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Research paper

Altered oxidative neurometabolic response to methylene blue in bipolar disorder revealed by quantitative neuroimaging

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ABSTRACT

Background: Cerebral mitochondrial and hemodynamic abnormalities have been implicated in Bipolar Disorder pathophysiology, likely contributing to neurometabolic vulnerability–leading to worsen clinical outcomes and mood instability. To investigate neurometabolic vulnerability in patients with BD, we combined multi-modal quantitative MRI assessment of cerebral oxygenation with acute administration of Methylene Blue, a neuro-metabolic/hemodynamic modulator acting on cerebral mitochondria.

Methods: Fifteen euthymic patients with chronic BD-type 1, and fifteen age/gender-matched healthy controls underwent two separate MRI sessions in a single-blinded randomized cross-over design, each after intravenous infusion of either MB (0.5 mg/kg) or placebo. MRI-based measures of Cerebral Blood Flow and Oxygen Extraction Fraction were integrated to compute Cerebral Metabolic Rate of Oxygen in Frontal Lobe, Anterior Cingulate, and Hippocampus–implicated in BD neurometabolic pathophysiology. Inter-daily variation in mood rating was used to assess mood instability.

Results: A decrease in global CBF and $CMRO_2$ was observed after acutely administrating MB to all participants. Greater regional $CMRO_2$ reductions were observed after MB, in patients compared to controls in FL (mean = −14.2 ± 19.5 % versus 2.3 ± 14.8 %), ACC (mean = −14.8 ± 23.7 % versus 2.4 ± 15.7 %). The effects on $CMRO_2$ in those regions were primarily driven by patients with longer disease duration and higher mood instability.

Limitations: Sample size; medications potentially impacting on response to MB.

Conclusions: An altered neurometabolic response to MB, a mitochondrial/hemodynamic modulator, was observed in patients, supporting the hypothesis of vulnerability to neurometabolic stress in BD. Integrating quantitative imaging of cerebral oxygen metabolism with a mitochondrial-targeting pharmacological challenge could provide a novel biomarker of neurometabolic and cerebrovascular pathophysiology in BD.

Abbreviations: BD, Bipolar Disorder; MRI, Magnetic Resonance Imaging; MB, Methylene Blue; CBF, Cerebral Blood Flow; $CMRO_2$, Cerebral Metabolic Rate of Oxygen; FL, Frontal Lobe; ACC, Anterior Cingulate Cortex.

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1. Introduction

Bipolar Disorder (BD) is a common, chronic, and devastating mental illness. Despite the prophylactic effect of long-term pharmacological treatments, a significant proportion of patients continue to experience disabling residual symptoms such as mood instability, cognitive impairment, and progressive course of illness (Berk et al., 2011).

Converging data from neuroimaging and post-mortem studies suggest that BD pathophysiology implicates an inadequately compensated neurometabolic stress: this is indicated by evidence of impaired cerebral perfusion (Toma et al., 2018) and metabolism (Ketter et al., 2001; Wu et al., 2021); alterations of mitochondrial electron transport chain (ETC) components associated with increased tissue damage from oxidative stress (Andreazza et al., 2010; Konradi et al., 2004; Wang et al., 2009); and a shift in cerebral energy production from oxidative phosphorylation towards less-efficient glycolysis, corroborated by observation of brain accumulation of lactate (Andreazza et al., 2010; Dogan et al., 2018).

Predisposing and precipitant factors for BD are represented by dysregulated peripheral metabolism, systemic inflammation, substance abuse, sleeps deprivation, as well as manic hyperactive states intrinsically associated to increased excitatory neurotransmission (Campbell and Campbell, 2024). Together, these challenges place demands on the brain's energy supply (Berk et al., 2011; Brown et al., 2014; Du et al., 2018; Pinna and Colasanti, 2021; Yüksel and Öngür, 2010) and necessitate a neurometabolic reserve capacity that patients' brains, operating near the limits of their metabolic envelope, might be unable to provide.

The notion of BD neurometabolic vulnerability is evidenced by the observation of deficits in the frontal lobe's high energy phosphate reservoir systems (i.e., creatine kinase forward reaction), critical for ATP regeneration when energy demand exceeds the available metabolic supply (Du et al., 2018; MacDonald et al., 2006; Shi et al., 2012). Furthermore, cellular density and numbers of the metabolically vulnerable hippocampal parvalbumin GABAergic interneuron population are reduced in the post-mortem BD brain (Pinna and Colasanti, 2021), and patients' mitochondria exhibit an abnormal adaptation to metabolic stress (Naydenov et al., 2007).

The BD neurometabolic vulnerability characteristics appear to directly impact clinical outcomes: evidence from Positron Emission Tomography studies shows a pattern of prefrontal hypometabolism in patients relative to healthy controls in brain areas involved in affective regulation, such as the anterior cingulate cortex (Ketter et al., 2001; Toma et al., 2018). These neurometabolic alterations have correlated with greater severity of depressive symptomatology (Ketter et al., 2001; Wu et al., 2021). Similarly, elevated lactate levels, especially in the cingulate cortex, have been associated with acute mood episodes of any polarity (Dogan et al., 2018).

Neuroimaging studies in patients with BD indicated abnormalities in prefrontal cortical regions implicated in both automatic and voluntary emotional regulation, such as altered neural responses in ventromedial prefrontal cortical regions, abnormal activities in dorsal and ventral aspects of the anterior cingulate, as well as gray matter structural alterations in anterior cingulate and orbitofrontal cortex (Phillips et al., 2008). Key functional abnormalities observed in patients with BD involve the dysfunction of dopamine-modulated prefrontal circuitries, particularly ventromedial prefrontal and orbitofrontal cortical regions, which support reward processing (Phillips and Swartz, 2014).

Neurometabolic abnormalities have been suggested to underlie progression of disease, as patients at later stages presented more marked redox alterations, relative to patients at earlier stages (Andreazza et al., 2009). It has been hypothesized that neurometabolic alterations contribute to the progressive clinical worsening, loss of treatment response and shortening of inter-episode interval, observed in a subgroup of patients with neuroprogressive characteristics (Berk et al., 2011).

In vivo measure of neurometabolism can be obtained using quantitative Magnetic Resonance Imaging (MRI) to estimate the Cerebral Metabolic Rate of Oxygen ($CMRO_2$) from brain measures of Cerebral Blood Flow (CBF) and Oxygen Extraction Fraction (OEF) (Kety and Schmidt, 1948; Simpson et al., 2022; Stone and Blockley, 2017; Yablonskiy and Haacke, 1994).

In the present study we combined neurometabolic MRI measures with acute administration of a pharmacological neurometabolic challenge: for this we selected Methylthioninium chloride, known as Methylene blue (MB), a compound with high brain penetration that modulates mitochondrial functions through actions at the level of mitochondrial transport chain. MB action is complex and consists of pleiotropic effects following a hormetic dose-response. Specifically, at low doses MB is proposed to act as an alternative electron carrier, accepting electrons from Complex I and transferring them directly to Complex IV. Thus, MB suppresses mitochondrial electron leakage and, in turn, reduces the production of reactive oxygen species (Alda, 2019; Atamna et al., 2012; Atamna et al., 2008). Furthermore, MB inhibits nitric oxide-induced vasodilation via blockade of guanylyl cyclase activation and nitric oxide synthase (NOS) enzymes, affecting cerebral blood flow and perfusion. Recent in vivo imaging indicated that intravenous (IV) MB administration dose-dependently reduced cerebral perfusion and metabolic rates in both rodents and humans (Singh et al., 2023), in contrast with earlier observations in rats (Huang et al., 2013; Lin et al., 2012), and in line with its NOS-inhibition effect.

By combining the use of an experimental neurometabolic challenge with brain metabolic measures acquired in vivo through MRI, we could obtain a more precise mechanistic understanding of neurometabolic vulnerability in BD. Furthermore, we could identify a novel biomarker of altered neurometabolism, potentially being used as a treatment endpoint for the development of novel mitochondrial-targeting treatments for BD. It is possible that due to underlying neurometabolic alterations in patients with BD, resulting from compromised perfusion and mitochondria dysfunction, the response to MB would likely result in a more evident reduction in cerebral metabolic measures than in healthy subjects.

2. Methods and materials

2.1. Participants

Patients with a DSM-5 diagnosis of BD-type 1 and age/gender-matched healthy controls (HCs) were recruited from mental health services (Sussex Partnership NHS Foundation Trust, South London and Maudsley NHS Foundation Trust), as well as through social networks (i.e., Facebook), peer support groups (Bipolar UK), and the universities of Sussex and Brighton. Patients were euthymic and had a chronic and active disease–chronic, being defined by duration of illness from first mood episode (i.e., the onset of overt affective symptoms recognized by patients and documented on their clinical notes) longer than 5 years; active, as demonstrated by at least one manic or depressive episode documented in the past 12 months. They did not have any current psychiatric comorbidities or medical conditions, nor any comorbid substance abuse or substance use disorder, and had no change in medications over the previous six weeks. HCs had no current or previous psychiatric or medical conditions, including no comorbid substance abuse or substance use disorder.

All participants were 18–60 years inclusive. Exclusion criteria included contra-indications for undergoing an MRI and for administration of MB, such as deficiency of glucose-6-phosphate dehydrogenase (G6PD), pregnancy, serotonergic medications. All participants had capacity to consent and gave their informed consent before any trial-related activity.

Ethical approval was obtained from the Brighton and Sussex Medical School Research Governance and Ethics Committee (19/ES/0018) and was in accordance with the Declaration of Helsinki.

2.2. Study design and procedures

Participants underwent two separate MRI sessions following a single-blinded randomized cross-over design, approximately 30 min each after an IV infusion of either 0.5 mg/kg MB (ProveBlue, Martindale Pharmaceuticals Ltd.), or a placebo solution (50 ml of glucose solution 5 %). MRI sessions took place between 11 am-5 pm, ensuring approximately the same timeframe for each participant on each session (mean difference = 32 ± 97 min).

The dose was based on a previous study showing similar effects to 1 mg/kg on brain metabolism (Singh et al., 2023). It is lower than the doses used for clinical application, and clinical recommendations were followed for its dilution and use (Joint Formulary Committee, 2024; Martindale Pharma, an Ethypharm Group Company, 2023).

Placebo (PLA) and MB conditions followed identical procedures, consisting of IV administration through a syringe pump with identical infusion rate. Participants could not see the syringe pump and were unaware of the blue color of MB solution being administered.

Clinical assessments included diagnostic interviews, neuropsychological tests and rating scales to assess depression and mania (see Supplementary Materials). Positive and Negative Affect Schedule (PANAS) (Watson et al., 1988) was used daily for seven days prior to MRI scan to assess mood instability as daily variation scores.

Blood samples were collected at screening and on both sessions before infusion to assess hemoglobin, hematocrit, and mean corpuscular hemoglobin concentration. Plasma glucose and Body Mass Index (BMI) were assessed at screening. Additionally, blood pressure, electrocardiogram (ECG) and heart rate were monitored pre-/post-infusions (Table S1). All participants underwent continuous ECG monitoring during the infusion, to monitor for any cardiovascular adverse events.

For further details on study design/procedures, and physiological measures, and assessment scales, refer to Supplementary Materials.

2.3. MRI acquisition, image processing and analysis

MRI data were acquired on a Siemens Prisma 3 T scanner equipped with a manufacturer provided 32-channel head coil.

For each participant, the following images were acquired on both sessions: an anatomical T1-weighted Magnetization-Prepared Rapid Gradient Echo (MPRAGE), used for segmentation and spatial normalization purposes; baseline CBF acquired with pseudo-Continuous Arterial Spin Labelling (pCASL), using the parameter recommendation of the consensus paper for this population (Alsop et al., 2015); and OEF, derived from the reversible transverse relaxation rate (R_2) acquired via a streamlined quantitative blood oxygenation level dependent (sqBOLD) sequence (Simpson et al., 2022).

$CMRO_2$ was derived using the approach illustrated in Singh et al. (Singh et al., 2023). Briefly, the CBF and OEF images, which are acquired quasi-simultaneously, are integrated into a single $CMRO_2$ image using the Fick's principle (Kety and Schmidt, 1948) as outlined in Yablonskiy et al. (Yablonskiy et al., 2013).

For full details of MRI acquisition and image processing and analysis, refer to Supplementary Materials.

2.4. Regions of interest (ROIs)

Prefrontal Cortex (PFC), Anterior Cingulate Cortex (ACC), Hippocampus (HPC) were selected as ROIs based on their relevance to BD neurometabolic pathophysiology as evidenced by post-mortem mitochondrial ETC abnormalities reported in these three regions (Andreazza et al., 2010; Konradi et al., 2004; Wang et al., 2009), and Magnetic Resonance Spectroscopy-derived lactate elevation reported in ACC (Machado-Vieira et al., 2017).

ROIs were identified using the Mindboggle atlas (Klein and Tourville, 2012). For each ROI, only voxels surviving the threshold for gray matter (GM) tissue content>80 % were included.

CBF and OEF images were normalized into MNI space and for each participant, an ROI-wise averaged OEF (rOEF) and CBF (rCBF) was computed for each session. These values were then used to estimate regional $CMRO_2$ ($rCMRO_2$) as described above.

In addition to the 3 ROIs, whole-brain GM CBF, OEF, and $CMRO_2$ were also computed to test for global effects of MB.

2.5. Statistics

Statistical analyses were performed with IBM SPSS Statistics (version 29.0.1.0). An a-priori value of $p = 0.05$ was used as threshold for statistical significance. All data are reported as mean ± SD.

Mood instability was obtained from the inter-daily coefficient of variation of PANAS positive/negative scores (PANAS Cv). Mood instability and Disease Duration were binarized into low/high mood instability and short/long disease duration groups, respectively, using median scores (26 and 24 years, respectively) as cut-off.

Between-groups comparisons of continuous demographic variables, baseline hematological parameters, physiological measures, neuropsychological tests, and baseline imaging measures were done using two-tailed *t*-tests for independent samples. Analysis of variance (ANOVA) with Least Significance Difference for post-hoc corrections, was used for comparisons between>2 groups. Between-groups difference in gender distribution was studied using Chi-Square.

We used the Pearson's *r* correlation coefficient for the study of correlation between cognitive measures and baseline imaging measures.

The effects of Treatment (MB versus PLA control condition) and Treatment × Group interactions, on clinical and imaging variables were studied using repeated measures ANOVA.

Any test involving Disease Duration was corrected for age adding it as covariate.

As exploratory tests, repeated measures ANOVA of Treatment × Group interaction on $CMRO_2$ was repeated after adding Glucose and BMI as covariates.

A paired sample Student's t-test was used to test the association of the randomization order with imaging measures.

Correction for multiple comparisons using the False Discovery Rate (FDR) was conducted for all tests involving analysis of imaging measures across the three ROIs. Statistical significance is expressed as $p_{corr} < 0.05$ if survived FDR correction, and $p_{uncorr} < 0.05$ if it does not remain significant after FDR correction.

3. Results

3.1. Demographics, physiological, neuropsychological, and clinical measures

Of 46 recruited participants, only 15 patients with BD and 15 HCs completed the study (BD: 3 = ineligible, 9 = withdrawn; HCs: 2 = ineligible, 2 = withdrawn). The sociodemographic and clinical characteristics of all included participants are presented in Table 1.

There was no difference in age/gender distribution between patients and HCs, nor in plasma glucose or BMI. Education, qualification, employment status and premorbid neuropsychological functions were similar between groups (Table 1). At screening and scanning sessions, physiological measures did not differ between patients and controls (see Supplementary Materials).

Performance at cognitive tests administered at screening also did not differ between groups, although patients exhibited a trend-level worse performance in the Rey Auditory Verbal Learning Test (RAVLT), relative to HCs ($p = 0.091$).

At screening, all patients were euthymic, as indicated by clinical assessment scales scoring well below validated cut-off scores. Twelve out of 15 participants were on prophylactic mood stabilizing treatment. The time-delay between first affective episode in patients and diagnosis was 13 ± 10.9 years.

Table 1
Demographic, physiological, cognitive, and clinical parameters at screening.

Screening parameters	Patients with BD (n = 15) [mean ± SD]	HCs (n = 15) [mean ± SD]	*p*
Demographics			
Age	41.4 ± 11	37 ± 9.7	>0.05
Sex, Male/Female	5/10	5/10	
Education, years[a]	16.38 ± 4.4	18.67 ± 3.16	
Qualifications			
	9 (60 %)	12 (80 %)	
- Degree, n (%)	6 (40 %)	3 (20 %)	
- Other, n (%)			
Employment			
	9 (60 %)	11 (73 %)	
- Employed, n (%)	6 (40 %)	4 (27 %)	
- Unemployed/other, n (%)			
Physiological measures			
Weight (kg)	78.07 ± 13.94	77.33 ± 21.66	>0.05
Height (cm)	167 ± 8.51	170.47 ± 13.56	
BMI	28.11 ± 5.33	26.39 ± 5.69	
Plasma Glucose (mmol/L)	5.01 ± 0.84	4.77 ± 0.53	
Hb (g/L)	142.27 ± 8.75	140.73 ± 11.52	
Hct (L/L)	0.44 ± 0.04	0.43 ± 0.03	
MCHC (g/L)	327.47 ± 12.63	325.20 ± 13.34	
Systolic BP (mmHg)	125.2 ± 12.59	119.43 ± 10.57	
Diastolic BP (mmHg)	79.8 ± 11.17	75.07 ± 7.58	
Heart rate (bpm)	72.13 ± 10.24	67.73 ± 10.17	
QTc (msec)	401.14 ± 16.60	400.21 ± 32.64	
PR (msec)	146.43 ± 23.03	156.71 ± 22.97	
Cognitive measures			
TMT-B (sec)	78.87 ± 42.16	66.6 ± 28.63	>0.05
NART (predicted WAIS-IV, FSIQ)	111.1 ± 11.17	110.84 ± 4.87	
RAVLT (mean)	19.07 ± 2.13	20.65 ± 2.78	

	Patients with BD (mean ± SD)
Clinical characteristics	
First episode (age)	17.33 ± 6.37
Diagnosis (age)	30.33 ± 9.612
Duration of disease	24.13 ± 11.66
Pharmacotherapy (n)	12
	5 (41.6 %)
- SGA, n (%)	2 (16.6 %)
- Lithium, n (%)	1 (8.3 %)
- Anti-epileptic, n (%)	4 (33.3 %)
- Other[b], n (%)	
HDRS17 (screening)	3.13 ± 2.70
YMRS (screening)	2 ± 2.48
PANAS (Positive)	31.21 ± 5.38
PANAS (Negative)	15.64 ± 7.2
PANAS C_V (Positive)	0.17 ± 0.12
PANAS C_V (Negative)	0.19 ± 0.11
PANAS Total C_V	0.31 ± 0.21

BD, Bipolar Disorder; HCs, Healthy Controls; BMI, Body Mass Index; TMT-B, Trial Making Test B; NART (predicted WAIS-IV, FSIQ), National Adult Reading Test (predicted Wechsler Adult Intelligence Scale 4th Edition, Full Scale Intelligent Quotient); RAVLT, Rey Auditory Verbal Learning Test; Hb, hemoglobin; Hct, hematocrit; MCHC, mean corpuscular hemoglobin concentration; BP, blood pressure; SGA, Second Generation Antipsychotics; HDRS17, 17 item-Hamilton Rating Scale-Depression; YMRS, Young Mania Rating Scale; PANAS, Positive and Negative Affect Schedule; Cv, coefficient of variation.

[a] Range is 6 (primary school), 5 (secondary school), 2 (further education college), >13 overall (higher education).

[b] Combination of one antipsychotic with one antiepileptic (n=3); combination of one antipsychotic with lithium (n=1).

3.2. Effect of MB on clinical variables

There were neither significant MB effects on clinical measures, nor effect of the interaction between groups and condition on any clinical measures (Table 2). Furthermore, patients did not differ in clinical presentation, between MB versus placebo (Table 2).

Table 2
Clinical measures during scanning sessions.

Scanning sessions parameters	Patients with BD (mean ± SD)		HCs (mean ± SD)		*p*
Clinical measures	*Delta PLA*	*Delta MB*	*Delta PLA*	*Delta MB*	
VAS Energy (score 0–10)	−2.50 ± 14.77	−6.07 ± 20.59	0.15 ± 13.61	−0.54 ± 18.99	>0.05
VAS Mood (score 0–10)	−0.21 ± 11.33	−0.86 ± 9.70	2.46 ± 5.95	3.08 ± 6.63	

	Patients with BD (mean ± SD)		***p***
Clinical measures	*PLA*	*MB*	
HDRS17	3.13 ± 3.25	2.87 ± 2.64	
YMRS	1.40 ± 1.24	1.47 ± 2	
PANAS (Positive)[a]	3.6 ± 7.54	29.93 ± 7.13	>0.05
PANAS (Negative)[a]	13.67 ± 3.48	14.27 ± 5.89	

BD, Bipolar Disorder; HCs, Healthy Controls; Delta PLA, difference between after and before PLA sessions; Delta MB, difference between after and before MB sessions; VAS, visual analogue scale; PLA, placebo; HDRS17, 17 item-Hamilton Rating Scale-Depression; YMRS, Young Mania Rating Scale; PANAS, Positive and Negative Affect Schedule.

[a] Daily measure a week prior to the first scanning session.

For details on MB effect on physiological measures, refer to Supplementary Materials.

3.3. Imaging measures

3.3.1. Post-placebo (i.e., basal) measures

We observed no differences in global or regional CBF/OEF/$CMRO_2$ values after placebo between HCs and patients as a whole (Supplementary Table S2). However, when subdividing patients into short/long disease duration groups (median = 24 years)–after accounting for any age difference, patients with longer disease duration had higher $rCMRO_2^{PLA}$ in ACC relative to HCs (p_{uncorr} = 0.033) (Fig. 1). Similarly,

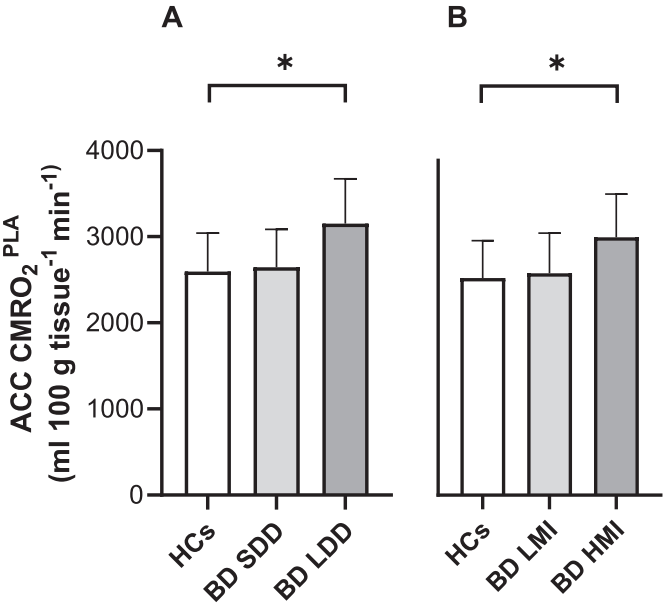

Fig. 1. Baseline $CMRO_2$ differences in ACC for disease duration (A) and mood instability (B) between HCs and subgroups of patients with BD. Patients with long disease duration (LDD) and high mood instability (HMI) had higher $CMRO_2$ in ACC relative to HCs.
SDD, short disease duration; LMI, low mood instability; *, *p* significant, but not surviving correction for multiple comparisons.

after dividing patients into low/high PANAS Cv groups (median = 0.26), patients with higher mood instability displayed higher $rCMRO_2^{PLA}$ in ACC relative to controls (p_{uncorr} = 0.026). Neither of these findings survived correction for multiple comparisons. Differences within patient subgroups (i.e., short versus long disease duration, and low versus high PANAS Cv), as well as between short disease duration/low PANAS Cv subgroups and HCs, did not reach significance (Fig. 1).

rCBF and rOEF in ACC did not differ between either short/long disease duration or low/high mood instability subgroups, and HCs.

Post-placebo imaging measures in other ROIs are reported in the Supplementary Material, Table S2.

3.3.2. Effects of MB

The acute administration of MB was associated to a global reduction in GM $CMRO_2$ across the whole sample of patients and HCs ($CMRO_2^{PLA}$ = 3.57 ± 0.65 $ml^{100\ g\text{-}1\ min\text{-}1}$, $CMRO_2^{MB}$ = 3.33 ± 0.67 $ml^{100\ g\text{-}1\ min\text{-}1}$, p = 0.043). This effect was driven by a reduction in GM CBF (CBF^{PLA} = 56.80 $ml^{100\ g\text{-}1\ min\text{-}1}$, CBF^{MB} = 51.88 $ml^{100\ g\text{-}1\ min\text{-}1}$, p = 0.005), whilst there was no change in OEF associated with the administration of acute MB.

At the ROI-level, we observed an effect of the interaction between groups and treatment conditions on $rCMRO_2$ in PFC (p_{corr} = 0.010) and ACC (p_{uncorr} = 0.044), whilst it did not reach significance in HPC (p = 0.071). After repeating the tests with BMI and Glucose added as covariates, the effect of Treatment × Group interaction was significant and survived multiple comparisons correction in all regions (PFC, p_{corr} = 0.001; ACC, p_{corr} = 0.024; HPC, p_{corr} = 0.035). These results suggest a greater reduction of $rCMRO_2$ after MB compared to placebo, in patients relative to HCs (Fig. 2). In patients, MB reduced $rCMRO_2$ of −14.2 ± 19.5 % in PFC (versus 2.3 ± 14.8 % in HCs), −14.8 ± 23.7 % in ACC (versus 2.4 ± 15.7 % in HCs), and -12.8 ± 20.9 % in HPC (versus 3.3 ± 15.7 % in HCs). The effects on $CMRO_2$ corresponded to those on CBF, as we observed greater reductions in rCBF after MB in patients versus HCs in ACC (p_{uncorr} = 0.027), with PFC not reaching significance (p = 0.062). After repeating the tests with BMI and glucose added as covariates, the effect of the interaction between groups and treatment on rCBF was significant and surviving multiple comparison in ACC (p_{corr} = 0.009) and PFC (p_{corr} = 0.016).

No effect of interaction was observed between groups and MB treatment on rOEF in any ROI (Supplementary Materials, Table S2).

We then repeated the study of the interaction between groups and treatment conditions on $rCMRO_2$ and rCBF after dividing patients based on disease duration and mood instability: MB caused greater $rCMRO_2$ reductions in PFC (p_{corr} = 0.003) and ACC (p_{corr} = 0.010) in patients with higher disease duration (age-corrected) relative to HCs (Fig. 3), both surviving the multiple comparisons correction and both remaining significant after correction for BMI and glucose.

MB also induced greater reductions in $rCMRO_2$ in PFC (p_{uncorr} = 0.007) and ACC (p_{uncorr} = 0.047) in patients higher mood instability relative to controls. After correction for BMI and plasma glucose, the finding in the PFC, but not in ACC, survived correction for multiple comparisons (p_{corr} = 0.006) (Fig. 3).

Differences between disease duration and mood instability subgroups, as well as short disease duration/low mood instability subgroups and HCs, did not reach significance (Fig. 3).

No significant effect of interaction was observed between treatment and groups–divided according to disease duration or mood instability–on rCBF or rOEF.

None of the MB effects on $rCMRO_2$/rCBF/rOEF were associated with

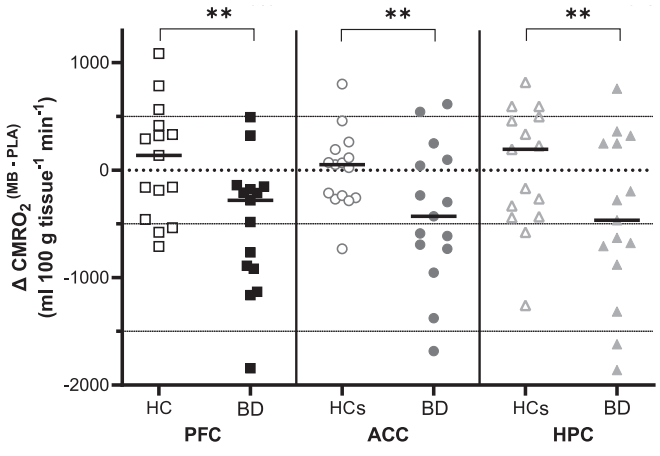

Fig. 2. MB effect on $rCMRO_2$ between groups. A significant reduction of $rCMRO_2$ was found in patients with BD relative to HCs, after adding BMI and Glucose as covariates.
**, p significant after correction for multiple comparisons.

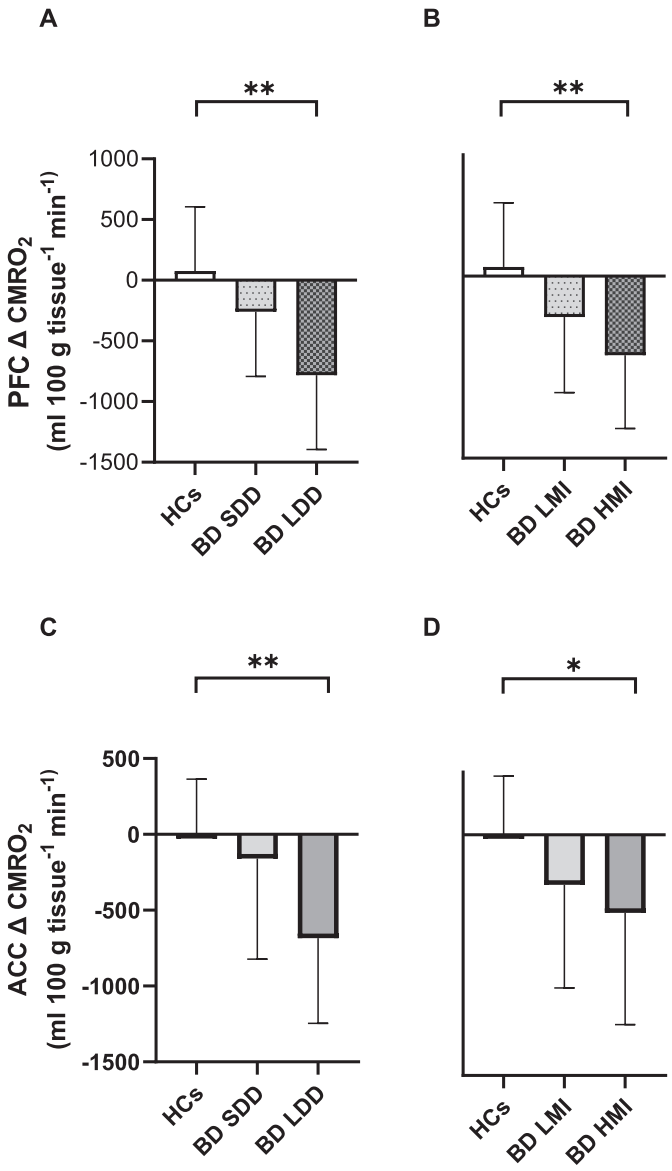

Fig. 3. $CMRO_2$ differences after MB in PFC and ACC for disease duration (A and C, respectively) and mood instability (B and D, respectively) between HCs, and subgroups of patients with BD. Patients with long disease duration (LDD) and high mood instability (HMI) had higher $CMRO_2$ in PFC and ACC relative to HCs, after adding BMI and Glucose as covariates.
SDD, short disease duration; LMI, low mood instability; ** p, significant after correction; * p, significant–not after correction.

any clinical rating scales, or performance on cognitive tests.

Furthermore, no significant effect of medications were found on the interaction between treatment and groups for any imaging measures, and no interaction was observed between the effect of treatment order (MB versus placebo) and any imaging measures.

4. Discussion

The main finding of this study was an altered response of $CMRO_2$ to the mitochondrial and cerebrovascular modulator MB, in patients with BD compared to HCs, in all the regions of interest. The finding survived statistical threshold for multiple comparisons and remained significant after correction for BMI and plasma glucose, suggesting that the observed effect reflects alterations in cerebral metabolism independent from any potential peripheral metabolic effects. Given our hypothesis that the altered BD neurometabolic reserve has specific relevance to mechanisms underlying neuroprogression, we chose to focus our recruitment on patients who were euthymic at the time of scanning but had disease duration longer than 5 years and experienced a recent symptom relapse. We also explored whether brain measures of oxygen metabolism were associated with clinical variables related to neuroprogression, such as disease duration, mood instability or cognitive impairment (Cardoso et al., 2015; Chen et al., 2022; Chrobak et al., 2023; Jones et al., 2022).

A separate study recently found a global $CMRO_2$ reduction induced by MB in both rodents and healthy human volunteers (Singh et al., 2023). This study replicates the observation of a whole brain $CMRO_2$ reduction induced by MB, expands it by focusing on effects on a regional level, and reports a greater regional reduction of cerebral oxygen metabolism by MB in patients with BD relative to non-affected participants. This is a novel, and we believe, important finding, consistent with other studies reporting an impaired response to other metabolic challenges in patients (Du et al., 2018; Yuksel et al., 2015). The observed differences between patients with BD and unaffected controls, were driven by patients with a longer disease duration and higher mood instability, supporting the idea that neurometabolic alterations in BD correspond to neuroprogressive pathophysiology.

The effect of MB on cerebral oxygen metabolism likely implicates two separate mechanisms: first, MB acts as an alternate electron donor at mitochondrial ETC level (Atamna et al., 2008), and second, MB reduces cerebral perfusion and oxygenation due to its inhibition of nitric oxide (NO) pathways (Saha and Burns, 2020).

Our observation of an altered response to MB in patients might involve both mechanisms. Firstly, mitochondrial ETC alterations, consistently observed in patients brain might alter their mitochondrial response to MB. These alterations include lower levels of a specific subunit of Complex I, namely NDUFS7, and an overall decreased activity of ETC complex I associated with increased protein oxidation and nitration of ETC components, particularly in the prefrontal cortex (Andreazza et al., 2010), and lipid peroxidation in cingulate cortex (Wang et al., 2009). Also, hippocampal mitochondrial ETC proteins were found to be defective due to a reduced expression of nuclear messenger RNA, resulting into a wider decrement of genes expression regulating oxidative phosphorylation and proteasome degradation induced by ATP (Konradi et al., 2004).

Secondly, the inhibiting action on the NO pathways could have had a greater effect in patients. NO contributes to regulate vascular tone, both at a macro-/micro-level (Carter et al., 2021). MB reduces NO production inhibiting both the soluble guanylate cyclase and all isoforms of NOS (Saha and Burns, 2020). In patients, systemic vascular impairment and endothelial dysfunction have been reported (Mio et al., 2023; Rybakowski et al., 2006; Toma et al., 2018), potentially altering in turn NO levels. Indeed, altered serum levels of NO metabolites–associated with a specific allele of *NOS1* gene encoding neuronal NO-synthase (nNOS) (Freudenberg et al., 2015; Kittel-Schneider et al., 2015; McNeill et al., 2022), and a reduced nNOS activity itself (Fontoura et al., 2012), have been reported in BD.

Overall, impairment in NO signaling in BD may also explain the finding of altered cerebral perfusion demonstrated in symptomatic patients regardless of mood episode polarity (Toma et al., 2018).

It is possible that in BD, cerebrovascular regulation is impaired and the maintenance of CBF relies particularly on the integrity of NO pathway, similarly to observations in ageing where NO pharmacological inhibition causes larger CBF reduction than in younger healthy brains (Kamper et al., 2004). Our finding of a greater effect of MB on $CMRO_2$, mostly driven by effect on CBF, is consistent with that observation, and with other reports indicating similarities between the pathophysiological alterations reported in BD and those observed in the ageing process (Kamper et al., 2004; Van Der Markt et al., 2023; Villa et al., 2023; Zovetti et al., 2023). Moreover, we observed that a reduction of CBF in patients was not compensated by an increase in OEF, resulting in an overall reduction in $CMRO_2$. This might reflect an inefficient compensatory response to reduced oxygen supply, in line with the findings suggestive of an altered neurovascular coupling in adolescents with BD (Karthikeyan et al., 2019). Neurovascular coupling varies between brain regions and as a function of neuronal stimuli (Fox et al., 1988; Fox and Raichle, 1986; He et al., 2019; Karthikeyan et al., 2019; Peng et al., 2014; Seitz and Roland, 1992; Shaw et al., 2021), and its alteration might confer vulnerability to neurodegeneration (He et al., 2019; Karthikeyan et al., 2019; Peng et al., 2014; Shaw et al., 2021).

The other major findings of our study, which survived statistical threshold for multiple comparisons, were the observation of higher baseline $rCMRO_2$ and greater MB-induced $rCMRO_2$ in prefrontal and cingulate areas in patients with a longer disease duration. These findings complement evidence that the duration of a single mood episode of any polarity is associated to greater cerebral blood flow, indicative of a greater brain oxygen supply (He et al., 2019; Wang et al., 2018). Extrapolating this observation made at a single episode level, we hypothesize that there is an analogue relationship between cerebral oxygen demands and the overall illness duration, which might reflect a relationship between neural activation–occurring during the characteristic emotional dysregulation–and the course of BD (He et al., 2019). Assuming that the brain tissue in patients with BD is working at the limit of its metabolic envelope, it is reasonable to expect that a longer duration of disease might be associated with enhanced response to the acute effects of MB, reflecting a reduced neurometabolic reserve capacity, and higher inability to provide an adequate metabolic response at times of high energy demand (Du et al., 2018).

An increase in cerebral metabolism–and thus oxygen consumption–has also been found in the ageing brain, possibly resulting from adaptation to neurodegenerative processes such as a loss of tissue volume or a reduction of efficiency of neural cellular processes–leading in turn to compensatory hypermetabolism and increased oxygen utilization to sustain the same amount and efficiency of neural processing (Peng et al., 2014).

Interestingly, our results of increased oxygen metabolism after longer illness duration may indicate compensatory mechanisms that are similar to those seen in the ageing process. However, we had corrected those results for age, showing their independence in patients from the ageing process itself. These findings, taken together, support the hypothesis that the bipolar brain undergoes accelerating ageing processes (Van Der Markt et al., 2023; Zovetti et al., 2023).

We also found higher baseline $rCMRO_2$ and higher $rCMRO_2$ response to MB to correspond to higher mood instability, but this finding survived correction for multiple comparisons only in the PFC. It is plausible that alterations in neural excitatory/inhibitory balance are associated with mood instability (Pinna and Colasanti, 2021), and that our finding might also reflect the increased neurometabolic demands resulting from an unstable excitatory pattern of neurons in the BD brain (Stern et al., 2020). Therefore, it is reasonable to hypothesize that the observed increase in oxygen metabolism, and the observed altered neurometabolic response to an acute metabolic challenge, might be both a predisposing

factor and a consequence of mood instability.

Lastly, we did not observe a correspondence between neurometabolic alterations and performance in cognitive tests, although we found across the whole sample a negative correlation between National Adult Reading Test (NART) and both $rCMRO_2^{PLA}$ and $rCBF^{PLA}$, and a positive correlation between RAVLT and $rCBF^{PLA}$, although both were not significant after multiple comparison correction. An inverse association between premorbid intelligence and both blood flow and the overall oxygen consumption is consistent with previous studies (Scarmeas et al., 2023). On the other hand, verbal learning and memory have been consistently reported as associated with a higher blood flow (Ragland et al., 2000).

To the best of our knowledge this is the first in-vivo neuroimaging study reporting an altered response to a mitochondrial modulator compound in patients with BD. Though providing novel and important data, some of our findings are to be considered preliminary when they did not survive correction for multiple comparisons. Our study presents with some limitations, such as a small sample size which was secondary to strict inclusion criteria (e.g., BD-type 1, at least 5 years after the first mood episode, with active episode(s) within the past year, on no serotonergic medications due to potential severe interactions with MB); as a result of this, we were unable to account for the effect of mood stabilizing pharmacological treatment on the neurometabolic response to MB. Since a longer disease duration might be associated with longer exposure to pharmacological treatment, this might have also had a confounding effect on the observed interactions between disease duration and MB effect. Future studies could investigate whether the neurometabolic response to a mitochondrial modulator such as MB is differentially impacted by specific treatments with known profile of mitochondrial effects (Rosell-Hidalgo et al., 2023). Furthermore, it is possible that participants were unblinded to the order of conditions of some participants who had noticed coloring of urine after MB administration–however, neither did we see an effect of the order of conditions, nor did we notice an interaction between randomization order and any of the main effects reported.

Interpretation of our results is complicated by the complexity of MB action, including its hormetic and pleiotropic effects. Our observation of MB reducing CBF and $CMRO_2$ is consistent with findings in healthy humans (Rodriguez et al., 2016; Singh et al., 2023) and rodents, although prior preclinical studies had reported that MB enhances oxygen metabolism in vitro (Atamna et al., 2008) and in vivo in rats (Atamna et al., 2008; Huang et al., 2013; Lin et al., 2012). Interestingly, six months adjunctive treatment with MB improved depressive symptoms in patients with BD (Alda et al., 2017).

A final comment relates to the multimodal and non-invasive nature of our MRI measure of $CMRO_2$, which derives from the integration of three separate measures (i.e., CBF, OEF, arterial oxygen content (Ca)) (Simpson et al., 2022). The advantage of our method is that it enables the study of regional distribution of the effects, not relying on CO_2 administration which can be panicogenic in vulnerable patients, such as patients with BD (Colasanti et al., 2012). However, $CMRO_2$ was derived from other physiologically relevant measures rather than directly measured. The observed effects on $CMRO_2$ were driven primarily by effects on CBF, whilst OEF and Ca were not altered between patients and HCs, and were not affected by MB.

In conclusion, our findings suggest that patients with BD present an altered neurometabolic response to a neurometabolic challenge acting at a cerebral mitochondrial level. The response we observed might reflect the presence of altered brain mitochondrial machinery in BD (Andreazza et al., 2010; Konradi et al., 2004; Wang et al., 2009) or systemic vascular impairment (Goldstein, 2017), or both.

Our findings of a larger effect of MB and of increased cerebral oxygen utilization in patients with longer disease duration and mood instability are both relevant to the concept of neuroprogression in BD, which is currently debated (Strejilevich et al., 2023). Overall, our study provides novel insight into the relationship between neurometabolism, cerebrovascular regulation and BD pathophysiology. Furthermore, it provides proof-of-concept support for employing a multimodal neuroimaging measure of $CMRO_2$ integrated with administration of a mitochondrial modulator, to develop a novel biomarker of neurometabolic pathophysiology in BD, and of response to treatments targeting cerebral mitochondria. Future studies on a wider population of patients with BD are warranted to replicate the results gathered in our study and expand them to other regions of the brain.

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CRediT authorship contribution statement

Alfonso Russo: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Balázs Örzsik:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Nefize Yalin:** Writing – review & editing, Methodology, Conceptualization. **Ivor Simpson:** Writing – review & editing, Formal analysis, Data curation. **Prince Nwaubani:** Writing – review & editing, Investigation. **Antonello Pinna:** Writing – review & editing, Investigation, Data curation. **Riccardo De Marco:** Writing – review & editing, Investigation. **Harriet Sharp:** Writing – review & editing, Investigation. **Amy Kartar:** Writing – review & editing, Investigation, Data curation. **Nisha Singh:** Writing – review & editing, Methodology, Conceptualization. **Nicholas Blockley:** Writing – review & editing, Methodology, Conceptualization. **Alan John Luke Stone:** Writing – review & editing, Methodology, Conceptualization. **Federico E. Turkheimer:** Writing – review & editing, Methodology, Conceptualization. **Allan H. Young:** Writing – review & editing, Methodology, Conceptualization. **Mara Cercignani:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Fernando Zelaya:** Writing – review & editing, Methodology, Conceptualization. **Iris Asllani:** Writing – review & editing, Formal analysis. **Alessandro Colasanti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have nothing to disclose.

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The article by Simpson et al. (Simpson et al., 2022) has been posted on arXiv preprint server.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jad.2024.07.029.

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