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Tourette's syndrome: the role of immunity and brain metabolism

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Preface

There is an increasing need for greater awareness, better understanding and more efficacious treatment in adult persisting Tourette's syndrome. This research is the product of a proficuous international collaboration between University of Turin, St George's, University of London and University of Calgary, Canada. This project underwent formal peer review in accordance with the requirements outlined by University of Turin. Considerable knowledge and expertise were placed in the project concept, task design and development and would not have possible without all the people who have generously contributed. This work has advanced the understanding of the immunological, inflammatory and clinical correlates of adult Tourette's patients and provides a theoretical basis for the future development of new therapies potentially based on immunomodulation. Here is presented evidence in support of immune dysregulation and alteration of the excitation and inhibition neural balance in adult patients affected by Tourette's, possibly contributing and/or predisposing to a state of chronic neuro-inflammation which might be a factor in abnormalities in the development and function of cortico-striato-thalamo-cortical circuits. In the arena of adult Tourette's syndrome, we have identified a novel putative biomarker which correlates with severity of motor symptoms. As well as encouraging further research into this hypothesis and an extension to other tic disorders, the measurement of immune cells and brain biomarkers may provide an objective marker of diagnosis, clinical assessment and rehabilitation of tic severity. Part of this work has been peer-reviewed and published in the *European Journal of Neurology* [1] and was recognised by L'Accademia LIMPE-DISMOV as article of the month for April 2021.

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List of abbreviations

1H-MRS	proton (hydrogen-1) magnetic resonance spectroscopy
ADHD	attention deficit hyperactivity disorder
ADRS	Adult ADHD Rating Scale
ASD	autism spectrum disorder
BAI	Beck Anxiety Inventory
BDI-II	Beck Depression Inventory II
CK	creatine kinase
CK-BB	creatine kinase B brain type
CNS	central nervous system
Cr	creatine
CSTC	cortico-striato-thalamo-cortical
df	degrees of freedom
DC	dendritic cell(s)
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5
FSC	forward scatter
FWHM	full width at half maximum
FWM	frontal white matter
GABA	gamma-aminobutyric acid
GAD	generalised anxiety disorder
GAS	group A Streptococcus
GLM	general linear model
Gln	glutamine
Glu	glutamate
Glx	combined glutamine and glutamate
GPe	globus pallidus pars externa
GPi	globus pallidus pars interna
GSH	glutathione
HLA	human leukocyte antigen
HV	healthy volunteers
Ins	inositol
Lac	lactate
MDC1	myeloid dendritic cell type 1
MDC2	myeloid dendritic cell type 2
MRI	magnetic resonance imaging
NAA	N-acetylaspartate

NAAG	N-acetylaspartyl glutamate
OCD	obsessive-compulsive disorder
PANS	paediatric acute-onset neuropsychiatric syndrome
PCr	phosphocreatine
PDC	plasmacytoid dendritic cell
PUT	putamen
SD	standard deviation
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticularis
SNR	signal-to-noise ratio
SSC	side scatter
STN	subthalamic nucleus
T1w	T1-weighted
tCho	total choline (combined glycerophosphocholine and phosphocholine)
tCr	total creatine (combined creatine and phosphocreatine)
TCR	T-cell receptor
tNAA	combined N-acetylaspartate and N-acetylaspartyl glutamate
TS	Tourette's syndrome
VTA	ventral tegmental area
XEASD	Xiao-Er-An-Shen Decoction
Y-BOCS	Yale-Brown Obsessive Compulsive Scale
YGTSS	Yale Global Tic Severity Scale
YGTSS-TSS	Yale Global Tic Severity Scale Tic Severity Score

CHAPTER 1

GENERAL INTRODUCTION

Diagnostic criteria, epidemiology and comorbidities

Gilles de la Tourette syndrome or Tourette's syndrome (TS) is a childhood-onset neurodevelopmental disorder characterized by the presence of several motor and phonic tics[2]. Diagnosis for TS, according to the current DSM-5 criteria, is based on the presence of both motor and phonic tics, although not necessarily concurrently, duration of tic symptoms longer than 1 year, onset before age 18 years, and absence of any known cause such as another medical condition or substance use[3]. The typical age of onset of tics is between four and six years old and severity typically peaks between 8 and 12 years of age. It affects males more frequently than females by a ratio of 3-4:1[4]. TS affects 0.3% to 0.9% of the under-18 general population[5].

By the end of the second decade of life, many individuals are virtually tic free[6]. Longitudinal studies demonstrated that fewer than 20% of cases continue to experience clinically impairing tics as adults[7,8]. The prevalence of TS in the adult population is controversial, ranging from 49 to 657 cases per million adults, likely related to differences in diagnostic criteria. Overall, it was recently estimated to be 118 cases of TS per million adults in a meta-analysis including 2,356,485 participants. By contrast, the male:female ratio of risk of adulthood TS was similar between studies with a risk ratio of 2.33[9].

Tics are defined as repetitive, sudden, brief, unwanted, non-goal-directed and non-rhythmical muscle contraction involving specific muscle groups. Tics are suppressible by volition and suppression normally results in subsequent tic rebound. They are suggestible and are usually preceded by a sensory phenomenon (urge) which is of diagnostic importance and distinguishes tics from myoclonus, stereotypies or

dystonia. Premonitory urges occur in about 90% of TS patients[10] and are not often recognised or associated with tics until the age of 10 years[11].

Up to 90% of TS patients are affected by comorbid neurodevelopmental or behavioural pathologies, including obsessive-compulsive behaviour and disorder (OCD), attention deficit hyperactivity disorder (ADHD), mood/anxiety disorders, and impulse control disorders[12]. The most commonly associated comorbidity is ADHD, followed by obsessive-compulsive behaviour and obsessive-compulsive disorder[13].

OCD is characterized by recurrent and intrusive obsessions which are egodystonic (perceived as uncomfortable thoughts) or compulsions, repetitive and apparently purposeful behaviours which are stereotyped in fashion or performed according to strict rules. Normally they are a significant source of distress to the individual or interfere with social or role functioning[3]. Genetic studies on heritability of TS and OCD showed a strong genetic correlation between these two conditions, supporting the hypothesis of some genetic overlap between these two phenotypically related neuropsychiatric disorders[14].

ADHD normally presents in childhood and parents are often the first to note clumsiness, excessive activity, low frustration tolerance and 'accident proneness'[15]. Although it is present in about 60% of patients, it exerts a negative effect on behaviour and psychosocial outcomes[16]. Differently from OCD, evidence does not support a genetic link between ADHD and TS[15].

Finally, several studies have highlighted the burden of anxiety and depression on disability in children and adolescents with TS[15,17-19]. Depression was significantly

associated with severity of tics and comorbid ADHD. Conversely, the presence of coexistent anxiety and behavioural problems was not related to obsessionality. Furthermore, TS patients showed a consistent positive family history of depression[17]. However, it remains unclear whether depression and anxiety constitute triggers for tics or, conversely, the social stigma associated with tics signifies a trigger for depression and anxiety.

Aetiopathogenesis, CSTC circuit and neurotransmitters

The aetiology of TS complex and involves a complex interplay of genetic and non-genetic factors. TS is considered to be a polygenic condition, involving multiple common and less common genetic variants and mutations. Amongst the non-genetic factors, perinatal events and immunological factors play a key role in the diversity of the clinical phenotype and in the structural and functional abnormalities of involved neural circuits[2].

Several genetic studies on twins and families demonstrated that while TS is one of the most inheritable neuropsychiatric disorders, it follows non-Mendelian heritability. Siblings of TS patients have a 15-fold increased risk of developing TS or chronic tic disorders when compared to the general population[20,21]. Similarly to other neurodevelopmental psychiatric conditions, no definitive risk gene has been associated with TS[22,23]. A milestone genome-wide association study which used multivariate modelling to provide the first direct genetic measure of aggregated TS genetic risk, demonstrated that TS is predominantly a polygenic disorder. Risk variants

were distributed widely across the genome, overlapping significantly with, but also distinct from, OCD genetic risk [14].

Amongst non-genetic factors, increasing evidence links TS pathogenesis to immune pathways and environmental causes which will be discussed in detail in the next paragraph. The current model is founded on the hypothesis that pre- and peri-natal factors such as infections, maternal stress and gestational smoking might trigger the activation of microglia, which constitute the brain's resident immune system, determining or contributing to abnormal synaptic plasticity on the background of a susceptible genetic profile. During life, subsequent similar triggers such as infections or psychosocial stressors could reactivate the microglia, determining symptom onset and waxing and waning of tic course[2,24].

Over the past 30 years, there has been a constant effort to unveil the pathophysiological mechanisms of TS and to identify the neuronal locus or networks involved in its emergence. Currently, there is no generally accepted model of TS as the main issue seems to lie in the lack of a coherent theoretical framework for tics and associated phenomena. Historically, TS has been considered a movement disorder characterized by a lack of inhibitory control whereas more recent evidence has shifted the pathogenetic model of TS towards a disorder of purposeful action selection and execution of movement [25].

There is good evidence of abnormalities in cortico-striato-thalamo-cortical (CSTC) circuits in TS[26,27] and three major circuits are involved: the premotor cortex–putamen circuit (habitual behavioural circuit), the ventral medial prefrontal cortex–

caudate nucleus circuit (the goal-directed circuit) and inputs from amygdala, prefrontal cortex, ventral striatum and anterior cingulate gyrus (related limbic circuits)[28,29].

Likely an imbalance of neurotransmitters, or combinations of neurotransmitters, located in these pathways is relevant to the developmental anomalies of the CTSC circuits[30]. Although the exact candidates and their role remain yet to be determined, neurochemicals involved include dopamine, glutamate, serotonin and acetylcholine[2].

Changes in the dopaminergic system play a crucial role in the pathophysiology of TS and strong evidence is available in support of it[31-33]. The frontal cortex is innervated by dopaminergic neurons from the ventral tegmental area (mesocortical and mesolimbic dopaminergic pathways) and in the striatum glutamatergic projections from the cortex synapse with dopaminergic outputs from the basal ganglia (substantia nigra pars compacta) and with intrinsic striatal direct and indirect gamma-aminobutyric acid (GABA)-ergic projections. The direct projections mainly involve excitatory dopamine D1 receptors, whereas the indirect pathway expresses inhibitory dopamine D2 receptors. It is assumed that dopaminergic changes are predominantly related to altered dopamine D2 receptors with dysfunction described at all levels in neurotransmission. Amongst the pre-synaptic changes, hyperinnervation and hypofunction have been described. At the intrasynaptic level, a dysfunctional stimulus-induced release of dopamine has been hypothesised following evidence from dopamine release after amphetamine stimulation. Finally, altered density of striatal and cortical dopamine receptors have been described at post-synaptic level[32-36]. The known efficacy of dopamine antagonists in TS further supports the primary role of

dopaminergic dysfunction in TS. See Figure 1 for a summary of CTSC circuits and their interaction with dopaminergic pathways.

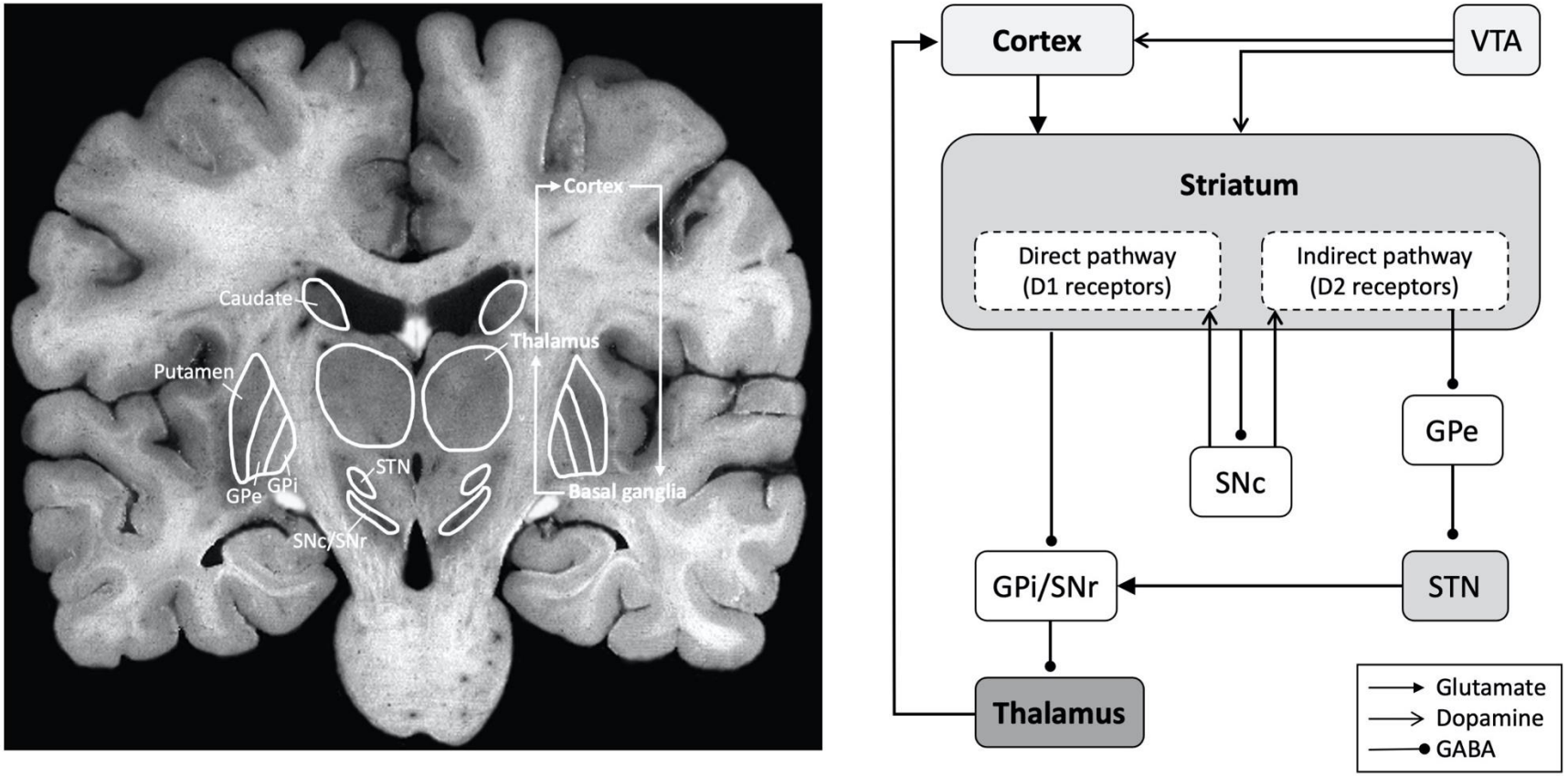

Figure 1. Cortico-striato-thalamo-cortical circuits. Coronal brain section showing relevant structures (left) and schematic diagram of connectivity and neurotransmitters (right). GPe, globus pallidus pars externa; GPi, globus pallidus pars interna; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticularis; STN, subthalamic nucleus; VTA, ventral tegmental area.

Some studies have also documented abnormalities in other neurotransmitter systems including the GABAergic system [37]. GABA is the primary neurotransmitter of striatal synaptic projection neurons and interneurons located in both the striatum and the cortex. Dysfunction of the GABAergic inhibitory system may conceivably underlie the symptoms of motor disinhibition presenting as tics and psychiatric manifestations[38]. Although post-mortem studies have identified a generalised reduction in the number of GABAergic interneurons, magnetic resonance spectroscopy studies have reported increased concentrations of GABA in the striatum likely representing a greater inhibitory tone [39,40]. In mice models of TS, the disruption of striatal and cortical GABAergic neurons by means of local injection of GABA antagonist drugs reproduced tic-like behaviours[41]. Furthermore, the therapeutic efficacy of benzodiazepines in TS, which eventually induces an overall increase of the GABAergic tone, confirms the key role of this neurotransmitter.

Glutamate is the primary and most represented excitatory neurotransmitter in the cortical and thalamic projection neurons and the subthalamic nucleus [42,43], and was first found reduced in the basal ganglia of TS patients in a post-mortem study by Anderson et al. in 1992 [44]. It plays an essential role in the CSTC circuit and is often co-transmitted with dopamine in the prefrontal cortex, midbrain, and striatum[45,46]. Raised levels of glutamate measured via magnetic resonance spectroscopy were reported in the striatum and premotor cortex of children affected by TS when compared with age and gender matched healthy controls [39]. Animal models support a role for cortico–striatal glutamatergic afferents in the generation of tic-like

movements [41]. Modulators of glutamatergic neurotransmission have been considered as potential pharmacological targets in TS, so far with unsatisfactory results [47,48].

Finally, several other neurotransmitters have been studied as possible players in the pathogenesis of TS including serotonin, acetycholine, noradrenealine and endogenous cannabinoids [49]. Median raphe nucleus serotonergic neurons project to the basal ganglia circuits and reduced levels of cerebrospinal fluid serotonin and tryptophan have been reported in TS patients when compared to healthy controls[50]. However, it is unclear whether these findings might be determined by the presence of comorbid OCD. Evidence for a potential role of noradrenaline is supported by the therapeutic efficacy of α2-adrenergic agonists such as clonidine and guanfacine. However, the efficacy of clonidine might be determined by the regulatory effect on glutamate firing activity in the cortical pyramidal neurons rather than solely by the effect on the adrenergic pathway [51]. Finally, placebo-controlled trials have suggested a role for oral δ-9-tetrahydrocannabinol in tic suppression in patients affected by TS and its effect is thought to be mediated by central cannabinoid receptors located in the striatum, hippocampus and thalamus, eventually modulating reward, nociceptive and appetitive pathways [52,53].

Neuro-immunological basis of Tourette's syndrome

A body of evidence links the crosstalk between neural and immune pathways to the pathogenesis of TS and other neurodevelopmental disorders, such as autism spectrum disorder (ASD). Similarly to a model previously proposed for psychosis[54], on

the background of a genetic predisposition, pre- and peri-natal stressors such as infection, maternal stress or gestational smoking could trigger the activation of microglia (the brain's resident immune system), thereby influencing synaptic plasticity. Around the time of symptom onset, similar triggers such as infections or psychosocial stress could reactivate the microglia inducing further synaptic rearrangements and enhancement of peripheral immune and inflammatory responses[55,56].

The contribution of immune mechanisms to the pathogenesis of TS was originally suggested by clinical studies aimed at exploring the association between TS and immune-precipitating factors like infections. As a result, there was interest in and extensive exploration of paediatric acute-onset neuropsychiatric syndromes (PANS) which manifest with sudden onset of OCD and/or tic symptoms in childhood. The symptoms are usually dramatic and can include motor and vocal tics, obsessions and compulsions. It has been hypothesised that symptoms may arise from the development of brain-reactive autoantibodies after infection with Group A Streptococcus (GAS; Paediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal Infections, or PANDAS)[57,58]. By analogy to the pathophysiology of Sydenham's chorea, a neuropsychiatric disorder that also occurs following GAS infection, PANDAS are thought to arise from cross-reactive antibodies directed against the Streptococcus M protein binding the dopamine receptor[59-61]. Numerous studies have sought to better characterise the clinical and pathophysiological mechanisms of PANDAS; however, its diagnosis remains controversial, and its pathophysiology remains to be clearly elucidated [62].

Data on infection exposure in TS is limited and not specific for prenatal or neonatal exposure and studies on childhood infections are controversial. Population-based data obtained from American health insurance organizations demonstrated a higher risk of Streptococcal infections in the year preceding tic onset[63]. Conversely, data retrieved from UK General Practitioners' databases did not confirm that exposure to similar infections preceded the onset of tics [64]. It is unknown whether infections facilitate a hyperimmune state that eventually determines behavioural and motor symptoms or, alternatively, whether infections might reflect an underlying primary immune dysregulation. Overall, despite the presence of some evidence in favour of a role for infection in TS and behavioural symptoms, the cause–effect relationship requires further exploration.[24]

The genetic basis of immune dysregulation in TS is thought to be polygenic, though the genetic variants predisposing to immune dysregulation in TS remain widely unexplored. Recent genome wide association studies demonstrated a positive genetic correlation between TS and allergy as well as a critical gene for neuro-immune interaction (FLT3) [65,66] which may contribute to the comorbidity between TS and allergic illnesses. Furthermore, a recent study reported the association between tics and a specific polymorphism of the tumour necrosis factor gene (−308 A/G) which was previously linked to other autoimmune diseases such as asthma and Graves' disease[67].

Familial concurrence of TS with autoimmune diseases points towards a genetic predisposition to immune dysregulation which might be complicated by pre-natal exposition to immune or inflammatory mechanisms such as vertical autoantibody transmission or abnormal cytokine environments[24]. In support of this hypothesis,

recent studies from a Swedish National Registry reported that mothers, fathers and siblings of TS patients were significantly more likely to be affected by autoimmune disorders [68].

Amongst the post-natal precipitating factors for the onset of tics, a body of evidence supports a possible role for pharyngotonsillitis related to GAS. Retrospective population studies about the association between GAS exposure and onset of tics are controversial with data from US[63,69] and Denmark[70] supporting the hypothesis and data from the UK[64] not replicating the findings. Moreover, a recent multicentre European study aimed at prospectively studying the association between GAS pharyngeal exposures and tics in a population of children with chronic tic disorders did not support a role for GAS exposure in tic exacerbation [71].

Despite some discrepancies, evidence supports a dysregulation of both peripheral and central inflammatory mechanisms in TS [72]. A few studies have shown a correlation between plasma levels of different interleukins, such as IL-12, IL-2 and TNF-α, and tic severity, regardless of medical treatment or concurrent infections[73,74], as well as reduced levels of circulating IL-2 and IL-12 only in patients with comorbid OCD. Furthermore, recent studies documented a dysfunctional innate response in TS after stimulation with lipopolysaccharide [75] and dysregulated synthesis of immunoglobulins with reduced IgG3 plasma levels [74]. TS patients appear to have a lower number of circulating regulatory T (Treg) cells in keeping with an overactivation of immune responses secondary to a lack of self-antigen monitoring and prevention of autoimmune responses mediated by these cells [76]. Overall, these findings are in support of an increased susceptibility to infections and, possibly, defective immune

regulatory mechanisms that prevent autoimmunity. However, the nature and severity of autoimmune processes in TS, and their role in the pathogenesis of TS, remains unclear.

Finally, recent evidence points towards a mild degree of inflammatory change in the neural tissue of TS patients. These seem mostly mediated by the activation of the microglia, the brain's resident immune system, possibly leading to altered neuronal and/or synaptic maturation and functioning [72]. Post-mortem studies on small numbers of TS patients documented a significant increase in the expression of genes coding for IL-2 and monocyte chemotactic factor-1 [77,78], as well as upregulated immune-related genes involved in the activation of microglia in the striatum [79] and increased CD45-positive cells and local microglial reaction within the caudate/putamen.

Overall, these findings suggest that peripheral and neuronal immune events co-occur albeit partially independently. The evidence for molecular pathways associated with microglia activation and functioning in TS places this disorder within the spectrum of neurodevelopmental disorders linked to microglial dysfunction[72].

The role of dendritic cells in neurodevelopmental disorders

Dendritic cells (DC) are specialised sentinel cells that constitute the bridge between the innate and adaptive immune systems. DC recognise pathogens using pattern recognition receptors and they subsequently migrate to lymphoid organs to present pathogen-derived antigens to antigen-specific T cells. When activated, DC

produce cytokines and upregulate co-stimulatory chemicals that drive T cell maturation and differentiation. In the absence of activation, antigen presentation by steady-state DC might lead to T cell unresponsiveness and might promote tolerance[80]. DC comprise two major classes: plasmacytoid DC (PDC) and myeloid DC types 1 and 2 (MDC1 and MDC2), which significantly differ in genetics and functionality. Within each subset, some DC may preferentially sense pathogens and secrete pro-inflammatory cytokines, whereas others may be more efficient at migration, antigen presentation and T cell priming, substantially dividing them into 'detector' and 'presenter' DC [81].

Around 40 years ago, a ground-breaking study on autoimmune neuro-inflammation in mouse models identified DC in close contact with T cells in inflammatory brain lesions[82]. Strikingly, when DC taken from mice manifesting neural autoimmune disease were transferred to naïve recipient mice, they were able to induce clinical evidence of disease[83]. These studies draw attention to the role of DC in immune tolerance and autoimmune diseases.

Medullary thymic cells are the major responsible elements for negative selection of auto-reactive T cells, however thymic DC have been shown to cross-present self-antigens in the thymus, possibly facilitating the generation of Treg cells. Furthermore, the tolerogenic effect of DC seem to be mostly mediated by their functional state and, in particular, by the absence of activation[84]. It is still unclear whether DC contribute to the development of autoimmunity. DC-deficient mice were reported to develop myeloproliferative syndrome in one study and autoimmune manifestations in a subsequent similar work[85]. On the one hand, increased DC numbers have been associated with Treg cell induction and development of a tolerogenic

environment[86,87]. On the other hand, it has been suggested that increased number of DC might impair apoptosis and cause autoimmunity[88,89]. Although the evidence is incomplete and somewhat controversial, substantial evidence exists for a pathogenic role for DC in autoimmune conditions, which is determined by the activation and effector differentiation of the T cell population[84].

So far, very few studies have assessed the role of DC in neurodevelopmental disorders. An increased frequency of MDC1 was found in autistic children compared to typically developing controls, supporting a role for DC-related immune dysfunction in this condition [90,91]. To date, no studies have investigated the role of DC in TS.

Magnetic resonance spectroscopy for *in vivo* quantification of brain metabolites

Hydrogen-1 (proton) magnetic resonance spectroscopy (1H-MRS) is a technique which allows estimation of absolute concentrations of biochemical compounds or 'metabolites' in tissues including brain *in vivo* [92]. The technique exploits the principle that hydrogen-1 nuclei in different molecules exhibit distinct magnetic resonance frequencies due to their unique chemical environments, giving rise to frequency spectra in which peaks signify the abundance of different metabolites [93]. Its diagnostic use is established clinically in neuro-oncology and seizure disorders[94] and it is practicable on most commercial magnetic resonance imaging (MRI) scanners.

Metabolites detectable in brain 1H-MRS can reflect cellular processes in brain parenchyma including myeloid/glial cell activation (creatine, Cr; inositol, Ins), energy metabolism (Cr; phosphocreatine, PCr; lactate, Lac), neurotransmission (glutamate, Glu; glutamine, Gln; GABA; Glu and Gln are sometimes indistinguishable on spectra and quantified together as 'Glx'), cell turnover (choline) and neuronal loss (N-acetyl aspartate, NAA) [94-96]. Evaluation of these markers has been utilised in the study of neuro-inflammation [95,97].

Numerous studies in adults and adolescents have used 1H-MRS to investigate the neurochemical properties of TS. Most have reported results on neurotransmitter abnormalities, often in relation to clinical features. In sensorimotor cortical areas, there is evidence of increased Glu [39], reduced GABA correlated with motor tic severity[38] and abnormal correlation between beta-band oscillatory power and GABA[98]. In anterior cingulate cortex, reduced Gln was found to correlate negatively with tic severity [99] and Glu correlated positively with obsessive-compulsive symptoms[39]. In the striatum, reduction in Glx and Gln/Glu ratio were demonstrated, while negative correlations were shown between striatal Gln and tic severity and between thalamic Glu and premonitory urges [100]. 1H-MRS in children with TS has also revealed evidence of reduced NAA and creatine in frontal cortex and putamen, interpreted as reduction in neuronal number or function and energy metabolism [101].

There is an extensive literature in 1H-MRS in ASD [96] which shares a number of overlapping features with TS in terms of classification as a neurodevelopmental disorder, childhood onset, male prevalence, comorbidity profiles, stereotyped behaviours and possible pathophysiological mechanisms[102,103]. Consistent findings in

children with ASD include reduced subcortical NAA and total creatine, cortical white matter total creatine, Glx, NAA and Ins, and grey matter total creatine, Glx, NAA and choline, suggestive of globally reduced neuronal and metabolic brain activity [96].

Aim of the work

Since the psychiatric hypothesis for tics onset, much progress has been made at clarifying the physio-pathological mechanisms of TS. As presented above, strong evidence supports a role for genetic and environmental factors as well as for the involvement of basal ganglia and related cerebral cortex. Neurochemistry and neuroimaging techniques have demonstrated abnormalities of neurotransmitters in these cortico-subcortical pathways. Furthermore, a body of evidence suggests a pivotal role of dysfunctional neuro-immunological mechanisms in the genesis of tic disorders. Briefly, it is currently hypostasized that on the background of a genetic predisposition stress factors could trigger the activation of microglia influencing synaptic plasticity and inflammatory responses leading to tic onset and periodic tics exacerbations.

Notwithstanding this progress, the exact pathogenesis of tic disorders remains largely unknown. In the present work, we investigated the hypothesis that TS is associated with a dysfunctional neuro-immune crosstalk and neuro-metabolic stress. We sought to compare findings in TS patients to age- and sex-matched healthy volunteers and theorised that TS patients would show different immune phenotypes and elevated biochemical brain markers of neuroinflammation and neuro-metabolic

stress when compared to controls. Furthermore, we examined the impact of behavioural comorbidities on the measures obtained and envisage that TS patents affected by different behavioural comorbidities might show a different brain metabolic profile.

Chapter 2 analyses the link between innate and adaptive immunity in TS by exploring immune phenotyping of circulating immune cells (DC). The study also aimed investigating a putative association between DC and *in vivo* brain metabolic markers of glial activation/inflammation obtained via quantitative MRS to determine whether this supports the hypothesis of an active crosstalk between central nervous and immune systems in TS.

Chapter 3 explored the concentrations of different MRS derived brain metabolites and their relationships with clinical measures of TS disease and common associated behavioural comorbidities to clarify whether these would be in support of cerebral redox imbalance and possible consequent brain oxidative stress.

CHAPTER 2

ANALYSES OF PERIPHERAL BLOOD DENDRITIC CELLS AND MAGNETIC RESONANCE SPECTROSCOPY SUPPORT DYSFUNCTIONAL NEURO-IMMUNE CROSSTALK IN TOURETTE'S SYNDROME

ABSTRACT

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

We explored in 18 TS patients (15 stable on pharmacological treatment, 3 unmedicated) and 18 age-matched healthy volunteers (HV) circulating blood-derived DC and their relationship with clinical parameters and brain metabolites measured via proton magnetic resonance spectroscopy (1H-MRS). DC subsets, including plasmacytoid (PDC) and myeloid type 1 and 2 (MDC1, MDC2), were studied with flow cytometry. 1H-MRS measured total choline (tCho), glutamate plus glutamine (Glx), total creatine (tCr), and N-acetylaspartate and N-acetylaspartyl-glutamate (tNAA) levels in frontal white matter (FWM) and putamen (PUT).

We did not observe differences in absolute concentrations of DC subsets or brain inflammatory metabolites between patients and HV. However, TS patients manifesting anxiety showed significant increase of MDC1 when 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 TS ($p=0.0015$), but not of HV.

Elevated frequencies of MDC1 subset in TS patients manifesting anxiety may reflect a pro-inflammatory status potentially facilitating an 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 a pro-inflammatory status and metabolic changes in sensitive brain regions.

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 [12]. A body of evidence supports a dysfunctional neuro-immune cross talk in TS and other neurodevelopmental disorders, such as autism and ADHD, which might contribute to abnormalities in the trajectory of development of cortico-basal ganglia and cortico-cortical connections [104,105]. 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 [78,79]. Population-based epidemiologic and genome-wide association studies converge in demonstrating co-occurrence and genetic correlation of TS with highly prevalent autoimmune diseases [68] and allergic illnesses [106]. At a systemic level, patients with TS have shown dys- or hyper-regulated cell-mediated pro-inflammatory responses suggestive of an inflammatory state, as well as altered distribution of some immune regulatory cell types (e.g. T regulatory lymphocytes) in keeping with predisposition to autoimmunity. Finally, active immunization by direct injection of cytokines or patients' serum anti-neuronal antibodies replicated TS-like behaviours in mice [107,108 24].

Circulating peripheral blood dendritic cells (DC) constitute a critical link between innate and adaptive immune responses. They represent a heterogeneous population of professional antigen-presenting cells comprising three major DC subsets:

plasmacytoid DC (PDC), myeloid type 1 DC (MDC1), and myeloid type 2 DC (MDC2) [109]. DC are implicated in the pathogenesis of a number of autoimmune conditions including multiple sclerosis, psoriasis, type-1 diabetes and systemic lupus erythematosus [84]. An increased frequency of MDC1 was found in autistic children compared to typically developing, supporting DC-related immune dysfunction [90,91]. Circulating peripheral blood DC subsets and their relationship to neuroinflammatory changes 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 [95], as markers of neuronal or glial damage in selected brain regions [97]. For example, N-acetylaspartate changes have been previously described in patients with neurological manifestations of lupus erythematosus [110,111], while choline and lactate compound abnormalities have been linked to active inflammatory demyelination and neuronal injury in multiple sclerosis [112,113].

In the present study, we first investigated the frequency and distribution of circulating peripheral blood-derived DC in TS patients, comparing them to age- and sex- matched healthy volunteers. Subsequently, we explored the relationship between DC subsets and the clinical severity of tics and comorbid behavioural symptoms, taking into account 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 an active crosstalk between central nervous and immune systems 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 DSM-5 diagnostic criteria for TS and had received stable pharmacological treatment for the previous 3 months. Healthy volunteers (HV) 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. All data collection was performed on the same day. 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)[114], Yale-Brown Obsessive-Compulsive Scale (Y-BOCS)[115], Adult ADHD-Rating Scale

(ADRS)[116], Beck Depression Inventory-II (BDI-II)[117] , and Beck Anxiety Inventory (BAI)[118]. The YGTSS, Y-BOCS and ADRS instruments were administered by the same trained neurologist. Comorbid OCD and ADHD were diagnosed using DSM-5 criteria. Presence of anxiety was defined by a BAI score $\geq$ 8 (the latter indicating the presence of mild, moderate or severe anxiety), and presence of depression by a BDI-II score $\geq$14 [117,118].

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 hours before being processed for immunophenotyping. Subsequently, participants underwent MRI scan to obtain 1H-MRS data.

Quantification of circulating peripheral blood DC subsets

DC subsets were identified using the Human Blood DC Enumeration kit (Miltenyi Biotec). As per manufacturer's protocol, fresh peripheral blood samples (300µl 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 MDC1 (BDCA-1+), MDC2 (BDCA-3+), and PDC (BDCA-2+) (Figure 2). Each sample was stained in parallel with an isotype control mouse antibody cocktail containing IgG1-FITC, IgG2a-PE, IgG1-APC. Dead cells were excluded using a dead cell discriminator dye (PE-Cy5) (Figure 2).

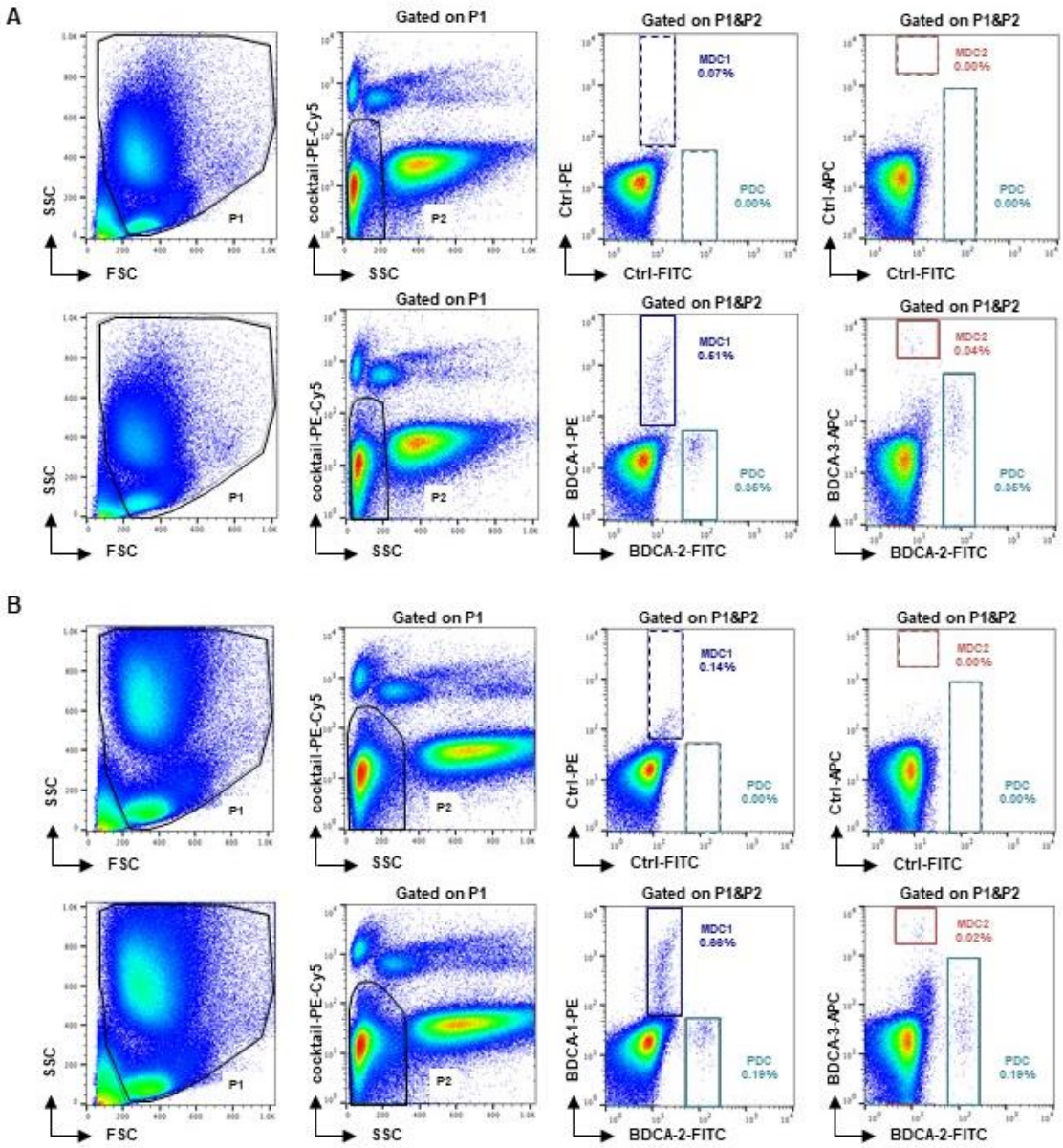

Figure 2. Quantification of circulating dendritic cell subsets. The frequency of circulating dendritic cell subsets was determined in fresh peripheral blood samples from healthy volunteers (n=16) and patients with Tourette syndrome (TS, n=17) by flow cytometry (detailed in Methods). Illustrative dot 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 dendritic cells type 1 (MDC1), myeloid dendritic cells type 2 (MDC2) and plasmacytoid dendritic cells (PDC). Dashed rectangular gates display staining with isotype control (Ctrl) antibodies (detailed in Methods) A) Healthy volunteer. B) Tourette's syndrome patient.

Samples were washed with a phosphate buffer saline (0.5% bovine serum albumin) and fixed. Flow cytometry data acquisition were performed within 3-6 hours from collection using a Navios (Beckman Coulter) and a FACSCalibur (BD Biosciences) flow cytometers, subsequently analysed with the FlowJo software (FlowJo, LLC).

1H-MRS data acquisition

1H-MRS data were acquired using a Philips 3T dual Tx Achieva MRI system with a 32-channel head coil. Sagittal 3D T1-weighted (T1w) images were acquired to provide high grey/white matter contrast that depicts brain anatomy and allows accurate MRS voxel placement (acquisition parameters: 1x1x1.5mm resolution, inversion time TI=998ms, TE=3.8ms, TR=7.8ms, flip angle 8 degrees, acquisition time 4.5 minutes). MRS voxel localisation was focused on left PUT (voxel size 30x12x10mm) and subcortical FWM (voxel size 20x12x12mm) of the right hemisphere. MRS voxel placement was performed always by the same operator, with voxels oriented obliquely to the three image planes to maximise tissue of interest and exclude surrounding tissue, as shown in Figure 3.

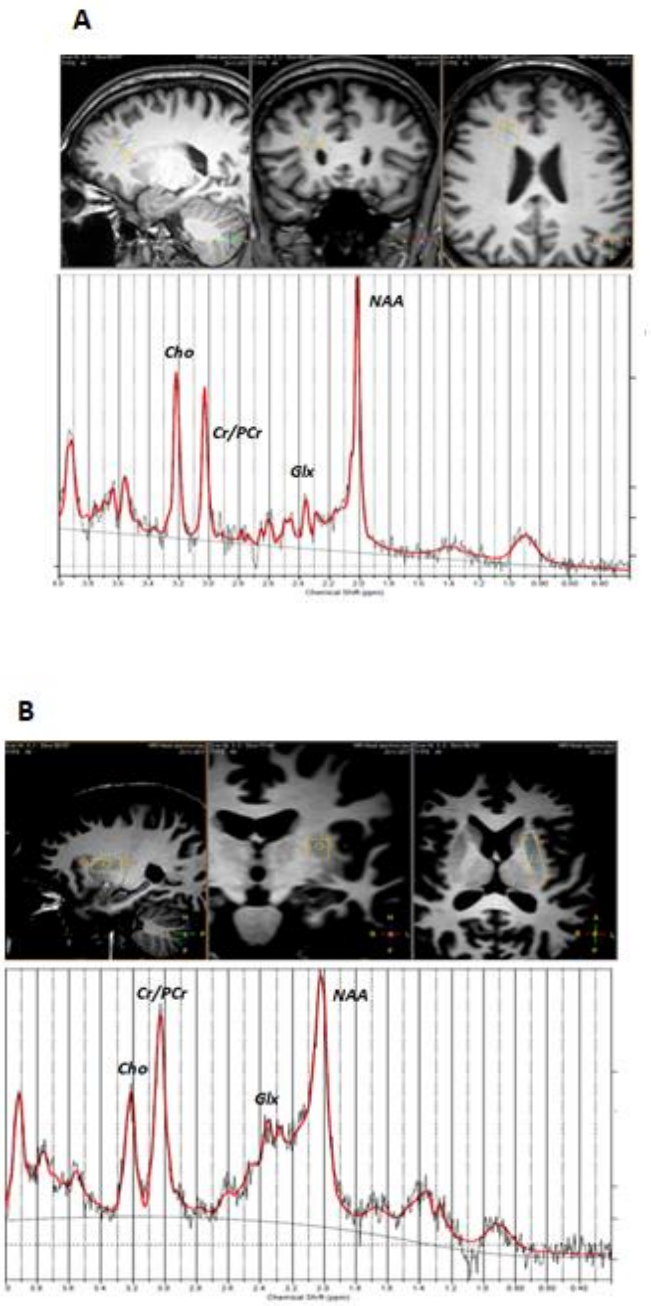

Figure 3. Voxel location and LCModel fit to the 1H MRS data in a Tourette's patient. A) frontal white matter; B) putamen. Voxel sizes were 20 x 12 x 12 mm for frontal white matter and 30 x12 x 10 mm for putamen. Voxels were obliquely positioned on the three orthogonal image planes to maximise the tissue 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: total NAA (tNAA); glutamate plus glutamine (Glx); total creatines (tCr); total cholines (tCho). 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.

1H-MRS data were obtained using the single volume Point-RESolved Spectroscopy sequence at short echo time of TE=32ms with repetition time TR=2000ms. 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 minutes. Patients alerted the operator to their own tics during scans, after which lower resolution 3D T1w images (acquisition time 51 seconds) were always acquired after each 1H-MRS acquisition to allow visual assessment of patient's movement, repeating 1H-MRS if deemed necessary. The total MRI scan time was on average approximately 30 minutes, including repetitions of 1H-MRS acquisitions.

1H-MRS data was analysed using LCModel version 6.31 [119] to determine the signal intensities of combined N-acetylaspartate and N-acetylaspartyl glutamate (tNAA), glutamate plus glutamine (Glx), total creatine plus phosphocreatine (tCr), total choline (tCho; combined phosphocholine and glycerophosphocholine). Results are reported as metabolite concentrations (mM) using the tissue water signal as a reference (assumed 41.7M). 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 TS and HV were compared using *t*-tests for independent samples. The level of significance was set at $p<0.05$ (two-tailed).

Relationships between DC subset frequencies, 1H-MRS metabolites and demographic and 1H-MRS quality parameters were first explored with bivariate correlations. As the initial analysis revealed significant correlations between metabolite concentrations and age and 1H-MRS linewidth (full width half maximum, FWHM), these parameters were used subsequently as covariates in bivariate correlation analysis and a General Linear Model (GLM) analysis.

We subsequently analysed the association of psychiatric comorbidities and drugs with DC subset frequencies and 1H-MRS brain metabolites in TS patients. TS were divided into subgroups with (TS+) and without (TS-) a specific pre-defined psychiatric comorbidity, i.e. anxiety, depression, ADHD and OCD. For each comorbidity, 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 the 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 WHO Smoking and Tobacco Use Policy definition[120]) 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 2 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 HV entered the study. The two groups were similar for demographic characteristics (Table 1). Scores for ADRS, BAI and BDI were significantly higher in TS patients compared to HV ($p=0.003$, $p=0.001$, $p=0.014$, respectively; see Table 1).

Table 1. Clinical and demographic variables

	TOURETTE'S PATIENTS (N=18)	HEALTHY VOLUNTEERS (N=18)	*p*
Age (years)	31.1 ± 8.6	28.7 ± 5.4	0.32
Sex (M/F)	14/4	9/9	0.09
Daily smokers	3†	1	0.22
Age at onset (years)	7.6 ± 2.6	-	NA
Yale Global Tic Severity Scale global score (0-100)	48.6 ± 20.4	0	NA
Yale Global Tic Severity Scale severity sub score (0-50)	22.0 ± 8.7	0	NA
Yale-Brown Obsessive - Compulsive Scale global score (0-40)	15.1 ± 9.3	0	NA
Adult ADHD rating scale (0-72)	31.1 ± 12.3	21.7 ± 14.3	**0.003***
Beck Depression Inventory-II score (0-63)	10.0 ± 9.5	3.5 ± 4.2	**0.014***
Beck Anxiety Inventory score (0-63)	18.4 ± 15.0	4.8 ± 5.0	**0.001***

*†Data not available for 3 patients; *p < 0.05*

Amongst comorbid disorders, OCD was diagnosed in 8 TS patients, ADHD in 8, anxiety in 12, and depression in 5. 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); 3 were chronic cannabis users; 3 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 errors. We did not observe significant between-group differences in frequency of MDC1 (TS, 0.60±0.20%, HV, 0.55±0.18%, p=0.41), MDC2 (TS, 0.049±0.02%, HV, 0.046±0.01%; p=0.52), and PDC (TS, 0.36±0.13%, HV, 0.41±0.16%, p=0.42) subsets. ANOVA comparing the DC subset frequency between TS patients with or without comorbidities and HV yielded a significant group effect when TS patients were subgrouped by anxiety comorbidity (p=0.025; Table 2); *post hoc* analysis showed significantly higher MDC1 frequency in TS with anxiety (TS+anxiety) compared to TS without anxiety (TS-anxiety) (p= 0.01; Figure 4).

Table 2. ANOVA sub-group analysis of DC frequencies between groups based on psychiatric comorbidities.

		Anxiety		ADHD		OCD		Depression	
		Mean ± SD	**N**	**Mean ± SD**	**N**	**Mean ± SD**	**N**	**Mean ± SD**	**N**
% of MDC1	**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
	HV	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 MDC2	**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 PDC	**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	

Abbreviations: TS + denotes Tourette's syndrome patients with comorbidity X; TS - denotes Tourette's syndrome patients without comorbidity X; HV, Healthy Volunteers. A= Anxiety, ADHD, Attention Deficit and Hyperactivity Disorder. OCD, Obsessive-Compulsive Disorder. Depression.

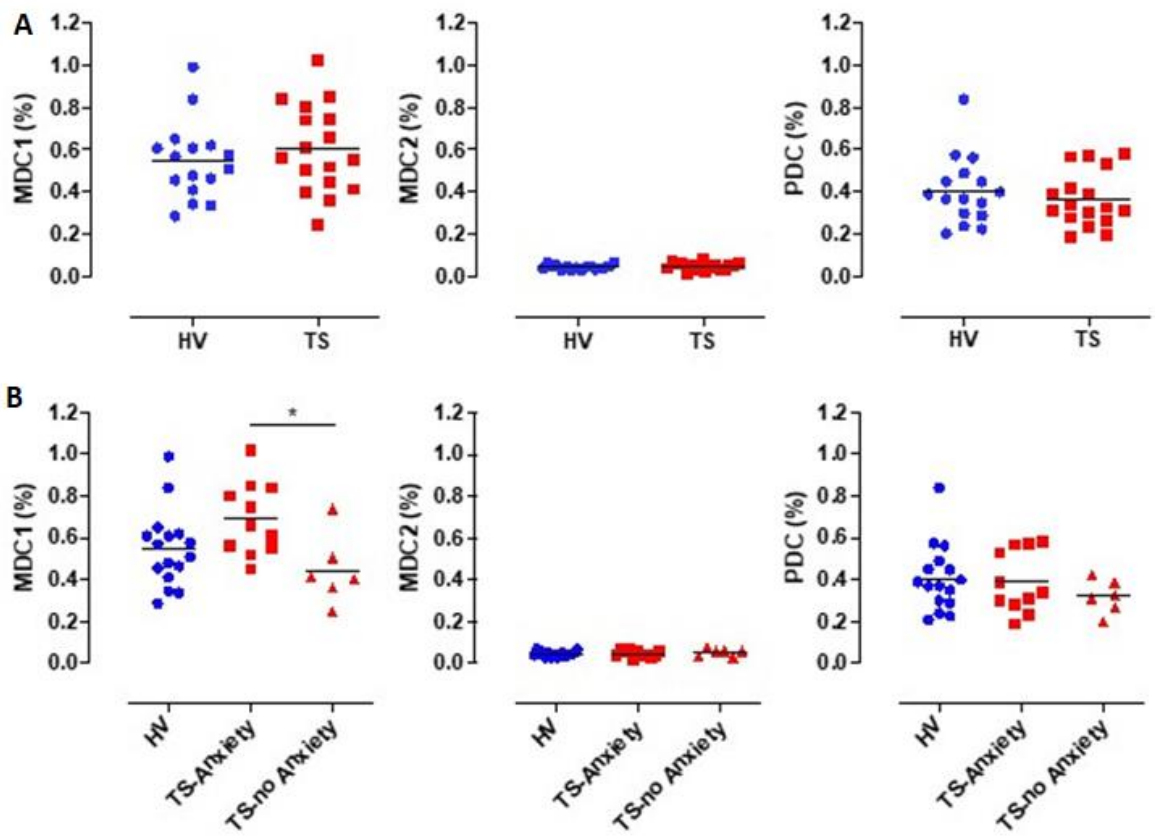

*Figure 4. Frequency of circulating dendritic cell subsets in patients with Tourette syndrome. The frequency of circulating dendritic cell subsets was determined in healthy volunteers (n=16) and patients with Tourette syndrome (TS, n=17) by flow cytometry (detailed in Methods). A. Graphs display the percentage of MDC1, MDC2 and PDC dendritic cell 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 dendritic cell 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. MDC1, myeloid dendritic cells type 1; MDC2, myeloid dendritic cells type 2; PDC, plasmacytoid dendritic cells.*

We did not detect any other significant association between other comorbidities and DC subset frequencies (Table 2). Similarly, we could not identify any significant correlation between severity of tics, obsessive-compulsive symptoms, depressive and anxiety symptoms, and frequency of DC subsets in TS patients (Table 3).

Table 3. Correlations between DC subsets and clinical variables in TS patients.

CLINICAL VARIABLES						
		YGTSS severity	**Y-BOCS**	**ADRS**	**BAI**	**BDI-II**
MDC1	**Pearson Correlation**	0.21	0.083	0.232	0.256	0.438
N=17	**Sig. (2-tailed)**	0.419	0.751	0.371	0.322	0.079
MDC2	**Pearson Correlation**	0.279	-0.1	-0.145	-0.097	-0.072
N=17	**Sig. (2-tailed)**	0.278	0.701	0.58	0.71	0.782
PDC	**Pearson Correlation**	0.462	0.034	0.248	0.34	0.124
N=17	**Sig. (2-tailed)**	0.062	0.897	0.336	0.181	0.636

**YGTTS severity= Yale Global Tic Severity Ratings items only; Y-BOCS= Yale-Brown Obsessive-Compulsive Scale; ADRS= Adult ADHD-Rating Scale; BAI= Beck Anxiety Inventory; BDI-II= Beck Depression Inventory-II*

Finally, ANOVA comparing TS patient subgroups divided according to current antipsychotic exposure and HV did not show any significant effect of clinical group (Table 4). Likewise, sensitivity analyses testing the potential impact of other drug classes on DC subsets frequency did not reveal significant associations (Table 4).

Table 4. DC subset sensitivity analysis between TS groups based on exposure to psychotropic medications and nicotine.

		ANOVA		**t-tests[a]**											
		Antipsychotics (N=7)			Excluding SSRI (N=3)		Excluding Benzodiazepines (N=3)		Excluding Cannabis (N=3)		Excluding Clonidine (N=1)		Excluding Atomoxetine (N=1)		Excluding Nicotine (N=3)	
		Mean± SD	N		Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N
% MDC1	**TS +**	0.63±0.19	6													
	TS-	0.59±0.22	11	**TS-**	0.57±0.22	14	0.56±0.18	14	0.59±0.22	14	0.60±0.21	16	0.60±0.21	16	0.56±0.18	12
	HV	0.55±0.18	16	**HV**	0.55±0.18	16	0.55±0.18	16	0.55±0.18	16	0.55±0.18	16	0.55±0.18	16	0.56±0.19	15
	F (2,30)	0.419		**t**	-0.593		-0.254		-0.586		-0.762		-0.691		0.016	
	P	0.662		**p**	0.558		0.802		0.563		0.452		0.495		0.987	
% MDC2	**TS +**	0.05±0.01	6													
	TS-	0.05±0.02	11	**TS-**	0.05±0.02	14	0.05±0.02	14	0.05±0.02	14	0.05±0.02	16	0.05±0.02	16	0.05±0.01	12
	HV	0.05±0.01	16	**HV**	0.05±0.01	16	0.05±0.01	16	0.05±0.01	16	0.05±0.01	16	0.05±0.01	16	0.05±0.02	15
	F (2,30)	0.366		**t**	-1.115		-0.07		-0.705		-1.044		-0.769		-0.282	
	P	0.697		**p**	0.274		0.945		0.487		0.305		0.448		0.780	
% PDC	**TS +**	0.33±0.13	6													
	TS-	0.38±0.13	11	**TS-**	0.39±0.13	10	0.34±0.12	14	0.34±0.12	14	0.38±0.12	16	0.35±0.12	16	0.40±0.16	12
	HV	0.41±0.16	16	**HV**	0.41±0.16	16	0.41±0.16	16	0.41±0.16	16	0.41±0.16	16	0.41±0.16	16	0.32±0.10	15
	F (2,30)	0.935		**t**	0.405		1.205		1.336		0.602		1.079		1.486	
	P	0.591		**p**	0.689		0.238		0.192		0.551		0.289		0.150	

1H-MRS data from TS patients (2 FWM, 3 PUT) and HV (3 FWM, 1 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. Assessment of quality of accepted data with LCModel gave mean and standard deviation of the water FWHM and metabolite signal-to-noise ratio (SNR) 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 putamen (n=32), without significant differences between patients and HV.

Metabolite concentrations change with age [121] and age-related changes in iron deposition in the basal ganglia [122] may also change water relaxation times [121], thus affecting metabolite estimates. PDC are also known to decrease with age [123]. 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 PDC 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 total creatine levels (tCr) and MDC1 subset in the frontal white matter (FWM) of TS patients (r=-0.784, p=0.0015), which survived a Bonferroni-corrected significance level of 0.0021 for 24 comparisons (four metabolites, three cell types and two regions), but not in putamen (r=-0.444, p=0.148) (Table 5). Other correlations significant at $p<0.05$ (not Bonferroni-corrected) were: NAA with PDC (r=-0.588, p=0.035) in FWM of TS, and Glx with PDC (r=0.651, p=0.022) in PUT of TS patients. No significant correlations were present for both FWM and PUT in HV (Table 5).

Table 5. Correlations between DC subsets and MRS metabolites in FWM and PUT in TS and HV.

			MRS METABOLITES									
			FRONTAL WHITE MATTER					PUTAMEN				
			N	tCho	tCr	NAA	Glx	N	tCho	tCr	NAA	Glx
TOURETTES	MDC1	Pearson Correlation	15	0.273	**-0.784****	-0.429	-0.202	14	0.085	-0.444	-0.240	0.425
		Sig. (2-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
		Sig. (2-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***
		Sig. (2-tailed)		0.699	0.307	**0.035**	0.250		0.364	0.231	0.411	**0.022**
VOLUNTEERS	MDC1	Pearson Correlation	13	0.531	0.090	0.212	-0.562	15	0.252	0.016	0.379	0.144
		Sig. (2-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
		Sig. (2-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
		Sig. (2-tailed)		0.517	0.795	0.665	0.253		0.401	0.452	0.858	0.788

*tCho= total Choline; tCr= total creatine; NAA= N-acetylaspartate; Glx= glutamate +glutamine; *correlation is significant at the 0.05 level; **correlation is significant at the 0.0021 level (corrected by multiple comparison factor); covariates: age and full-width half maximum (FWHM).*

The correlation of tCr with MDC1 in FWM of TS patients was also highly significant without covariates (r=-0.797, p<0.001; Figure 5A). Although not significant, there was a trend for a tCr decrease with MDC1 in putamen, which closely matches that of the correlation in FWM (Figure 5A); this correlation was not found in HV (Figure 5B).

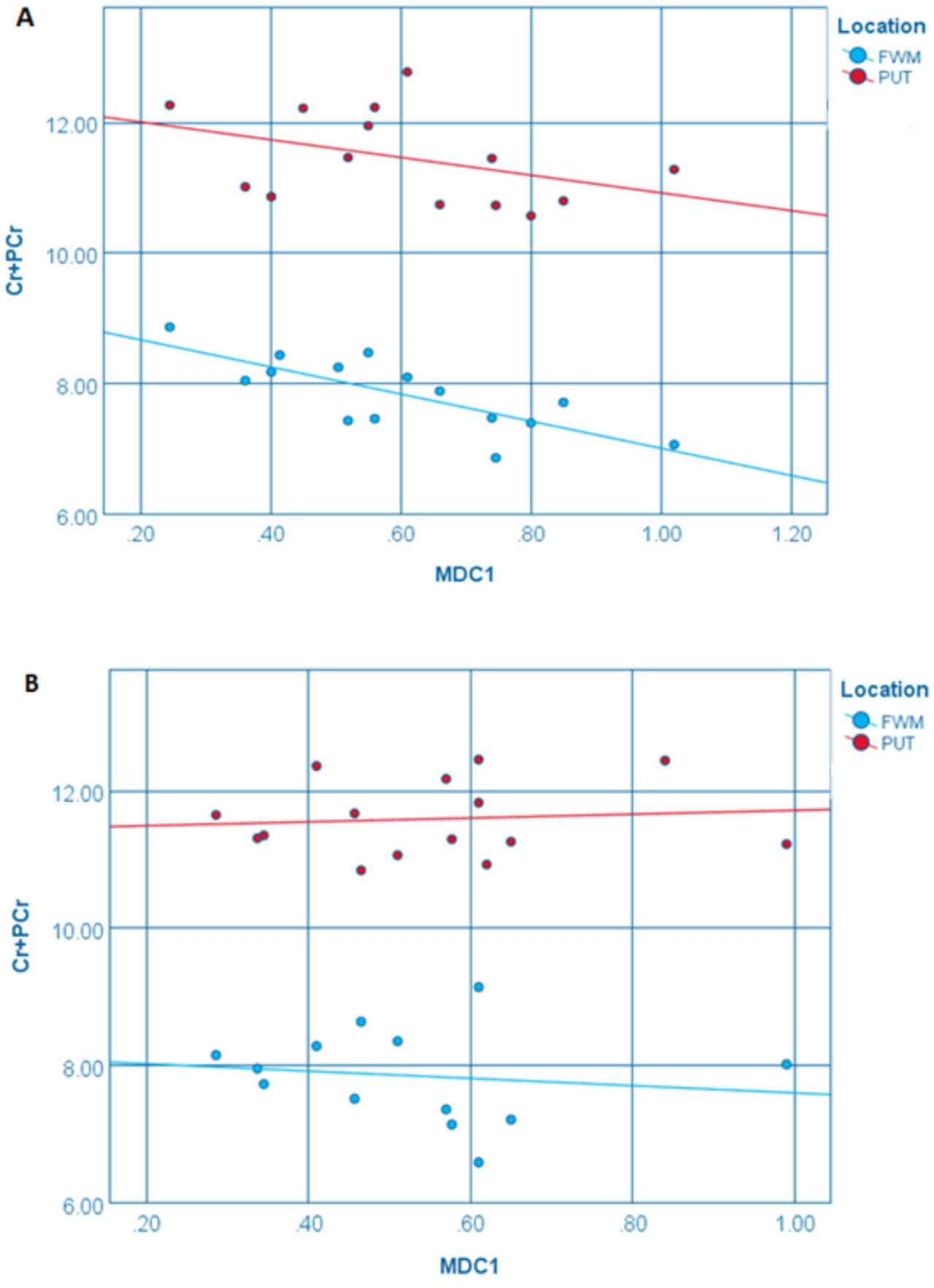

Figure 5. Scatter plot of MDC1 and total Creatine (Cr+PCr) correlation by location. A) TS and B) HV.

A general linear model investigated the relationship between tCr and MDC1 including both putamen and FWM data, with age and FWHM as covariates. tCr correlated to MDC1 across both anatomical regions with F=12.61, p=0.002, with also a significant age effect and highly significant effect size for location (Table 6).

Finally, sensitivity analyses testing the potential impact of drug classes on tested brain metabolites either in FWM and in PUT did not show significant associations (Table 7, A and B).

Table 6. General linear model aimed at investigating relationships between total creatine (tCr) and MDC1 by location including possible confounding factors (age and FWHM).

Dependent Variable:	**tCr**				
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	99.335[a]	4	24.834	94.92	0.000
Intercept	83.173	1	83.173	317.907	0.000
MDC1	3.299	1	3.299	12.61	0.002
FWHM	0.686	1	0.686	2.623	0.118
Age	1.161	1	1.161	4.438	0.046
Location	12.998	1	12.998	49.683	0.000
Error	6.279	24	0.262		
Total	2772.362	29			
Corrected Total	105.614	28			
[a] R-squared = 0.941 (Adjusted R-squared = 0.931)					

tCr= total creatine; MDC1= myeloid dendritic cells type 1; FWHM: water full-width half maximum.

Table 7A and 7B. MRS metabolites sensitivity analysis between TS groups based on exposure to psychotropic medications and nicotine in A) FWM and B) PUT.

7A		ANOVA			t-tests[a]											
			Excluding Antipsychotics (N=7)			Excluding Antidepressants (N=3)		Excluding Benzodiazepines (N=3)		Excluding Cannabis (N=3)		Exculding Clonidine (N=1)		Excluding Atomoxetine (N=1)		Excluding Nicotine (N=3)	
			Mean±SD	N		Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N
FRONTAL WHITE MATTER	tCho	TS +	2.89±0.56	6													
		TS-	2.79±0.47	10	TS-	2.86±0.52	14	2.76±0.50	13	2.78±0.53	13	2.84±0.50	15	2.86±0.49	15	2.79±0.54	10
		HV	3.04±0.46	15	HV	3.04±0.46	15	3.04±0.46	15	3.04±0.46	15	3.04±0.46	15	3.04±0.46	15	3.06±0.47	14
		F (2,28)	0.858		t	0.994		1.561		1.406		1.154		1.058		1.329	
		P	0.435		P	0.329		0.131		0.171		0.258		0.299		0.198	
	tNAA	TS +	11.71±1.12	6													
		TS-	12.10±1.06	10	TS-	11.87±1.07	14	12.23±0.98	13	12.06±1.11	13	11.97±1.10	15	12.03±1.05	15	11.96±1.05	10
		HV	11.83±0.53	15	HV	11.83±0.53	15	11.83±0.53	15	11.83±0.53	15	11.83±0.53	15	11.83±0.53	15	11.81±0.54	14
		F (2,28)	0.487		t	-0.113		-1.361		-0.723		-0.424		-0.662		-0.404	
		P	0.62		p	0.911		0.185		0.476		0.674		0.514		0.69	
	tCr	TS +	7.88±0.91	6													
		TS-	7.98±0.53	10	TS-	7.92±0.71	14	8.01±0.66	13	7.91±0.73	13	7.95±0.69	15	8.01±0.62	15	7.89±0.59	10
		HV	7.83±0.65	15	HV	7.83±0.65	15	7.83±0.65	15	7.83±0.65	15	7.83±0.65	15	7.83±0.65	15	7.81±0.67	14
		F (2,28)	0.161		t	-0.383		-0.736		-0.305		-0.484		-0.805		-0.263	
		P	0.852		P	0.705		0.468		0.763		0.632		0.428		0.795	
	Glx	TS +	13.06±3.32	6													
		TS-	13.69±1.47	10	TS-	13.49±2.41	14	13.92±2.04	13	13.42±2.49	13	13.44±2.33	15	13.64±2.21	15	12.88±2.33	10
		HV	12.13±1.42	15	HV	12.13±1.42	15	12.13±1.42	15	12.13±1.42	15	12.13±1.42	15	12.13±1.42	15	12.06±1.45	14
		F (2,28)	2.065		t	-1.875		-2.724		-1.721		-1.871		-2.231		-1.067	
		P	0.146		P	0.072		0.011		0.097		0.072		0.034		0.298	

7B		ANOVA			t-tests[a]											
PUTAMEN			Excluding Antipsychotics (N=7)			Excluding Antidepressants (N=3)		Excluding Benzodiazepines (N=3)		Excluding Cannabis (N=3)		Exculding Clonidine (N=1)		Excluding Atomoxetine (N=1)		Excluding Nicotine (N=3)	
	tCho		Mean±SD	N		Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	N
		TS +	2.57±0.25	6													
		TS-	2.45±0.38	9	TS-	2.50±0.34	14	2.50±0.33	12	2.54±0.35	12	2.50±0.34	14	2.49±0.34	14	2.56±0.39	9
		HV	2.39±0.21	17	HV	2.39±0.21	17	2.39±0.21	17	2.39±0.21	17	2.39±0.21	17	2.39±0.21	17	2.40±0.21	16
		F (2,29)	0.918		t	-1.05		-1.102		-1.475		-1.05		-0.989		-1.357	
		P	0.411		P	0.303		0.28		0.152		0.303		0.331		0.188	
	tNAA	TS +	12.76±0.62	6													
		TS-	12.68±1.03	9	TS-	12.63±0.84	14	12.80±0.86	12	12.70±0.87	12	12.63±0.84	14	12.73±0.89	14	12.83±0.86	9
		HV	12.73±0.70	17	HV	12.73±0.70	17	12.73±0.70	17	12.73±0.70	17	12.73±0.70	17	12.73±0.70	17	12.73±0.73	16
		F (2,29)	0.02		t	0.346		-0.25		0.102		0.346		-0.026		-0.314	
		P	0.98		p	0.732		0.804		0.919		0.732		0.98		0.756	
	tCr	TS +	11.27±0.67	6													
		TS-	11.62±0.71	9	TS-	11.54±0.69	14	11.54±0.72	12	11.39±0.63	12	11.54±0.69	14	11.54±0.69	14	11.35±0.72	9
		HV	11.69±0.59	17	HV	11.69±0.59	17	11.69±0.59	17	11.69±0.59	17	11.69±0.59	17	11.69±0.59	17	11.72±0.60	16
		F (2,29)	0.97		t	0.686		0.647		1.321		0.686		0.683		1.385	
		P	0.391		P	0.498		0.523		0.198		0.498		0.5		0.179	
	Glx	TS +	22.91±2.16	6													
		TS-	22.29±1.78	9	TS-	22.44±1.93	14	22.56±2.01	12	22.18±1.83	12	22.44±1.93	14	22.48±1.95	14	21.92±1.82	9
		HV	22.30±1.54	17	HV	22.30±1.54	17	22.30±1.54	17	22.30±1.54	17	22.30±1.54	17	22.30±1.54	17	22.17±1.48	16
		F (2,29)	0.307		t	-0.228		-0.39		0.195		-0.228		-0.28		0.368	
		P	0.738		P	0.821		0.7		0.847		0.821		0.781		0.716	

*Abbreviations: TS + denotes Tourette's syndrome patients on substance X; TS - denotes Tourette's syndrome patients not on substance X; tCho= total Choline; tCr= total creatine; tNAA= combined N-acetylaspartate and N-acetylaspartyl glutamate ; Glx= glutamate + glutamine; **correlation is significant at the 0.003 level (corrected by multiple comparison factor). [a] Two independent sample t-test applied where sample size < 6 in one TS subgroup.*

DISCUSSION

To our knowledge, our study is the first to investigate the distribution of circulating DC subsets in TS, and to explore its relationship with the comorbidity profile of TS. In contrast to results reported in autism[90,91] we did not observe differences in the frequency of circulating DC subsets between TS patients and age-matched healthy subjects. Whereas the frequency of DC subsets did not correlate with tic severity, we detected an increase in MDC1 subset frequency in TS patients affected by anxiety symptoms (mild, moderate or severe). Moreover, our analysis of a possible association between DC subset frequency and metabolite levels within fronto-subcortical network regions showed a strong correlation between MDC1 DC subset frequency and tCr levels in the FWM of TS patients, but not in HV. 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, DC on the frequency and intensity of the abnormal behaviours typical of the TS spectrum. Our findings support rather that MDC1 frequency may potentially be a 'trait' marker of pathological anxiety 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 [124,125] remain heavily under-investigated. They may involve peripheral effects, including abnormal interleukin production that may drive naïve self-reactive T cells to react against central nervous system (CNS) tissue, or failure to generate/maintain T-cell tolerance via negative selection in the thymus. The increased prevalence of generalized anxiety disorder (GAD) in the TS population is well recognized [126], as well as the increased level of circulating cortisol and pro-inflammatory cytokines in individuals with generalized or other types of anxiety disorders [127,128]. Mild anxiety symptoms have also been associated with altered gene expression patterns of innate and adaptive immune responses [129]. 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 [130]and antigen presentation functions and, therefore, their ability to generate an effective T-cytotoxic response [131,132]. 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, MDC1 cells have a specific capability to present antigens via MHC class II to activate naïve $CD4^+$ T cells, and to promote T helper 1 responses [109]. As previously demonstrated in animal models of multiple sclerosis, clinical manifestations of CNS autoimmunity are preceded by a phase of microglia expansion and myeloid DC peripheral proliferation [133,134]. Similarly, in TS adults affected by anxiety, the peripheral increase of MDC1 may reflect a chronic pro-inflammatory status possibly facilitating an altered neuro-immune crosstalk.

To date, 1H-MRS studies of TS highlighted neurochemical changes associated with this diagnosis and/or with tic severity with some inter-study heterogeneity [98,100,135]. In line with our findings, previous reports demonstrated a reduction of tCr in putamen, right frontal cortex and thalamus [100,101]. 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 recognised [136,137]. Genetically determined deficits of creatine phenotypically present with severe neurological symptoms from young age [138]. 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 and a systemic inflammatory state. Alternatively, this correlation could suggest a direct influence on immune regulatory mechanisms at a systemic level [139,140] exerted by a generalised 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 (TCR) signalling during negative selection in the thymus [141], a key mechanism for self-antigen tolerance and the pathogenesis of autoimmune disease. If altered creatine metabolism influences CK isoform activity, a potential effect of this could be a dysfunction in the regulatory effect of CK-BB upon TCR signalling in T cells, contributing to their dysregulation, promotion of a chronic inflammatory state, and predisposition to autoreactive immune processes, particularly in TS patients with co-existing pathological 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 comorbid anxiety or behavioural symptoms, 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[134,140,141], 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 our study. Levels of Glu and Gln may be influenced by nicotine[142] and sleep patterns[143,144]. Similarly, tNAA levels may be affected by nicotine use[142], lactate may increase following caffeine ingestion[145] and choline and Glx levels exhibit diurnal variations[144,146]. A region-dependent reduction in tCr was reported in middle-aged smokers compared to non-smokers[142]. Although controversial, a sex-dependent variation of all metabolites was previously suggested for specific brain

regions[147,148]. Yet, our sensitivity analyses did not detect a significant confounding effect of tobacco smoking on the tCr-MDC1 observed relationship and an effect of diurnal variation is unlikely as MRIs were all 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, here we report an increase of the MDC1 subset of DC 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.

CHAPTER 3

MAGNETIC RESONANCE SPECTROSCOPY REVEALS EVIDENCE OF BRAIN OXIDATIVE STRESS IN TOURETTE'S SYNDROME

ABSTRACT

The hypothesis of an excitatory-inhibitory neurotransmitter imbalance leading to a dysfunction of cortico-striato-thalamo-cortical (CSTC) circuits in Tourette's syndrome (TS) is supported by neuroimaging, electrophysiological and post-mortem studies. Although the exact neurochemicals involved and their role remain yet to be determined, neurotransmitter abnormalities play a key role in the development of the TS phenotype including tics and behavioural comorbidities. Proton magnetic resonance spectroscopy (1H-MRS) is a unique instrument to study *in vivo* brain metabolites. We enrolled 18 TS patients and 18 age-matched healthy volunteers (HV). 1H-MRS was acquired in frontal white matter (FWM) and putamen (PUT) to measure total choline (tCho), glutamate plus glutamine (Glx), total creatine (tCr), total N-acetyl aspartate and N-acetylaspartyl glutamate (tNAA), inositol (Ins) and glutathione (GSH) levels. We correlated concentrations of brain metabolites with clinical parameters, namely measures of severity of tics and psychiatric comorbidities. We observed a significantly reduced concentration of glutamate (Glu) in TS patients when compared to HV ($p=0.046$) as well as a positive linear correlation between measures of disease severity and GSH in FWM ($r^2=0.479$, $p=0.044$) when covarying for age and gender. Furthermore, TS patients not affected by anxiety showed significantly reduced levels of GSH in PUT when compared with TS with anxiety and HV.

Increased Glu levels in TS patients' FWM is in keeping with previous literature and in further support of an excitation-inhibition balance within CSTC circuits. The direct correlation between GSH levels and disease severity supports a compensatory mechanism in response to a hyper glutamatergic state which might increase redox

stress and chronic inflammatory response. Finally, the subgroup of TS patients not affected by anxiety showed a decrement of GSH concentration in the FWM supporting the influence of behavioural comorbidities on the neurometabolic profile of adult TS.

INTRODUCTION

Gilles de la Tourette syndrome or Tourette syndrome (TS) is a childhood-onset neurodevelopmental movement disorder characterized by the presence of several motor and phonic tics[2]. Up to 90% of TS patients are affected by comorbid neurodevelopmental or behavioural pathologies, including obsessive compulsive behaviour and disorder (OCD), attention deficit and hyperactivity disorder (ADHD), mood/anxiety disorders, and impulse control disorders[12].

Over the past 30 years, there has been a constant effort to unveil the pathophysiological mechanisms of TS and to identify the neuronal locus or networks involved in its emergence. Different neuroimaging techniques, electrophysiological studies, animal models, and post-mortem studies support the hypothesis of an excitatory-inhibitory neurotransmitter imbalance within cortico-striato-thalamo-cortical (CSTC) circuits [26,27]. Although the exact neurochemicals and their specific role remain yet to be determined, dopamine[31,149], glutamate[100], and GABA[37] seem to be primarily involved. The hyperactivity of the dopaminergic system in TS is widely accepted and is founded on demonstrated abnormalities in dopamine receptor binding and increased density of dopamine receptors in the prefrontal cortex and the striatum of TS patients [31-33]. GABA is the primary inhibitor neurotransmitter of both striatum and cortex and dysfunction of the GABAergic system may conceivably underlie the symptoms of motor disinhibition presenting as tics and psychiatric manifestations[38]. Moreover, evidence in support of a role for the glutamatergic system in TS include its fundamental role in the CSTC pathways and wide interaction with both the dopaminergic and GABAergic neurons at this level[45] as well as the well-known

therapeutic efficacy of glutamate antagonist drugs on OCD symptoms[47]. Similarly, neurotransmitter abnormalities and anomalies of cellular redox compounds (namely, glutathione) have been described in other neurodevelopmental and psychiatric diseases such as autism spectrum disorder, OCD and schizophrenia as well as autoimmune conditions[150-153].

Proton magnetic resonance spectroscopy (1H-MRS) is a non-invasive method of exploring *in vivo* cerebral metabolic changes[95] and has been widely used to determine alterations of neurochemicals in several neurodevelopmental[96], neurodegenerative[154] and neuroimmune conditions[155], including TS[101,135]. So far, functional and structural imaging findings are heterogeneous and sometimes conflicting, often leaving researchers puzzled and leading to different interpretations[26].

In this study, we first examined the concentrations of different MRS derived brain metabolites in TS patients and subsequently compared them to age- and sex-matched healthy volunteers. Second, we explored the relationship between brain metabolites and clinical measures of disease and common behavioural comorbidities, taking into account the potential influence of exposure to psychotropic medications. We hypothesized that TS patients would manifest altered biochemical markers of brain oxidative stress measured *in vivo* via 1H-MRS to determine whether this would be in support of a cerebral redox imbalance. Finally, we explored the hypothesis that TS patents affected by different behavioural comorbidities (anxiety, depression, OCD and ADHD) might show a different brain metabolic profile.

MATERIALS AND METHODS

Participants

Patients were recruited from the St. George's University Hospital Tic Disorder and Movement Disorders clinic if they fulfilled DSM-5 diagnostic criteria for TS and had received stable pharmacological treatment for the previous 3 months. Healthy volunteers (HV) 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

All participants were administered the Yale Global Tic Severity Scale (YGTSS)[114], Yale-Brown Obsessive-Compulsive Scale (Y-BOCS)[115], Adult ADHD-Rating Scale (ADRS)[116], Beck Depression Inventory-II (BDI-II)[117] , and Beck Anxiety Inventory (BAI)[118]. The YGTSS, Y-BOCS and ADRS instruments were administered by the same trained neurologist. The Yale Global Tic Severity Scale Tic Severity Score (YGTSS-TTS) (items 0-50) was used as the measure of clinical severity of tics. Comorbid OCD and ADHD were diagnosed using DSM-5 criteria. Presence of anxiety was defined by a BAI score $\geq$ 16, and presence of depression by a BDI-II score $\geq$14 [117,118]. Subsequently, participants

underwent MRI scan to obtain 1H-MRS data, which was performed in the afternoon in all cases.

1H-MRS data acquisition

1H-MRS data were acquired using a Philips 3T dual Tx Achieva MRI system with a 32-channel head coil. Sagittal 3D T1-weighted (T1w) images were acquired to provide high grey/white matter contrast that depicts brain anatomy and allows accurate MRS voxel placement (acquisition parameters: 1x1x1.5mm resolution, inversion time TI=998ms, TE=3.8ms, TR=7.8ms, flip angle 8 degrees, acquisition time 4.5 minutes). MRS voxel localisation was focused on left PUT (voxel size 30x12x10mm) and subcortical FWM (voxel size 20x12x12mm) of the right hemisphere. MRS voxel placement was performed always by the same operator, with voxels oriented obliquely to the three image planes to maximise tissue of interest and exclude surrounding tissue, as shown in Figure 3.

1H-MRS data were obtained using the single volume Point-RESolved Spectroscopy sequence at short echo time of TE=32ms with repetition time TR=2000ms. 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 minutes. Patients alerted the operator to their own tics during scans, after which lower resolution 3D T1w images (acquisition time 51 seconds) were always acquired after each 1H-MRS acquisition to allow visual assessment of patient's

movement, repeating 1H-MRS if deemed necessary. The total MRI scan time was on average approximately 30 minutes, including repetitions of 1H-MRS acquisitions.

1H-MRS data was analysed using LCModel version 6.31 [119] to determine the signal intensities of combined N-acetylaspartate and N-acetylaspartyl glutamate (tNAA), glutamate plus glutamine (Glx), total creatine plus phosphocreatine (tCr), total choline (tCho, phosphocholine plus glycerophosphocholine), glutathione (GSH) and inositol (Ins) in both FWM and PUT. In FWM glutamate (Glu) and glutamine (Gln) were also measured individually. Results are reported as metabolite concentrations (mM) using the tissue water signal as a reference (assumed 41.7M). 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 ($p>0.05$). 1H-MRS metabolite brain levels in TS and HV were compared using t-tests for independent samples. The level of significance was set at $p<0.05$ (two-tailed).

Relationships between 1H-MRS metabolites and demographic and 1H-MRS quality parameters were first explored with bivariate Pearson correlations. As the initial analysis revealed significant correlations between metabolite concentrations and age and 1H-MRS linewidth (full width half maximum, FWHM), these parameters were used subsequently as covariates in bivariate correlation analysis.

We subsequently analysed the association of psychiatric comorbidities and drugs with 1H-MRS brain metabolites in TS patients. TS were divided into subgroups with (TS+) and without (TS-) a specific pre-defined psychiatric comorbidity, i.e., anxiety, depression, ADHD, and OCD. For each comorbidity, ANOVA was used to assess the effect of 'group' (TS+, TS- and HV); where significant, post hoc t-tests corrected by relevant correlation factor were used to perform pairwise comparisons between the groups (significance level $p<0.05$). A similar approach was used to explore associations with medication exposure. TS patients were divided into TS with and without exposure to antipsychotic drugs, and the effect of 'group' was explored with ANOVA. Potential effects of other medication classes were evaluated conducting sensitivity analyses after exclusion of TS patients with each specific medication class (any antidepressant, SSRIs only, benzodiazepines, cannabis, clonidine, and atomoxetine). For each drug category, between-group differences between HV, TS+ and TS- groups were explored using ANOVA with post hoc t-test corrected by relevant correlation factor where significant (significance level $p<0.05$). In cases where one group contained fewer than 2 patients, an independent t-test comparing the remaining two groups was performed instead of ANOVA (significance level $p<0.05$).

RESULTS

Eighteen TS patients and 18 HV entered the study. The two groups were similar for demographic characteristics (Table 1). Scores for ADRS, BAI and BDI were significantly higher in TS patients compared to HV (p=0.003, p=0.001, p=0.014, respectively; see Table 1). Amongst comorbid disorders, OCD was diagnosed in 8 TS patients, ADHD in 8, anxiety in 7, and depression in 5. 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); 3 were chronic cannabis users and none was on behavioural treatment for tics or had undergone functional brain surgery.

1H-MRS data from TS patients (2 FWM, 3 PUT) and HV (3 FWM, 1 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. Assessment of quality of accepted data with LCModel gave mean and standard deviation of the water FWHM and metabolite signal-to-noise ratio (SNR) 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 putamen (n=32), without significant differences between patients and HV.

In the FWM, we observed a significantly increased concentration of Glu in TS patients when compared to HV (t=-2.01; p=0.046). Conversely, we did not observe between-groups differences in any brain metabolite levels in the PUT (see Table 8).

*Table 8. Comparison of brain metabolite concentrations (mmol/l) in healthy controls (HV) and Tourette's patients (TS) in frontal white matter (FWM) and putamen (PUT) using unpaired 2-tailed t-test. T-statistic (t), degrees of freedom (df) and p-value (p) shown. The only significant difference was found for Glu in FWM (*p<0.05).*

	n (HV/TS)	HV	TS	t	df	p
FWM						
Gln	15/16	3.0 ± 0.6	3.3 ± 1.0	-0.855	29	0.400
Glu	15/16	9.1 ± 1.3	10.2 ± 1.6	-2.087	29	**0.046***
GSH	15/16	3.1 ± 1.1	3.4 ± 1.0	-0.753	29	0.458
Ins	15/16	6.8 ± 2.4	7.1 ± 2.4	-0.362	29	0.720
tCho	15/16	3.0 ± 0.5	2.8 ± 0.5	1.264	29	0.216
tNAA	15/16	11.8 ± 0.5	12.0 ± 1.1	-0.405	29	0.689
Glx	15/16	12.1 ± 1.4	13.5 ± 2.3	-1.949	29	0.061
tCr	15/16	7.8 ± 0.6	7.9 ± 0.7	-0.485	29	0.631
PUT						
GSH	17/16	5.9 ± 1.0	5.5 ± 1.5	0.962	31	0.344
Ins	17/16	5.2 ± 0.8	4.8 ± 1.0	1.240	31	0.224
tCho	17/16	2.4 ± 0.2	2.5 ± 0.3	-1.074	31	0.291
tNAA	17/16	12.7 ± 0.7	12.7 ± 0.8	0.028	31	0.978
Glx	17/16	22.3 ± 1.5	22.0 ± 2.9	0.414	31	0.682
tCr	17/16	11.7 ± 0.6	11.3 ± 1.0	1.393	31	0.174

We found positive univariate correlations between YGTSS-TSS and GSH in FWM (r= 0.601, p=0.023) and between YGTSS-TSS and Glx in PUT (r=0.547, p=0.043). Linear regressions between YGTSS-TTS and these correlated metabolites, considering age and gender as covariates, yielded a significant association with GSH in FWM (r^2=0.479, p=0.044; Table 9) but not with Glx in PUT (r^2=0.423, p=0.77). Furthermore, GSH showed a trend of significance as a single predictor (p=0.055). (See Table 9).

Table 9. Linear regression of YGTSS-TSS with GSH in FWM, with age and gender as covariates. GSH in FWM was a significant predictor of YGTSS-TSS in this model (p=0.044).

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,692[a]	.479	.349	7.33261

a. Predictors: (Constant), Age, Gender, GSH

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	592.732	3	197.577	3.675	,044[b]
	Residual	645.206	12	53.767		
	Total	1237.938	15			

a. Dependent Variable: YGTSS-TSS

b. Predictors: (Constant), Age, Gender, GSH

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	20.010	9.107		2.197	.048
	GSH	4.555	2.138	.507	2.130	.055
	Gender01	4.939	5.117	.219	.965	.354
	Age	-.538	.251	-.480	-2.147	.053

a. Dependent Variable: YGTSS-TSS

Finally, ANOVA comparing brain metabolite concentrations between TS patients with or without psychiatric comorbidities and HV showed a significant group effect only in concentrations of GSH in PUT when TS patients were sub-grouped by anxiety comorbidity (p=0.003; Table 10 and Table 11).

Table 10. Brain metabolites in FWM of HV and TS patients subgrouped by psychiatric comorbidity (TS+, with comorbidity; TS-, without comorbidity) and tested for group effects using ANOVA. No significant group effect is demonstrated.

FRONTAL WHITE MATTER		Anxiety		ADHD		OCD		Depression	
		Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N
Gln	TS+	3.5 ± 1.0	6	3.6 ± 1.0	7	3.7 ± 1.0	7	3.6 ± 1.1	4
	TS-	3.1 ± 1.0	10	3.0 ± 0.9	9	3.0 ± 0.9	9	3.2 ± 0.9	12
	HV	3.0 ± 0.6	14	3.0 ± 0.6	15	3.0 ± 0.6	15	3.0 ± 0.6	15
	df	29		30		30		30	
	p	0.493		0.178		0.169		0.422	
Glu	TS+	10.2 ± 1.7	6	10.2 ± 1.5	7	10.2 ± 1.5	7	11.0 ± 1.3	4
	TS-	10.2 ± 1.6	10	10.2 ± 1.7	9	10.1 ± 1.8	9	9.9 ± 1.6	12
	HV	9.0 ± 1.3	14	9.1 ± 1.3	15	9.1 ± 1.3	15	9.0 ± 1.3	15
	df	29		30		30		30	
	p	0.129		0.141		0.139		0.057	
GSH	TS+	3.8 ± 0.9	6	3.5 ± 1.1	7	3.5 ± 1.2	7	4.1 ± 0.7	4
	TS-	3.1 ± 1.0	10	3.2 ± 1.0	9	3.2 ± 0.9	9	3.1 ± 1.0	12
	HV	3.1 ± 1.2	14	3.1 ± 1.1	15	3.1 ± 1.1	15	3.1± 1.1	15
	df	29		30		30		30	
	p	0.329		0.637		0.628		0.222	
Ins	TS+	7.5 ± 3.6	6	7.0 ± 3.5	7	6.0 ± 1.7	7	6.3 ± 1.1	4
	TS-	6.8 ± 1.5	10	7.1 ± 1.3	9	7.9 ± 2.6	9	7.3 ± 2.7	12
	HV	6.9 ± 2.4	14	6.8 ± 2.4	15	6.8 ± 2.4	15	6.8 ± 2.4	15
	df	29		30		30		30	
	p	0.852		0.932		0.258		0.738	
tCho	TS+	2.9 ± 0.6	6	3.0 ± 0.6	7	2.8 ± 0.4	7	3.0 ± 0.7	4
	TS-	2.8 ± 0.5	10	2.7 ± 0.4	9	2.8 ± 0.6	9	2.8 ± 0.4	12
	HV	3.0 ± 0.5	14	3.0 ± 0.5	15	3.0 ± 0.5	15	3.0 ± 0.5	15
	df	29		30		30		30	
	p	0.449		0.299		0.472		0.366	
tNAA	TS+	11.6 ± 0.9	6	11.8 ± 1.0	7	12.0 ± 1.2	7	12.0 ± 0.7	4
	TS-	12.2 ± 1.1	10	12.1 ± 1.1	9	11.9 ± 1.0	9	11.9 ± 1.2	12
	HV	11.8 ± 0.5	14	11.8 ± 0.5	15	11.8 ± 0.5	15	11.8 ± 0.5	15
	df	29		30		30		30	
	p	0.305		0.705		0.921		0.915	
Glx	TS+	13.7 ± 2.4	6	13.8 ± 2.3	7	13.9 ± 2.1	7	14.7 ± 2.3	4
	TS-	13.3 ± 2.3	10	13.2 ± 2.3	9	13.1 ± 2.4	9	13.1 ± 2.2	12
	HV	12.0 ± 1.4	14	12.1 ± 1.4	15	12.1 ± 1.4	15	12.1 ± 1.4	15
	df	29		30		30		30	
	p	0.156		0.142		0.127		0.062	
tCr	TS+	7.5 ± 0.6	6	7.8 ± 0.9	7	8.1 ± 0.8	7	7.4 ± 0.3	4
	TS-	8.2 ± 0.6	10	8.1 ± 0.5	9	7.8 ± 0.6	9	8.1 ± 0.7	12
	HV	7.8 ± 0.7	14	7.8 ± 0.6	15	7.8 ± 0.6	15	7.8 ± 0.6	15
	df	29		30		30		30	
	p	0.106		0.602		0.593		0.159	

*Table 11. Brain metabolites in PUT of HV and TS patients subgrouped by psychiatric comorbidity (TS+, with comorbidity; TS-, without comorbidity) and tested for group effects using ANOVA. There is a significant group effect for GSH when TS patients are subgrouped by anxiety (*p<0.05).*

PUTAMEN		Anxiety		ADHD		OCD		Depression	
		Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N
GSH	TS+	6.6 ± 0.4	7	6.0 ± 1.3	7	6.0 ± 1.3	7	6.5 ± 0.4	4
	TS-	4.6 ± 1.4	9	5.1 ± 1.5	9	5.1 ± 1.5	9	5.1 ± 1.6	12
	HV	5.9 ± 1.0	17	5.9 ± 1.0	17	5.9 ± 1.0	17	5.9 ± 1.0	17
	df	32		32		32		32	
	p	**0.003***		0.365		0.222		0.127	
Ins	TS+	5.0 ± 0.9	7	5.2 ± 1.0	7	5.2 ± 1.0	7	4.6 ± 0.6	4
	TS-	4.7 ± 1.0	9	4.5 ± 0.8	9	4.5 ± 0.8	9	4.9 ± 1.0	12
	HV	5.2 ± 0.8	17	5.2 ± 0.8	17	5.2 ± 0.8	17	5.2 ± 0.8	17
	df	32		32		32		32	
	p	0.354		0.236		0.159		0.388	
tCho	TS+	2.5 ± 0.4	7	2.4 ± 0.2	7	2.4 ± 0.2	7	2.6 ± 0.5	4
	TS-	2.5 ± 0.3	9	2.6 ± 0.4	9	2.6 ± 0.4	9	2.5 ± 0.3	12
	HV	2.4 ± 0.2	17	2.4 ± 0.2	17	2.4 ± 0.2	17	2.4 ± 0.2	17
	df	32		32		32		32	
	p	0.573		0.364		0.256		0.365	
tNAA	TS+	12.4 ± 0.7	7	12.5 ± 0.7	7	12.5 ± 0.7	7	12.3 ± 0.8	4
	TS-	13.0 ± 0.9	9	12.9 ± 0.9	9	12.9 ± 0.9	9	12.9 ± 0.8	12
	HV	12.7 ± 0.7	17	12.7 ± 0.7	17	12.7 ± 0.7	17	12.7 ± 0.7	17
	df	32		32		32		32	
	p	0.247		0.704		0.489		0.379	
Glx	TS+	22.9 ± 2.2	7	21.7 ± 4.2	7	21.7 ± 4.2	7	23.2 ± 2.5	4
	TS-	21.2 ± 3.3	9	22.2 ± 1.7	9	22.2 ± 1.7	9	21.6 ± 3.0	12
	HV	22.3 ± 1.5	17	22.3 ± 1.5	17	22.3 ± 1.5	17	22.3 ± 1.5	17
	df	32		32		32		32	
	p	0.318		0.832		0.860		0.449	
tCr	TS+	11.5 ± 0.6	7	11.2 ± 1.2	7	11.2 ± 1.2	7	11.4 ± 0.6	4
	TS-	11.1 ± 1.2	9	11.4 ± 0.8	9	11.4 ± 0.8	9	11.3 ± 1.1	12
	HV	11.7 ± 0.6	17	11.7 ± 0.6	17	11.7 ± 0.6	17	11.7 ± 0.6	17
	df	32		32		32		32	
	p	0.247		0.434		0.337		0.369	

Post hoc analysis of this group effect showed significantly lower GSH concentration in TS patients without anxiety (TS-anxiety) compared to TS patients with anxiety (TS+anxiety) and HV in PUT (p= 0.001 and p= 0.007, respectively; see Figure 6).

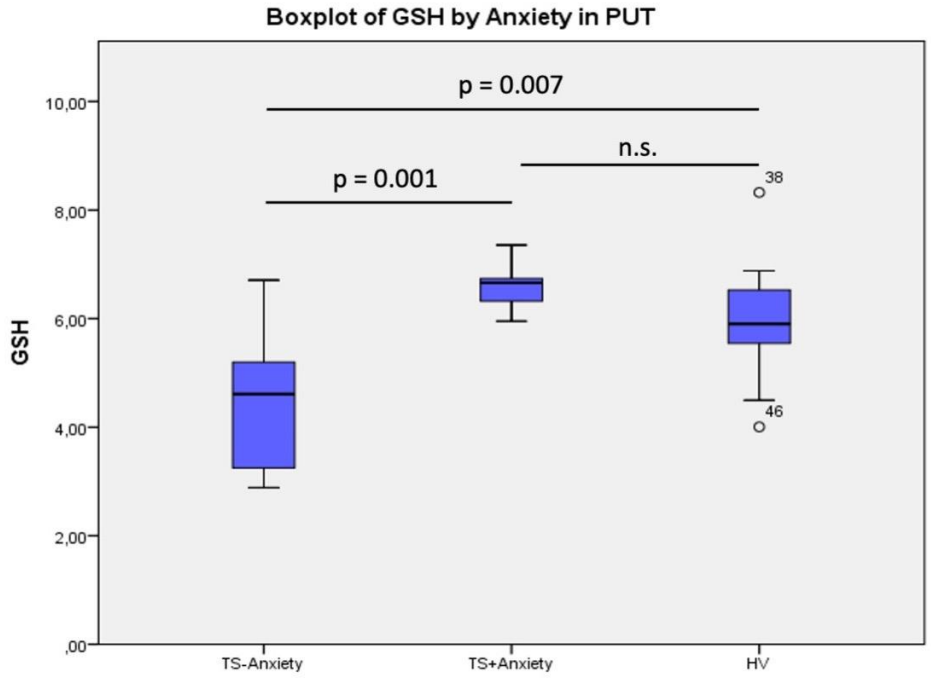

Figure 6. Box and whisker plot of GSH concentration (mmol/l) in PUT of HV and TS patients with and without anxiety. TS patients without anxiety have significantly lower GSH than TS patients with anxiety and HV (two-tailed t-test). n.s. = not significant.

We did not detect any other significant association between other comorbidities and brain levels of MRS metabolites (Table 10 and Table 11). Similarly, we could not identify any significant correlation between severity of tics, obsessive-compulsive symptoms, depressive and anxiety symptoms, and brain levels of MRS metabolites.

Finally, sensitivity analyses testing the potential impact of drug classes on tested brain metabolites either in FWM and in PUT did not show significant associations.

DISCUSSION

In this study we investigated levels of MRS-derived brain metabolites in TS patients, and we explored their relationship with measures of disease severity and comorbidity profile. In line with the previous literature[100], we observed a reduction of levels of Glu in cortico-striato-thalamo-cortical circuits and, specifically, in the FWM of TS patients when compared to age matched HV. Although changes in brain levels of GSH were previously demonstrated in other neurodevelopmental diseases such as autism[156], we observed reduced levels of GSH in the FWM of TS patients not affected by anxiety when compared to both TS patients affected by anxiety and HV. Furthermore, to our knowledge, this is the first study to demonstrate a correlation between measures of disease severity and GSH levels in the FWM of TS patients.

Recent MRS studies suggest that TS may be related to a complex interplay between different neurotransmitters[39,99,135]. While dopamine dysfunction has for long been considered the primary abnormality in TS[31,47,157,158], other neurotransmitters such as GABA and glutamate contribute to the pathophysiology of TS[39]. Glutamate is the primary and most represented excitatory neurotransmitter in the brain[42,43] and was first found reduced in the basal ganglia of TS patients in a post-mortem study by Anderson et al in 1992[44]. It plays an essential role in the cortico-striato-thalamo-cortical circuit and is often co-transmitted with dopamine in the prefrontal cortex, midbrain, and striatum[45,46] and modulators of glutamatergic neurotransmission have been considered as potential pharmacological targets in TS, so far with unsatisfactory results[47,159]. Our recent finding of increased Glu levels in TS patients' FWM is in further

support of the current evidence of dysfunctional excitatory and inhibitory neurotransmission within the cortico-subcortical circuit of TS patients [39,100,160].

Interestingly, in this study we first demonstrated a direct correlation between of GSH levels and measures of disease severity in the FWM of TS patients when covarying for age and gender. Glutathione (L- c-glutamyl-L-cysteinylglycine) is a tripeptide consisting of glutamate, cysteine, and glycine which is present virtually in all cells. It is involved in oxidation-reduction reactions, is an enzymatic cofactor, protects against reactive oxygen species and regulates several cellular functions, including synthesis and degradation of proteins and the formation of the deoxyribonucleotides, and is involved in immune responses[161]. In the brain, it is the main cellular free radical scavenger and is fundamental in protecting cells from exogenous and endogenous toxins[162]. Abnormalities of the cerebral GSH compound have been previously described in patients affected by other neurodevelopmental disorders such as autism spectrum disorder (ASD)[156,160,163-165], and other neurological conditions including multiple sclerosis[166], motor neuron disease[167] and epilepsy[168]. Furthermore, evidence supports a role of GSH in several psychiatric and autoimmune conditions. Post-mortem studies comparing frozen cerebellum and temporal cortex samples from ASD patients with those from healthy controls showed a significantly reduced GSH redox/antioxidant capacity in affected subjects. The authors hypothesized that an increased oxidative stress in ASD patients' brains may result in an altered inflammatory response, increased mitochondrial superoxide production with consequent oxidative protein and DNA damage[165,169]. On the other hand, two recent MRS studies aimed at measuring cortical levels of GSH in frontal and prefrontal cortex

as well as basal ganglia failed to detect significant differences between ASD patients and non-affected controls[156,164]. It is known that concentration differences in brain metabolites are regionally specific in ASD[170] but, overall, these contradictory findings are in support of the need for further clarifying studies.

In TS, a study of the variants of Glutathione-S-transferase P1, a gene codifying proteins involved in detoxification of oxidative stress products, showed that TS patients were more likely to express a single-nucleotide polymorphism of this gene (rs6591256) which negatively affects the function of the proteins leading to oxidative DNA damage and consequent abnormal cellular proliferation and apoptotic activity[171]. The authors therefore postulated that oxidative stress might be a relevant mechanism in the pathophysiology of TS. In further support of this hypothesis, Xiao-Er-An-Shen Decoction (XEASD), a herbal substance thought to enhance brain antioxidant status and used clinically for the treatment of TS children in Chinese medicine was found to significantly ameliorate the severity of motor and behavioural symptoms in mouse models of TS[172].

Interestingly, a key role for GSH metabolism anomalies as marker of oxidative stress has also been implicated in the pathophysiology of several neuropsychiatric conditions including psychosis, bipolar disorder, and schizophrenia [150,173,174]. Both MRS and peripheral studies aimed at evaluating levels of GSH in these groups of patients showed a significant reduction in both central nervous system and peripheral plasma levels of GSH[151,174]. The serum reduction of GSH also correlated with measures of disease severity of both schizophrenia and bipolar disorder, pointing towards the role of oxidative stress as a common molecular signature of both these conditions[174].

Finally, evidence also supports a key role of GSH in several autoimmune conditions[152]. Highly reactive oxygen-based molecules are produced by activated neutrophils during the inflammatory response in immune-mediated diseases ultimately causing collateral damage to surrounding tissue[175]. GSH, as the principal cell antioxidant, exerts a critical role during the lymphocyte activation process[176]. Several inflammatory/immune mediated disorders have been associated with reduced GSH levels and lowered cellular redox potential[177], including systemic lupus[178], rheumatoid arthritis[179] and autoimmune thyroiditis[180]. Clinical, epidemiological and experimental studies suggest the presence of a systemic and CNS low-grade chronic inflammatory state in TS[24,72]. At a systemic level, patients with TS have shown alteration of inflammatory responses and altered distribution of immune regulatory cells in keeping with predisposition to autoimmunity[107,108]. At the CNS level, studies using different methodologies have shown increased lymphocytic activation and increased markers of glial activation suggestive of neural immune dysregulation[181,182]. Our findings of a direct correlation between TS symptom severity and concentration of GSH might be in keeping with a compensatory, possibly transient, increase in antioxidant responses triggered by increased levels of inflammatory metabolites and related oxidative stress in the CNS of TS patients. Longitudinal studies should be conducted to verify if this compensatory rise of GSH level is indeed driven by a transient surge of tic severity and anxiety symptoms, or whether our findings indicate the existence of two subgroups of TS patients, who differ for GSH redox/antioxidant capacity.

In conclusion, the direct correlation between GSH levels and measures of disease severity in the FWM of TS patients demonstrated in the present study does not clearly

support a dysfunction of the GSH pathway in the general TS population but rather a compensatory production of GSH in response to a hyper glutamatergic state which might increase redox stress and chronic inflammatory response[183,184]. Finally, in agreement with our original hypothesis, and similarly to ASD and other psychiatric and immunological conditions, TS patients, particularly those not manifesting a higher burden of comorbid anxiety, showed a decrement of GSH concentration in the FWM supporting the influence of behavioural comorbidities on the neurometabolic profile, at least in the adult TS population.

CHAPTER 4

CONCLUSIONS

Exploring neuro-immune dysfunction in neurodevelopmental disorders

There is emerging evidence for the role of neuro-immune dysfunction in the pathogenesis of neurodevelopmental disorders including TS (see Chapter 1). Flow cytometry of circulating leukocytes is an established method which has been applied previously to characterise peripheral immune cell phenotypes in other neurodevelopmental disorders such as autism spectrum disorder, while 1H-MRS provides a unique and non-invasive tool to detect quantitatively levels of metabolites in brain parenchyma *in vivo*. Changes in these metabolites can reflect energetic, inflammatory, synaptic, oxidative or injurious processes occurring at a cellular level.

In this work, we set out to use these methods in order to investigate the hypothesis that TS is associated with dysfunctional neuro-immune crosstalk. We focused our immune phenotyping on subtypes of circulating DCs which form the critical link between innate and adaptive immunity, having been motivated by recent studies demonstrating altered DC frequencies in autism spectrum disorder. MRS quantification of brain metabolites was directed to frontal white matter (FWM) and putamen (PUT), two major hubs in cortico-striato-thalamo-cortical networks thought to be dysfunctional in TS (see Chapter 1). We sought to compare findings in TS patients to those in age- and sex-matched healthy volunteer control participants and hypothesised that TS patients would have different DC phenotypes and elevated biochemical brain markers of neuroinflammation relative to controls. Furthermore, given the prevalence of associated behavioural comorbidities, we examined the impact of these comorbidities on the measures obtained.

Immune dysregulation, neuro-inflammation and metabolic stress in Tourette's syndrome

We found abnormally elevated levels of the circulating DC subtype, MDC1, specifically in TS patients with comorbid anxiety, a potential substrate for immune dysregulation (Figure 4). Across the TS patient cohort, MDC1 frequency was shown to correlate inversely with total creatine concentration in FWM (Figure 5), suggesting a link between immunity and brain energetic status. At the same time, glutamate levels in FWM were found to be elevated in TS patients compared to healthy controls (Table 8).

Levels of glutathione (GSH), the principal cellular antioxidant, have previously been examined in other neuropsychiatric and neurodevelopmental disorders such as autism, psychosis, bipolar disorder and schizophrenia, as a putative marker of cellular oxidative stress. This is, however, the first time that the role of brain GSH has been investigated in TS (Chapter 3). In TS patients, GSH in FWM increases in correlation with clinical measures of disease severity (Table 9), while GSH levels in PUT are elevated in TS patients with comorbid anxiety compared to those without anxiety and are comparable to those in healthy volunteers (Figure 6).

Synthesising these major findings, we hypothesise that TS is characterised by a chronic pro-inflammatory, hyper glutamatergic and energetically impaired state. We propose that this induces oxidative stress to which the cellular response is a compensatory rise in GSH, increasing with greater disease severity. The presence of

comorbid anxiety appears to play a significant role, both immunologically and metabolically. It is possible that, in TS patients with greater burden of anxiety symptoms, an increase in circulating glucocorticoids, which is well documented in anxiety states, expands the number and impairs the function of DC subsets, contributing further to a pro-inflammatory state. We speculate that metabolic stress in cortico-striato-thalamo-cortical circuits plays a contributory role in circuit dysfunction manifesting as lack of inhibitory control in action selection, leading to the behavioural phenotype of TS.

Outstanding questions

The population of adult TS patients studied here represents the minority of patients in whom tics persisted beyond childhood, examined typically many years after the onset of symptoms. It may not be possible to generalise our findings to the whole population of patients with TS. The pathogenetic role of immune and metabolic changes could be further investigated with comparison groups of child or adolescent patients earlier in the course of the disease, adult patients without persistent symptoms, as well as with longitudinal studies to chart the temporal evolution of changes in relation to disease status.

The strong correlation of frontal white matter glutathione and motor tic severity demonstrated here is novel and worthy of further investigation. In addition to its putative mechanistic role in pathogenesis, we speculate that it has the potential to develop use as an imaging biomarker of disease activity.

Methodological limitations include the fact that MRS brain metabolite levels can be influenced by many factors such as sleep patterns, caffeine, nicotine, time of day and gender, many of which are difficult to control fully.

DC phenotype is only one aspect of the immune profile which may be potentially altered in TS. Further investigations to link our findings with the role of cytokines, T cells, human leukocyte antigens (HLA) and antibodies may yield further insights into immune system dysregulation.

Further work is required to elucidate the role of behavioural comorbidities. Behavioural comorbidity profiles here were determined with investigator-administered questionnaires and we recognise that a semi-structured psychiatric interview would provide a more rigorous assessment of comorbidities.

Closing remarks

While TS and other neurodevelopmental disorders may not be completely immunological in aetiology, a picture is emerging of a role for dysregulated immunity and cellular metabolic stress, the surface of which is only just beginning to be scratched. The novel work presented here reinforces this notion and adds a small piece to this growing puzzle.

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