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ORIGINAL ARTICLE

Analyses of peripheral blood dendritic cells and magnetic resonance spectroscopy support dysfunctional neuro-immune crosstalk in Tourette syndrome

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Abstract

Background: Evidence supports that neurodevelopmental diseases, such as Tourette syndrome (TS), may involve dysfunctional neural-immune crosstalk. This could lead to altered brain maturation and differences in immune and stress responses. Dendritic cells (DCs) play a major role in immunity as professional antigen-presenting cells; changes in their frequency have been observed in several autoimmune conditions.

Methods: In 18 TS patients (15 on stable pharmacological treatment, three unmedicated) and 18 age-matched healthy volunteers (HVs), we explored circulating blood-derived DCs and their relationship with clinical variables and brain metabolites, measured via proton magnetic resonance spectroscopy (1H-MRS). DC subsets, including plasmacytoid and myeloid type 1 and 2 dendritic cells (MDC1, MDC2), were studied with flow cytometry. 1H-MRS was used to measure total choline, glutamate plus glutamine, total creatine (tCr), and total N-acetylaspartate and N-acetylaspartyl-glutamate levels in frontal white matter (FWM) and the putamen.

Results: We did not observe differences in absolute concentrations of DC subsets or brain inflammatory metabolites between patients and HVs. However, TS patients manifesting anxiety showed a significant increase in MDC1s compared to TS patients without anxiety ($p = 0.01$). We also found a strong negative correlation between MDC1 frequency and tCr in the FWM of patients with TS ($p = 0.0015$), but not of HVs.

Conclusion: Elevated frequencies of the MDC1 subset in TS patients manifesting anxiety may reflect a proinflammatory status, potentially facilitating altered neuro-immune crosstalk. Furthermore, the strong inverse correlation between brain tCr levels and MDC1 subset frequency in TS patients suggests a potential association between proinflammatory status and metabolic changes in sensitive brain regions.

KEYWORDS
anxiety, dendritic cells, inflammation, spectroscopy, Tourette syndrome

INTRODUCTION

Tourette syndrome (TS) is a childhood-onset neurodevelopmental disorder characterized by the coexistence of motor and phonic tics. Approximately 90% of TS patients manifest one or more neurodevelopmental and psychiatric comorbidities, in particular attention deficit/hyperactivity disorder (ADHD), obsessive–compulsive disorder (OCD), anxiety, and depression [1]. Evidence supports dysfunctional

neuro-immune crosstalk in TS and other neurodevelopmental disorders, such as autism and ADHD, which might contribute to abnormalities in the trajectory of the development of cortico-basal ganglia and cortico-cortical connections [2,3]. Microglia, the brain-resident mononuclear phagocytic cells, are thought to play a central role in these interactions. Transcriptomic studies revealed an association between microglial hyperactivation and dysfunction and TS [4,5]. Population-based epidemiological and genome-wide association studies converge in demonstrating the co-occurrence and genetic correlation of TS with highly prevalent autoimmune and allergic conditions [6,7]. At a systemic level, patients with TS have shown dys- or hyperregulated cell-mediated proinflammatory responses suggestive of an 'inflammatory' state, as well as altered distribution of some immune regulatory cell types (e.g., T-regulatory lymphocytes), consistent with predisposition to autoimmunity. Finally, active immunization by direct injection of cytokines or patients' serum anti-neuronal antibodies replicated TS-like behaviours in mice [8-10].

Circulating peripheral blood dendritic cells (DCs) constitute a critical link between innate and adaptive immunity. They represent a heterogeneous population of professional antigen-presenting cells comprising three major DC subsets: plasmacytoid (PDCs), myeloid type 1 (MDC1), and myeloid type 2 (MDC2) [11]. DCs are implicated in the pathogenesis of numerous autoimmune conditions including multiple sclerosis (MS), psoriasis, type 1 diabetes and systemic lupus erythematosus [12]. An increased frequency of MDC1s was found in autistic children compared to typically developing controls, supporting DC-related immune dysfunction [13,14]. Circulating peripheral blood DC subsets and their relationship to neuroinflammation remain under-investigated in other neurodevelopmental disorders, including TS.

Amongst several applications to the study of brain metabolism, proton magnetic resonance spectroscopy (1H-MRS) has the potential to provide insight into *in vivo* neuroinflammatory changes through the quantification of different metabolites [15], as markers of neuronal or glial damage in selected brain regions [16]. For example, N-acetylaspartate changes were previously described in patients with neurological manifestations of lupus erythematosus [17,18], while choline and lactate compound abnormalities were linked to active inflammatory demyelination and neuronal injury in MS [19,20].

In the present study, we first investigated the frequency and distribution of circulating peripheral blood-derived DCs in TS patients, comparing them to age- and sex-matched healthy volunteers (HVs). Subsequently, we explored relationships between DC subsets and clinical severity of tics and comorbid behavioural symptoms, accounting for the potential influence of exposure to psychotropic medications. Finally, we aimed to investigate the relationship between brain metabolites associated with glial activation/inflammation obtained via quantitative MRS and peripheral blood DC frequency to determine whether this supports the hypothesis of active crosstalk between the central nervous system (CNS) and immune system in TS. Our primary hypothesis was that TS patients would exhibit an abnormal distribution of the different DC subsets, and that this abnormality would be greater in patients with a greater burden of behavioural comorbidities.

METHODS

Participants

Patients were recruited from the St George's University Hospital Tic Disorder and Movement Disorders clinic if they fulfilled Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) diagnostic criteria for TS and had received stable pharmacological treatment for the previous 3 months. HVs without neurological diagnoses were enrolled amongst patients' friends or partners. Exclusion criteria were: autoimmune disorders; ongoing acute/chronic infections; chronic obstructive pulmonary disease; malignancies and chronic endocrinological, cardiovascular, pulmonary, liver or kidney diseases; treatment with corticosteroids or immunosuppressant drugs within the previous 12 months. The study was approved by the London-Westminster Research Ethics Committee (project ID 216892).

Clinical assessment and sample collection

All participants were administered the Yale Global Tic Severity Scale (YGTSS) [21], the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) [22], the Adult ADHD-Rating Scale (ADRS) [23], the Beck Depression Inventory-II (BDI-II) [24] and the Beck Anxiety Inventory (BAI) [25]. The YGTSS, Y-BOCS and ADRS instruments were administered by the same trained neurologist (M.S.). Comorbid OCD and ADHD were diagnosed using DSM-5 criteria.

The presence of depressive and anxiety symptoms was determined based on a BDI-II score ≥14 and a BAI score ≥8 (the latter indicating the presence of mild, moderate or severe anxiety) [24,25].

After clinical assessment, 10 ml of venous EDTA-anticoagulated blood were collected from each participant for immunological characterization. Samples were transferred to the laboratory and stored at 4°C for ≤3 h before being processed for immunophenotyping. On the same afternoon, participants underwent a magnetic resonance imaging (MRI) scan to obtain 1H-MRS data.

Quantification of circulating peripheral blood DC subsets

Dendritic cell subsets were identified using the Human Blood DC Enumeration kit (Miltenyi Biotec, Auburn, CA, USA). As per the manufacturer's protocol, fresh peripheral blood samples (300 µl of EDTA-blood) were stained with an antibody cocktail containing: antibodies directed against CD19 (CD19-PE-Cy5) for exclusion of B cells; antibodies directed against CD14 (CD14-PE-Cy5) for exclusion of monocytes; and antibodies against BDCA-1 (CD1c-PE), BDCA-2 (CD303-FITC) and BDCA-3 (CD141-APC) to identify MDC1s (BDCA-1+), MDC2s (BDCA-3+) and PDCs (BDCA-2+ [Figure 1]). Each sample was stained alongside an isotype control mouse antibody cocktail containing IgG1-FITC, IgG2a-PE and IgG1-APC. Dead cells were

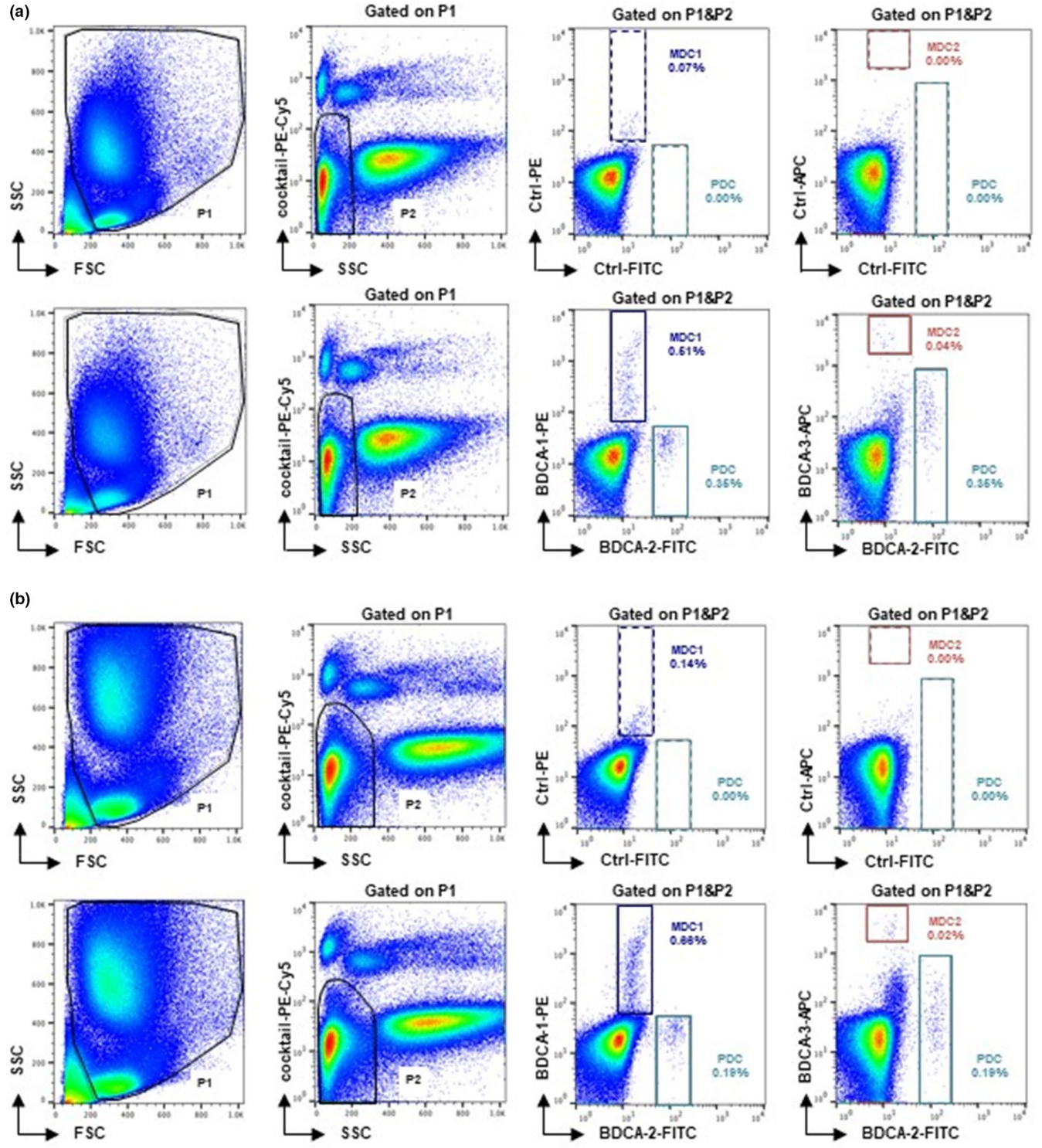

FIGURE 1 Quantification of circulating dendritic cell (DC) subsets. The frequency of circulating DC subsets was determined in fresh peripheral blood samples from healthy volunteers (HVs; $n = 16$) and patients with Tourette syndrome (TS; $n = 17$) by flow cytometry (detailed in Methods). Illustrative dot plots show the gating strategy: forward scatter (FSC) and side scatter (SSC) parameters were used to create a gate (P1) that excluded debris and platelets. Next, SSC and CD19/CD14/dead cell discriminator (cocktail-PE-Cy5) parameters were used to generate a gate (P2) that excluded B cells, monocytes, granulocytes and dead cells. Then, expression of BDCA-1 (CD1c), BDCA-2 (CD303) and BDCA-3 (CD141) was used to identify myeloid DC type 1 (MDC1), myeloid DC type 2 (MDC2) and plasmacytoid DCs (PDC). Dashed rectangular gates display staining with isotype control (Ctrl) antibodies (detailed in Methods) (a) HVs. (b) TS patients [Colour figure can be viewed at wileyonlinelibrary.com]

excluded using a dead cell discriminator dye (PE-Cy5 [Figure 1]). Samples were washed with phosphate buffer saline (0.5% bovine serum albumin) and fixed. Flow cytometry data acquisition was performed within 3–6 h from collection using Navios (Beckman Coulter, High Wycombe, UK) and FACSCalibur (BD Biosciences, San Jose, CA, USA) flow cytometers, and subsequently analysed with the FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Data acquisition by 1H-MRS

The 1H-MRS data were acquired using a Philips 3-T dual Tx Achieva MRI system (Philips Healthcare, Eindhoven, The Netherlands) with a 32-channel head coil. Sagittal three-dimensional T1-weighted images were acquired to provide high grey/white matter contrast that depicts brain anatomy and allows accurate MRS voxel placement (acquisition parameters: 1 ×1 × 1.5 mm resolution, inversion time [TI] = 998 ms, echo time [TE] = 3.8 ms, repetition time [TR] = 7.8 ms, flip angle 8°, acquisition time 4.5 min). MRS voxel localization was focused on the left putamen (PUT; voxel size 30 × 12 × 10 mm) and subcortical frontal white matter (FWM; voxel size 20 × 12 × 12 mm) of the right hemisphere. MRS voxel placement was always performed by the same operator, with voxels oriented obliquely to the three image planes to maximize tissue of interest and exclude surrounding tissue, as shown in Figure 2. 1H-MRS data were obtained using the single-volume Point-RESolved Spectroscopy sequence at a short TE (32 ms), with TR = 2000 ms. Metabolite spectra were acquired with 192 averages and a non-water suppressed acquisition of the tissue water, acquired with 16 averages. Each acquisition lasted 6.5 min. Patients alerted the operator to their own tics during scans, after which lower-resolution three-dimensional T1-weighted images (acquisition time 51 s) were always acquired after each 1H-MRS acquisition to allow visual assessment of the patient's movement, repeating 1H-MRS if deemed necessary. The total MRI scan time was approximately 30 min, including repetitions of 1H-MRS acquisitions.

The 1H-MRS data were analysed using LCModel version 6.31[26] to determine the signal intensities of combined N-acetylaspartate and N-acetylaspartyl glutamate (tNAA), glutamate plus glutamine (Glx), total creatine plus phosphocreatine, total choline and phosphocoline and glycerophosphocoline. Results are reported as metabolite concentrations (mM) using the tissue water signal as a reference (assumed 41.7 M). No corrections for relaxation time effects or tissue partial volumes within the MRS voxel were made.

Data analysis

All data were analysed using IBM SPSS Statistics 23. The normality assumption for all measures was confirmed by Kolmogorov–Smirnov test ($p > 0.05$). Frequencies of DC and 1H-MRS metabolite brain levels in patients with TS and HVs were compared using two-sample *t*-tests. The level of significance was set at $p < 0.05$ (two-tailed).

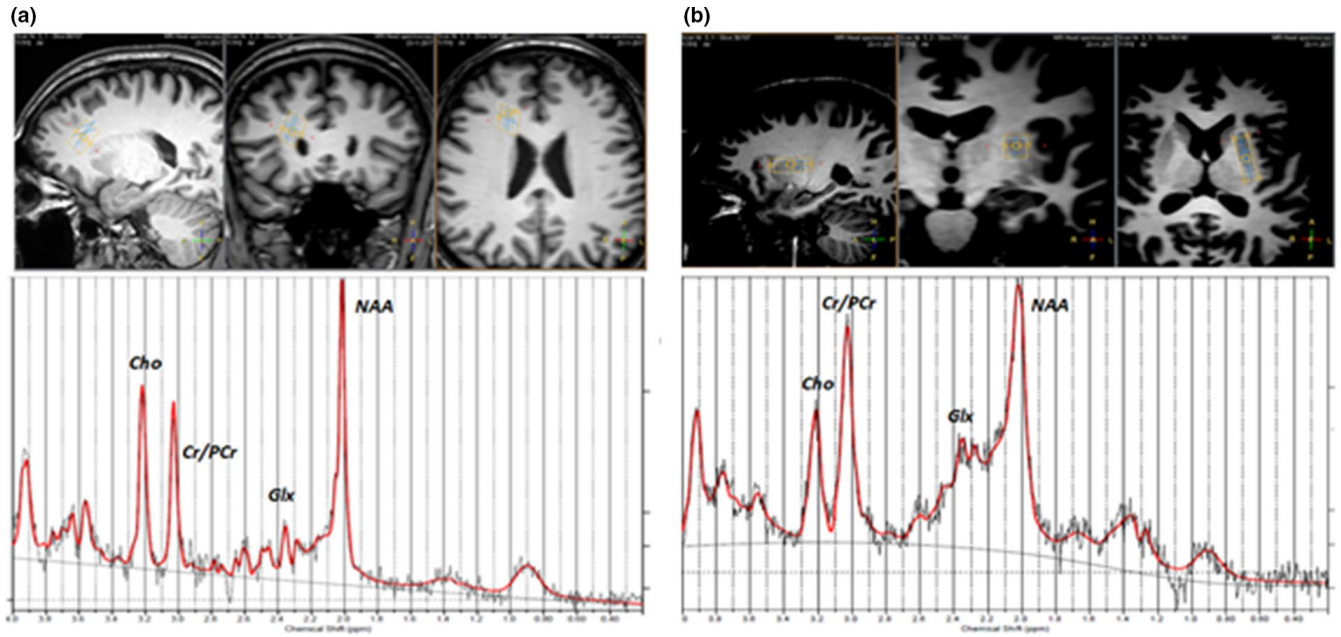

FIGURE 2 Voxel location and LCModel fit to the proton magnetic resonance spectroscopy (1H MRS) data in a patient with Tourette syndrome (TS). (a) Frontal white matter (FMW); (b) Putamen (PUT). Voxel sizes were 20 × 12 × 12 mm for FMW and 30 × 12 × 10 mm for PUT. Voxels were obliquely positioned on the three orthogonal image planes to maximize the tissue of interest within each voxel. Yellow boxes indicate the localization for tNAA, and the white box that for the water resonance for the metabolite acquisition. The tissue water reference signal was obtained from the same region as that of the tNAA signal. Labelled metabolite peaks are: tNAA; glutamate plus glutamine (Glx); total creatine (Cr); and total choline (Cho). In the spectra, the red line indicates the LCModel fit to the raw data, the lower line indicates the baseline and the upper plot the residual signal. NAA: N-acetyl-aspartate [Colour figure can be viewed at wileyonlinelibrary.com]

Relationships between DC subset frequencies, 1H-MRS metabolites and demographic and 1H-MRS quality variables were first explored with bivariate correlations. As a preliminary analysis revealed significant correlations between metabolite concentrations and age and 1H-MRS line width (full width half maximum [FWHM]), these parameters were used subsequently as covariates in bivariate correlation and general linear model (GLM) analyses.

We subsequently analysed the association of psychiatric comorbidities and drugs with DC subset frequencies and 1H-MRS brain metabolites in TS patients. TS patients were divided into subgroups with (TS+) and without (TS−) a specific predefined psychiatric comorbidity, i.e., ADHD and OCD, or psychiatric symptom domain, i.e., anxiety and depression. For each comorbidity/comorbid symptom domain, ANOVA was used to assess the effect of 'group' (TS+, TS− and HV); where significant, post hoc *t*-tests with Bonferroni correction were used to perform pairwise comparisons between groups (significance level $p < 0.05$). Similarly, to explore associations with drug exposure, TS patients were divided into TS with (TS+) and without (TS−) exposure to antipsychotic drugs, and the effect of 'group' was explored with ANOVA. Effects of other medication classes and daily tobacco smoking (according to the World Health Organization Smoking and Tobacco Use Policy definition [27]) were evaluated, conducting sensitivity analyses after exclusion of TS patients with each specific drug class. For each class, between-group differences between HV, TS+ and TS− groups were explored using ANOVA with post hoc *t*-test with Bonferroni correction where significant (significance level $p < 0.05$). In cases where one group contained fewer than two patients, an independent *t*-test comparing the remaining two groups was performed instead of ANOVA (significance level $p < 0.05$).

Finally, unilinear GLMs were used to test possible explanatory and confounding factors or adjust for covariates where significant correlations were found according to our predefined cut-offs. Statistical significance at GLMs was defined as $p < 0.05$.

RESULTS

Eighteen TS patients and 18 HVs entered the study. The two groups were similar for demographic characteristics. Scores for ADRS, BAI and BDI were significantly higher in TS patients compared to HVs ($p = 0.003$, $p = 0.001$, $p = 0.014$, respectively; Table S1). Amongst comorbid disorders/symptoms, OCD was present in eight TS patients, ADHD in eight, anxiety in 12 and depression in five. Fifteen patients were treated for tics or other behavioural symptoms with the following medications: aripiprazole ($n = 5$); botulinum toxin ($n = 4$); clonidine, pimozide, sulpiride and clonazepam ($n = 1$ each); sertraline ($n = 2$); amitriptyline, clomipramine and atomoxetine ($n = 1$ each);

TABLE 1 ANOVA subgroup analysis of dendritic cell frequencies between groups based on psychiatric comorbidities

	Anxiety		ADHD		OCD		Depression	
	Mean ± SD	*N*	Mean ± SD	*N*	Mean ± SD	*N*	Mean ± SD	
% of MDC1s								
TS+	0.69 ± 0.17	11	0.67 ± 0.21	7	0.62 ± 0.23	7	0.76 ± 0.21	5
TS−	0.44 ± 0.17	6	0.56 ± 0.20	10	0.60 ± 0.20	10	0.54 ± 0.17	12
HVs	0.55 ± 0.18	16	0.55 ± 0.18	16	0.55 ± 0.18	16	0.55 ± 0.18	16
Df	32		32		32		32	
p	**0.025**		0.36		0.702		0.072	
% of MDC2s								
TS+	0.05 ± 0.02	11	0.05 ± 0.02	7	0.05 ± 0.02	7	0.05 ± 0.01	5
TS−	0.05 ± 0.02	6	0.05 ± 0.02	10	0.05 ± 0.02	10	0.05 ± 0.02	12
HV	0.05 ± 0.01	16	0.05 ± 0.01	16	0.05 ± 0.01	16	0.05 ± 0.01	16
Df	32		32		32		32	
p	0.457		0.733		0.816		0.576	
% of PDCs								
TS+	0.39 ± 0.15	11	0.42 ± 0.14	7	0.37 ± 0.14	7	0.38 ± 0.11	5
TS−	0.32 ± 0.08	6	0.33 ± 0.11	10	0.36 ± 0.13	10	0.36 ± 0.14	12
HV	0.41 ± 0.16	16	0.41 ± 0.16	16	0.41 ± 0.16	16	0.41 ± 0.16	16
df	32		32		32		32	
p	0.519		0.295		0.716		0.681	

Note: Bold indicates statistically significant between-groups difference.

Abbreviations: ADHD, attention deficit and hyperactivity disorder; HV, healthy volunteer; MDC1, myeloid dendritic cell type 1; MDC2, myeloid dendritic cell type 2; OCD, obsessive–compulsive disorder; PDC, plasmacytoid dendritic cell; TS−, Tourette syndrome patients without comorbidity X; TS+, Tourette syndrome patients with comorbidity X.

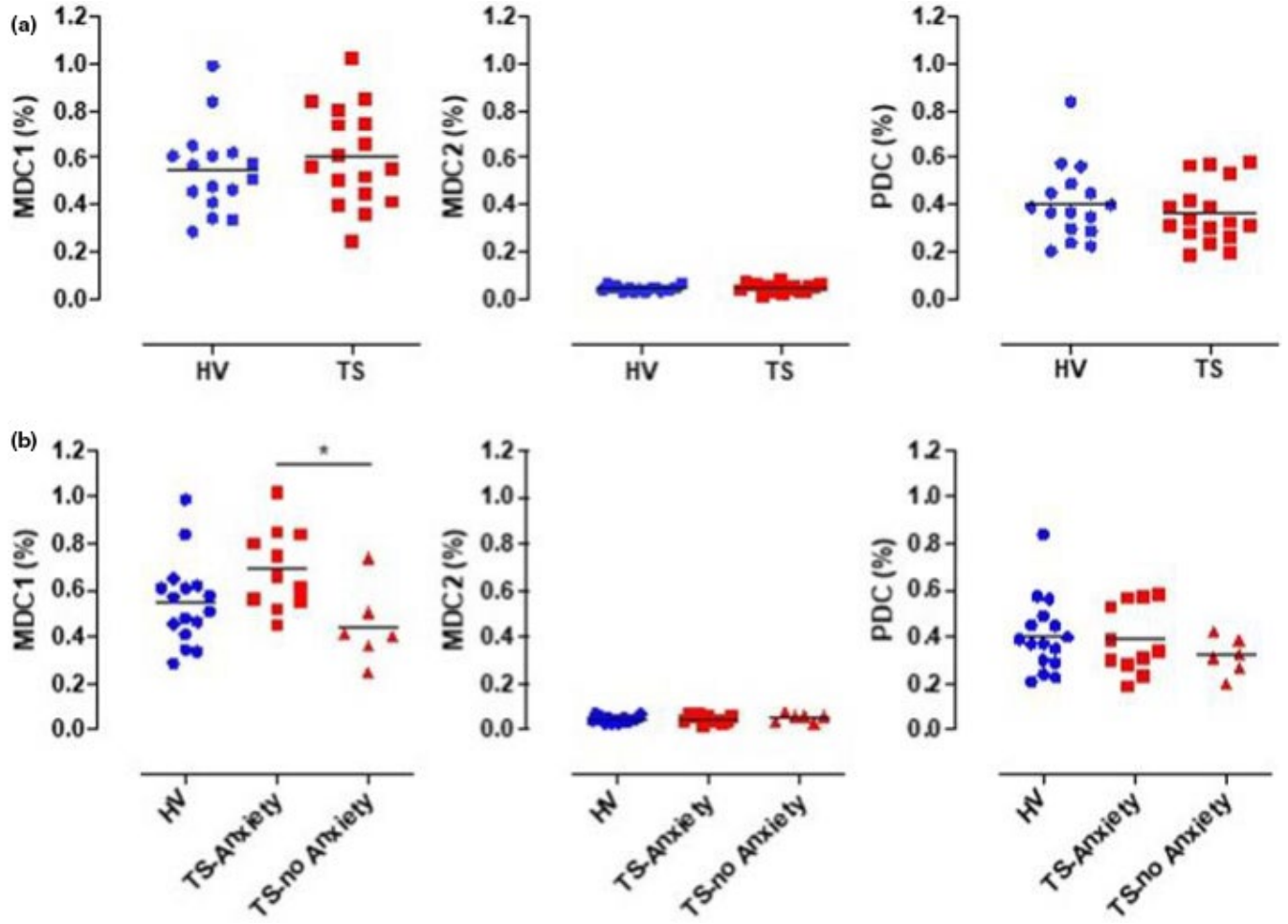

FIGURE 3 Frequency of circulating dendritic cell (DC) subsets in patients with Tourette syndrome (TS). The frequency of circulating DC subsets was determined in healthy volunteers (HV; *n* = 16) and patients with TS (*n* = 17) by flow cytometry (detailed in Methods). (a) Graphs display the percentage of myeloid type 1 (MDC1), myeloid type 2 (MDC2) and plasmacytoid dendritic cell (PDC) subsets in the two study groups (horizontal bars, mean). No significant differences were identified (unpaired two-tailed Student's *t*-test) (b) Graphs display the frequency of the three DC subsets in healthy volunteers (*n* = 16), patients with TS and anxiety (TS-Anxiety, *n* = 11), and patients with TS without anxiety (TS no Anxiety, *n* = 6); (horizontal bars, mean). **p* = 0.01 [Colour figure can be viewed at wileyonlinelibrary.com]

three were chronic cannabis users; three were daily tobacco smokers and none was on behavioural treatment or had undergone functional brain surgery.

Data from one patient and two HVs were excluded because of staining failure. We did not observe significant between-group differences in frequency of MDC1 (TS patients: 0.60% ± 0.20%; HVs: 0.55% ± 0.18%; $p = 0.41$), MDC2 (TS patients: 0.049% ± 0.02%; HVs: 0.046% ± 0.01%; $p = 0.52$), and PDC (TS patients: 0.36% ± 0.13%; HVs: 0.41% ± 0.16%; $p = 0.42$) subsets. ANOVA comparing DC subset frequencies between TS patients with or without behavioural comorbidities and HVs yielded a significant 'group' effect when TS patients were subgrouped by anxiety symptoms ($p = 0.025$; Table 1); post hoc analysis showed significantly higher MDC1 frequency in TS patients with anxiety compared to TS patients without anxiety ($p = 0.01$; Figure 3). We did not detect any other significant association between other comorbidities and DC subset frequencies Table 1). Similarly, we could not identify any significant correlation between severity of tics, ADHD, OCD, depressive and anxiety symptoms, and frequency of DC subsets (Table S2). Finally, ANOVA comparing TS patient subgroups divided according to current antipsychotic exposure and HVs did not show any significant effect of clinical group (Table S3). Likewise, sensitivity analyses testing the potential impact of other drugs on DC subset frequency did not reveal significant associations (Table S3).

The 1H-MRS data from TS patients (two FWM, three PUT) and HV (three FWM, one PUT) were excluded after visual assessment of spectrum quality prior to any analysis, due to poor water suppression, excessive baseline roll, artefactual peaks, poor peak resolution or low signal to noise. LCModel assessment of the quality of accepted data gave a mean and standard deviation of the water FWHM and metabolite signal-to-noise ratio of 0.036 ± 0.005 ppm and 14.8 ± 2.5, respectively, in FWM (*n* = 31) and 0.069 ± 0.02 ppm, 14 ± 1.7, respectively, in the PUT (*n* = 32), without significant differences between TS patients and HVs.

Metabolite concentration change with age [28] and age-related changes in iron deposition in the basal ganglia [29] may also

change water relaxation times [28], thus affecting metabolite estimates. PDCs are also known to decrease with age [30]. In a preliminary correlation analysis, we observed significant correlations ($0.05 > p > 0.011$) between several metabolites, age and FWHM in the putamen of HVs and patients, as well as an inverse correlation of PDCs with age ($r = -0.400$, $p = 0.021$). Hence, FWHM and age were used as covariates to assess correlations between metabolite concentrations and cell counts.

We found a strong negative correlation between tCr and the MDC1 subset in the FWM of TS patients ($r = -0.784$, $p = 0.0015$), which survived a Bonferroni-corrected p value of 0.0021 for 24 comparisons (four metabolites, three cell types and two regions), but not in PUT ($r = -0.444$, $p = 0.148$ [Table 2]). Other correlations significant at $p < 0.05$ (not Bonferroni-corrected) were: tNAA with PDCs ($r = -0.588$, $p = 0.035$) in the FWM of TS patients, and Glx with PDCs ($r = 0.651$, $p = 0.022$) in the PUT of TS patients. No significant correlations were present for both the FWM and PUT in HVs (Table 2). The correlation of tCr with MDC1 in the FWM of TS patients was also highly significant without covariates ($r = -0.797$, $p < 0.001$; Figure 4a). Although not significant, there was a trend for a tCr decrease with MDC1 in the PUT, which closely matches that of the correlation in the FWM (Figure 4a); this correlation was not found in HVs (Figure 4b). A GLM was used to investigate the relationship between tCr and MDC1s including both PUT and FWM data, with age and FWHM as covariates. tCr correlated to MDC1s across both anatomical regions, with $F = 12.61$, $p = 0.002$, with a significant age effect also and a highly significant effect size for location (Table S4). Finally, sensitivity analyses testing the potential impact of drug classes on tested brain metabolites either in the FWM or the PUT did not show significant associations (Tables S5A,B).

DISCUSSION

To our knowledge, the present study is the first to investigate the distribution of circulating DC subsets in TS patients and to explore its relationship with the comorbidity profile of TS. In contrast to

TABLE 2 Correlations between dendritic cell subsets and magnetic resonance spectroscopy metabolites in frontal white matter and putamen in patients with Tourette syndrome and healthy volunteers

	MRS metabolites									
	Frontal white matter					Putamen				
	N	tCho	tCr	NAA	Glx	*N*	tCho	tCr	NAA	Glx
TS patients										
MDC1										
Pearson correlation	15	0.273	**−0.784****	−0.429	−0.202	14	0.085	−0.444	−0.240	0.425
Significance (two-tailed)		0.367	**0.002**	0.144	0.507		0.794	0.148	0.453	0.168
MDC2										
Pearson correlation	15	0.173	−0.124	0.053	0.076	14	−0.100	−0.206	−0.008	−0.076
Significance (two-tailed)		0.572	0.686	0.864	0.805		0.756	0.520	0.981	0.814
PDC										
Pearson correlation	15	0.119	−0.307	**−0.588***	−0.344	14	−0.288	−0.374	−0.262	**0.651***
Significance (two-tailed)		0.699	0.307	**0.035**	0.250		0.364	0.231	0.411	**0.022**
HVs										
MDC1										
Pearson correlation	13	0.531	0.090	0.212	−0.562	15	0.252	0.016	0.379	0.144
Significance (two-tailed)		0.093	0.792	0.532	0.072		0.406	0.958	0.201	0.639
MDC2										
Pearson correlation	13	0.264	−0.179	0.377	−0.429	15	0.248	−0.026	0.347	0.224
Significance (two-tailed)		0.433	0.598	0.253	0.188		0.414	0.933	0.246	0.462
PDC										
Pearson correlation	13	−0.219	0.089	−0.148	0.377	15	−0.255	−0.229	−0.550	−0.830
Significance (two-tailed))		0.517	0.795	0.665	0.253		0.401	0.452	0.858	0.788

Note: Bold indicates statistically significant correlations.

Covariates: age and full-width half maximum.

Abbreviations: Glx, glutamate + glutamine; HV, healthy volunteer; MDC1, myeloid dendritic cell type 1; MDC2, myeloid dendritic cell type 2; MRS, magnetic resonance spectroscopy; NAA, N-acetylaspartate; PDC, plasmacytoid dendritic cell; tCho, total choline; tCr, total creatine; TS, Tourette syndrome.

*Correlation is significant at the 0.05 level.

**Correlation is significant at the 0.0021 level (corrected by multiple comparison factor).

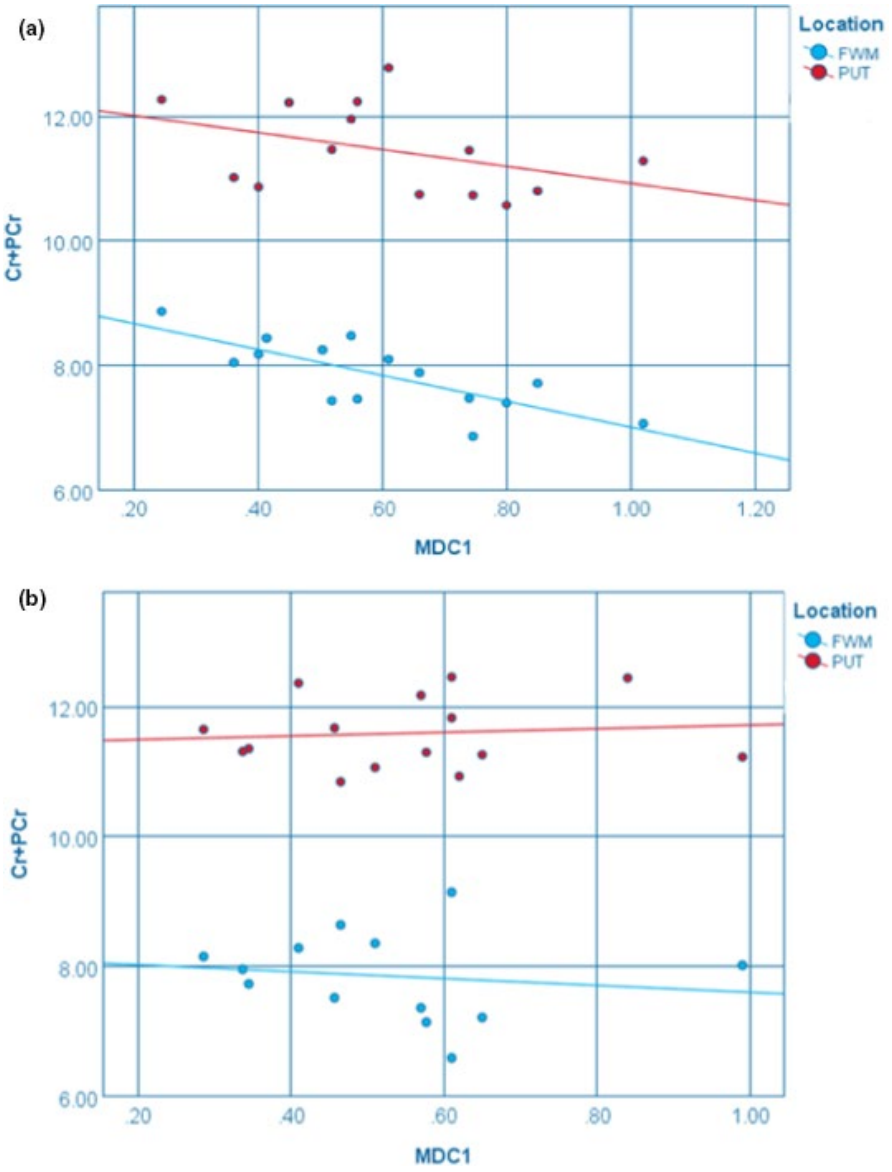

FIGURE 4 (a,b) Scatter plot of myeloid dendritic cell type 1 (MDC1) and total creatine (Cr + phosphocreatine [PCr]) correlation by location. (a) Tourette syndrome patients and (b) healthy volunteers. FWM, frontal white matter; PUT, putamen [Colour figure can be viewed at wileyonlinelibrary.com]

results reported in autism [13,14], we did not observe differences in the frequency of circulating DC subsets between TS patients and age-matched HVs. Whereas the frequency of DC subsets did not correlate with tic severity, we detected an increase in MDC1 frequency in TS patients manifesting anxiety symptoms (mild, moderate or severe). Moreover, our analysis of a possible association between DC subset frequencies and metabolite levels within fronto-subcortical network regions showed a strong correlation between MDC1 frequency and tCr levels in the FWM of TS patients, but not in HVs. This observation was independent of anxiety and supports a possible relationship between systemic immunoregulatory mechanisms and brain metabolism in adults with TS.

The lack of correlation between DC subset frequency and clinical severity of tics and comorbid diagnoses or symptoms (i.e., OCD and anxiety) does not support a direct influence of immune mechanisms regulated by, or influencing the activity of, DCs on the frequency and intensity of the abnormal behaviours typical of the TS spectrum.

Our findings support, rather, that MDC1 frequency could be associated with the presence of anxiety symptoms in TS patients. Longitudinal observations would add more clarity on whether MDC1-regulated immune mechanisms promote the development of anxiety symptoms, are accelerated by stress responses and anxiety, or represent epiphenomena that lack a direct mechanistic relationship with behavioural features.

The relationship between stress responses, anxiety symptoms and systemic immune regulation in chronic tic disorders, and the related contribution of autonomic and neuroendocrine signalling mechanisms [31,32] remain heavily under-investigated. They may involve peripheral effects, including abnormal interleukin production that may drive naïve self-reactive T cells to react against CNS tissue, or failure to generate/maintain T-cell tolerance via negative selection in the thymus. The increased prevalence of generalized anxiety disorder in the TS population is well recognized [33], as well as the increased level of circulating cortisol and proinflammatory cytokines in individuals with generalized or other anxiety disorders

[34,35]. Mild anxiety symptoms have also been associated with altered gene expression patterns of innate and adaptive immune responses [36]. It has been shown that the increase in circulating corticosteroid levels during stressful events can precipitate a dysfunction of cell-mediated immune processes also by disrupting DC maturation [37] and antigen presentation functions and, therefore, their ability to generate an effective T-cytotoxic response [38,39]. To our knowledge, however, the effect of corticosteroids on peripheral blood circulating DC subsets has never been investigated in the context of neuropsychiatric or neurodevelopmental disorders. Among DC subsets, MDC1s have a specific capability to present antigens via major histocompatibility complex class II to activate naïve $CD4^+$ T cells, and to promote T-helper 1 responses [11]. As previously demonstrated in animal models of MS, clinical manifestations of CNS autoimmunity are preceded by a phase of microglia expansion and myeloid DC peripheral proliferation [40,41]. The peripheral increase of MDC1s in TS patients with increased anxiety symptoms may reflect a proinflammatory state, possibly facilitating altered neuro-immune crosstalk.

To date, 1H-MRS studies of TS have highlighted neurochemical changes associated with this diagnosis and/or with tic severity, with some inter-study heterogeneity [42-44]. In line with our findings, previous reports demonstrated a reduction of tCr in the PUT, right frontal cortex and thalamus [43,45]. The central role played by creatine and creatine kinase/phosphocreatine in high metabolism cells, such as brain and muscle, by regenerating adenosine triphosphate from adenosine diphosphate is widely recognized [46,47]. Genetically determined deficits of creatine phenotypically present with severe neurological symptoms from young age [48]. The strong inverse correlation between brain tCr levels and MDC1 subset frequency in our TS patients lends support to a potential association between metabolic changes of brain regions that are directly involved in the generation and control of pathological behaviours in TS patients and a systemic inflammatory state. Alternatively, this correlation could suggest a direct influence on immune regulatory mechanisms at a systemic level [49,50], exerted by a generalized alteration of creatine metabolism, expressed here by lower concentrations of tCr in different brain regions. In particular, creatine kinase B (CK-BB; brain type) has been reported as a regulator of T-cell development and activation through the control of T-cell receptor signalling during negative selection in the thymus [51], a key mechanism for self-antigen tolerance and the pathogenesis of autoimmune disease. If altered creatine metabolism influences creatine kinase isoform activity, a potential effect of this could be a dysfunction in the regulatory effect of CK-BB upon T-cell receptor signalling in T cells, contributing to their dysregulation, promotion of an inflammatory state, and predisposition to autoreactive immune processes, particularly in TS patients with co-existing anxiety. A more focused exploration of creatine metabolism in TS and related disorders is needed to appraise this alternative interpretation. Furthermore, the observed correlation between brain tCr concentrations and peripheral blood MDC1 frequency was not influenced significantly by anxiety and depressive symptoms or behavioural comorbidities, suggesting that this link is not directly related to concomitant emotional or behavioural abnormalities in TS patients.

We acknowledge several limitations of our study. First, our sample may not be representative of the general TS population, as it involves a subgroup of patients whose tics persisted in adulthood. Second, to avoid skewing our sample towards milder forms of disease, as in previous neuroimaging studies on TS [41,50,51], we included patients on stable but disparate pharmacological treatments. Although our sensitivity analyses did not detect any major influence of drugs on outcomes of interest, we recognize that analyses might have missed smaller effects of drug exposure on DC frequencies and brain metabolite spectra. Third, the presence of anxiety symptoms in our TS patients was determined only on the basis of the BAI score, and the majority of them scored in the range of mild-to-moderate symptoms. The small TS patient group size precluded sufficient statistical power to assess associations between MDC1 frequency and severity of anxiety symptoms or a clinical diagnosis of comorbid anxiety disorder. Fourth, other factors might have influenced the brain metabolites explored in the present study. Levels of Glutamate (Glu) and Glutamine (Gln) may be influenced by nicotine [52] and sleep patterns [53,54]. Similarly, tNAA levels may be affected by nicotine use [52], lactate may increase following caffeine ingestion [55] and choline and Glx levels exhibit diurnal variations [54,56]. A region-dependent reduction in tCr was reported in middle-aged smokers compared to non-smokers [52]. Although controversial [57,58], a sex-dependent variation of all metabolites was previously suggested for specific brain regions [59,60]. Yet, our sensitivity analyses did not detect a significant confounding effect of tobacco smoking on the observed correlation between tCr and MDC1 and an effect of daily variation is unlikely as all MRI was performed in the afternoon. We nevertheless acknowledge that undetected effects of caffeine, poor sleep quality and lack of strict matching by sex might have increased the variability of metabolite levels in both groups, potentially obscuring subtle inter-group differences. Finally, the limited availability of cerebrospinal fluid specimens from this patient population and lack of access to *in vivo* molecular imaging markers of neuroinflammation did not allow us to correlate DC frequencies to neuroinflammatory processes beyond the information that could be provided by metabolic spectra.

In conclusion, we report an increase of the MDC1 subset of DCs in adults with TS and concurrent anxiety symptoms (mild, moderate or severe), which might be associated with a systemic inflammatory state described in patients with this neurodevelopmental disorder. Moreover, the strong correlation between this DC subset and decreased tCr in the FWM of TS patients could originate from immune dysregulation predisposing/contributing to inflammation, suggesting tCr as a marker of inflammatory changes in TS. Finally, our results support the importance of exploring the influence of the whole array of behavioural symptoms, beyond the primary diagnostic feature when investigating immunological and other regulatory mechanisms in complex neurodevelopmental disorders.

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CONFLICT OF INTEREST

None of the Authors has any conflict of interest to disclose in relation to this manuscript.

AUTHOR CONTRIBUTIONS

Marianna Sarchioto: Data curation (lead); Formal analysis (lead); Investigation (lead); Project administration (lead); Validation (equal); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Franklyn Howe:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (equal); Resources (equal); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – review and editing (supporting). **Ingrid E Dumitriu:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (equal); Resources (equal); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – review and editing (supporting). **Francesca Morgante:** Project administration (supporting); Resources (supporting); Writing – review and editing (supporting). **Jeremy Stern:** Resources (supporting); Writing – review and editing (supporting). **Mark Edwards:** Conceptualization (supporting); Funding acquisition (equal); Project administration (supporting); Resources (equal); Writing – review and editing (supporting). **Davide Martino:** Conceptualization (lead); Formal analysis (supporting); Funding acquisition (lead); Methodology (lead); Project administration (equal); Resources (equal); Supervision (lead); Validation (equal); Visualization (supporting); Writing-original draft (supporting); Writing – review and editing (lead).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author (D.M.), upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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