tests between T0 and T1 patients' groups (by means of SPSS), in order to detect potential longitudinal changes across the different phases of illness.

Results

The frequency of circulating CD8+CD28- T cells subset showed a significant increase ($Z=-2.203$; $p=0.028$) in BD patients passing from the active phases (T0) to euthymia (T1). No other significant results were detected. See **Figure 2**.

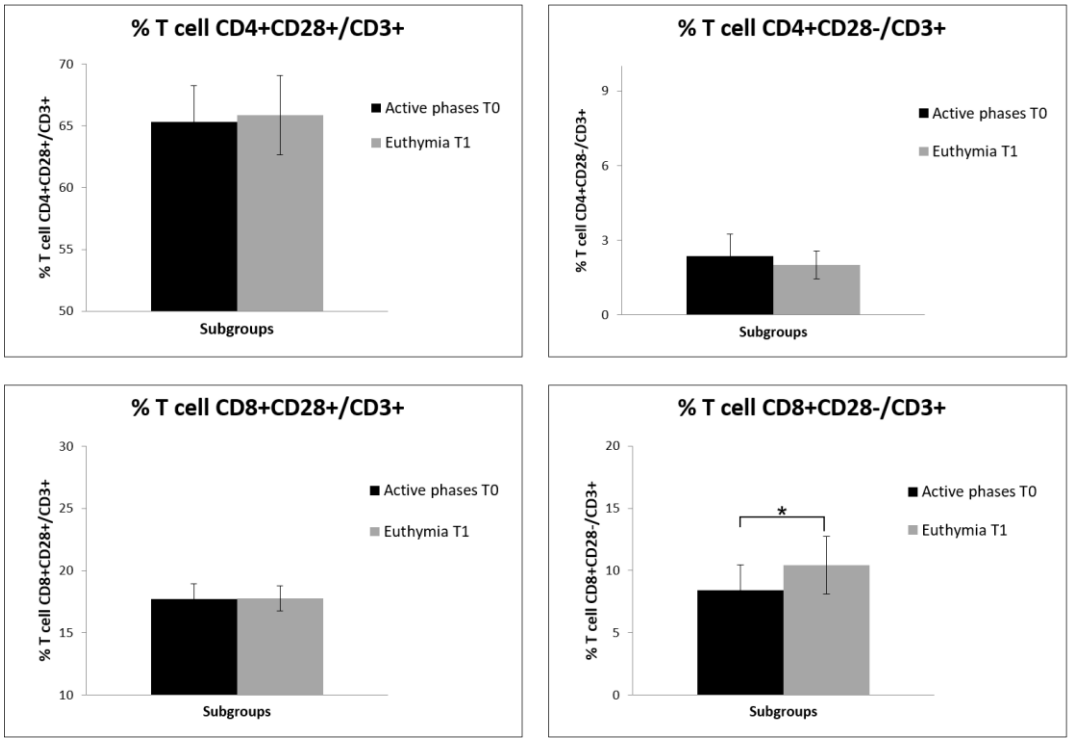


Figure 2. Differences of circulating T cell subsets in BD patients passing from the active phases to euthymia

Comparative analysis (Wilcoxon tests) on different CD4+ and CD8+ T cell subsets (i.e., CD4+CD28+ and CD4+CD28- T cells, as well as CD8+CD28+ and CD8+CD28- T cells), in BD patients passing from the active phases (T0) to euthymia (T1); $p<0.05$*.

Abbreviations: BD, bipolar disorder.

Discussion

Although preliminary, these results seem to confirm our previous findings in a longitudinal perspective, by showing increased FA/decreased RD in midline structures complemented by an increase in the circulating activated CD8+ T cell subsets, in BD patients passing from active phases to euthymia. These results might suggest that WM and immunological alterations occurring in mania and depression partially normalize in the euthymic phase. However, further work is needed to confirm and extend these preliminary data.

CHAPTER VI

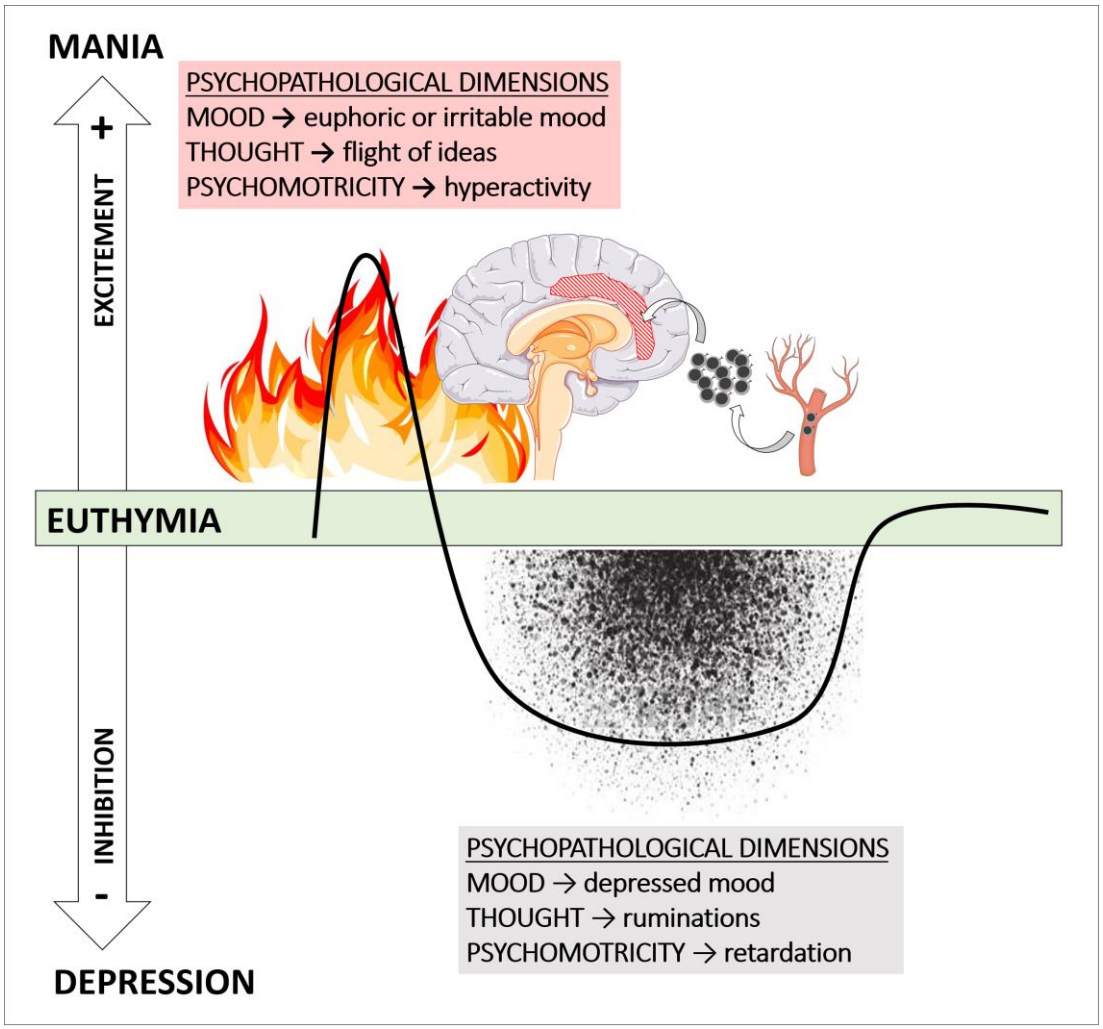
Epilogue – Is mania the fire of bipolar disorder?

Our work demonstrated consistent widespread WM microstructure alterations, mainly affecting the midline regions, in the active phases of BD, and in mania especially. Furthermore, we detected immune changes in mania again, characterized by increase in circulating early generated CD4+ T cells and reduction of activated CD8+ T lymphocytes, in turn correlating with WM alterations. These combined WM and immunological abnormalities of manic phase partially normalize in euthymia, as also confirmed in a longitudinal perspective. Finally, we observed similarities in WM microstructural alterations between mania and MS (but with different spatial distribution).

Collectively, these findings suggest a new pathophysiological model of mania. Accordingly, an immune response may occur in mania, resulting in an activation of CD8+ T cells that migrate in the brain, where exert their cytotoxic action, finally leading to WM damage.

In turn, WM damage could be related to functional brain abnormalities in BD. In our previous work on resting state fMRI data in BD, we have detected changes in the large-scale functional architecture of intrinsic brain activity in manic ad depressive phases, showing an opposing networks dysbalancing with a predominance of the sensorimotor network in mania while a predominance of the default mode network in depression [39, 41, 43, 51]. Differently from the absence of correlation between WM alterations and clinical symptoms, the functional alterations of intrinsic brain activity were found to be related to manic-depressive symptomatology. Moreover, recent data from our group suggested that such networks alterations may be traced to an abnormal modulation by neurotransmitters nuclei via subcortical-cortical loops. Composing the data, we suppose that a potential immune-mediated WM damage may lead to a functional disconnection of neurotransmitter areas, resulting in networks dysbalncing, which finally manifests in the psychopathological alterations of BD.

In conclusion, we hypothesize that manic-depressive symptomatology of BD may be related to alterations in the functional architecture of intrinsic brain activity which may be ultimately traced back to an immune-mediated WM damage. Our model thus supports a relationship between BD and immune-inflammatory neurological diseases such as MS. Moreover, our results suggest a prominent role of mania in BD and, interestingly, seem to be in accordance with the "primacy of mania" hypothesis, where mania is described as the *fire* of BD and seen as the core of the pathophysiology of the illness [5, 162]. Finally, according to our data, we could speculate that a potential immunotherapy that effectively target these specific and relevant immunological mechanisms, especially during the manic phase, could play a role in blocking the immune-mediated damage of WM, representing an important future aid in the treatment of BD.



Final schema. White matter and immunological alterations occurring in mania as potential biological basis for the pathophysiology of BD and the primacy of mania hypothesis.

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