**Disturbed Iron Homeostasis in Gilles de la Tourette syndrome**

Ahmad Seif Kanaan1,2 Dongmei Yu3, Alfred Anwander1, Berkin Bilgic4, Isabel Garcia Garcia5, Karla Claudio6, Andreas Schäfer7, Riccardo Metere1, Torsten Schlumm1, Rachel Szido1, Julia Sacher1, Arno Villringer1, Jamie Near4, Boris Bernhardt5,

Tourette Syndrome Association International Consortium for Genetics, Carol Mathews6, Jeremiah Scharf3, Kirsten Müller-Vahl2\* & Harald E. Möller1\*

1. *Max Planck Institute for Human Cognitive and Brain Sciences, Leipzig, Germany*
2. *Department of Psychiatry, Social Psychiatry and Psychotherapy, Hannover Medical School, Hannover, Germany*
3. *Departments of Psychiatry and Neurology, Center for Human Genetics Research, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA*
4. *Athinoula A. Martinos Center for Biomedical Imaging and Department of Radiology, Harvard Medical School, Boston, MA, USA*
5. *Montreal Neurological Institute, McGill University, Montreal, QC, Canada*
6. *Department of Psychiatry, Center for OCD, Anxiety, and Related Disorders, College of Medicine, University of Florida*
7. *Siemens Healthcare GmbH, Diagnostic Imaging, Magnetic Resonance, Research & Development, Erlangen, Germany*

\*These authors contributed equally to this work.

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**Introduction**

**Materials and Methods**

**Magnetic Resonance Imaging**

**Population Sampling**

The study was embedded in a larger multi-parametric investigation, parts of which have been published elsewhere (Kanaan *et al.*, 2017). All participants gave written informed consent following approval by the local ethics committee. *T*1-weighted and susceptibility weighted data were available from 28 GTS patients (5 female, 18-65 years) and 26 healthy controls (8 female, 18-65 years). Data collection from one patient was incomplete due to claustrophobia. Patients using psychoactive substances underwent a four-week washout period before participation and were deemed ineligible if they exhibited severe tics to the head and face, a history of other neurological disorders, current abuse of drugs/alcohol, or the presence of MRI contraindications. All patients were diagnosed based on DSM-5 criteria and underwent a thorough clinical assessment battery (Gerasch et al. 2016; Kanaan et al. 2017). Healthy controls without a history of neuropsychiatric and tic disorders were assessed in a similar manner. All subjects were instructed to not drink coffee or tea, abstain from smoking for at least 2h before the examination and adhere to a regular sleeping cycle the night before the scan. To minimize variability from circadian physiological effects, the time of day of the exam was matched between patients and controls with the majority of acquisitions falling between 10am and 4pm. When possible, a 10ml blood sample was collected from the subjects (*N*=37) for quantitative determination of serum ferritin (Supplementary Information).

**Clinical assessment**

All patients underwent a thorough clinical assessment battery (Gerasch *et al.*, 2016; Kanaan *et al.*, 2017) that included measurements of *(a)* tics using the Yale Global Tic Severity Scale (YGTSS) (Leckman *et al.*, 1989) and the modified Rush Video-Based Tic Rating Scale (RVTRS) (Goetz *et al.*, 1999); *(b)* obsessive compulsive behavior using the Yale-Brown Obsessive Compulsive Scale (Y-BOCS) (Goodman *et al.*, 1989) and the Revised Obsessive Compulsive Inventory (OCI-R) (Foa *et al.*, 2002); *(c)* attention-deficit/hyperactivity symptoms using the DSM-IV symptom list for ADHD and the Conners' Adult ADHD Rating Scale (CAARS) (Conners *et al.*, 1999); *(d)* depression using the Montgomery Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) and the Beck Depression Inventory II (BDI-II) (Beck, 1961); and *(e)* anxiety using the Beck Anxiety Inventory (BAI) (Beck *et al.*, 1988).

**Measurement of serum ferritin levels**

A 10-ml blood sample was collected from the majority of the subjects (*N*=37) for the *in vitro* quantitative determination of serum ferritin as a representative measure of the body’s iron reserves. The sample was first centrifuged at 24,000 rpm for a period of 10 min to separate hematocrit from plasma, which was subsequently stored in 1000μl aliquots at −70 °C. Serum ferritin levels were quantified based on the electochemiluminescence immunoassay, in which a voltage applied to a sample containing tagged ferritin molecules induces chemiluminescent emissions that are measured by a photomultiplier (Elecsys 2010, Roche Diagnostics GmbH, Mannheim, Germany).

**MR data acquisition**

All measurements were performed on a 3T MAGNETOM Verio (Siemens Healthineers, Erlangen, Germany) with a 32-channel head coil. Three-dimensional *T*1- and susceptibility-weighted data were acquired using MP2RAGE (repetition time, TR=5s; echo time, TE=3.93ms; inversion times, 0.7 and 2.5s; sagittal slab orientation; matrix 256×256×176; 1mm isotropic nominal resolution) and flow-compensated FLASH (flip angle 13°; TR=30ms; TE=17ms; matrix 256×256×160; 0.8mm isotropic nominal resolution), respectively.

**Quantitative Susceptibility Mapping**

Phase maps were reconstructed from multi-channel complex signals using an automated, data-driven coil combination method (SVD-ESPIRiT). Specifically, a conjugate virtual-body-coil map was reconstructed by computing the singular value decomposition (SVD) across the channels, and then taking the dominant singular vector as the body-coil reference (Chatnuntawech et al. 2016). SVD-compressed data were sorted in decreasing order of virtual channel eigenvalues, and input into ESPIRiT for the estimation of coil sensitivities (Uecker et al. 2014). Susceptibility maps were computed using the superfast dipole inversion approach, which employs *(a)* sophisticated harmonic artifact reduction for phase (SHARP) to eliminate background field contributions and *(b)* threshold k-space division (TKD) for calculating Δχ (Fig. 1) (Schweser et al. 2013). All QSM data were referenced to median cerebrospinal fluid susceptibility, measured within a subject-specific mask of the lateral ventricles (Straub et al. 2016). Carefully delineated masks of deep GM nuclei were generated (Supplementary Information) including the striatum (caudate/putamen), pallidum, thalamus, substantia nigra (SN), subthalamic nucleus (STN), red nucleus (RN), and dentate nucleus (DN) (Fig. 1). Given that patients with GTS are ultimately characterized by movement, head motion may influence voxel intensities and bias group comparisons. Consequently, we used a multivariate outlier-detection approach (Korkmaz, Goksuluk, and Zararsiz 2014) to remove low-quality data based on image-quality indices and susceptibility values (Supplementary Information).

**Masking of deep grey matter nuclei**

Masks of the striatum (caudate-putamen), globus pallidus and thalamus were obtained via the FSL-FIRST Bayesian model-based subcortical segmentation algorithm (18), which was applied on optimized hybrid-contrast MP2RAGE-QSM images (19, 20). Robust co-registration between skull-stripped MP2RAGE and FLASH data was achieved using rigid-body linear transformation of the *T*1-weighted data onto N4 bias-field-corrected FLASH magnitude data (http://github.com/stnava/ANTs). Given the difficulty of segmenting brainstem and cerebellar nuclei on *T*1-weighted data due to lack of contrast, in addition to the infeasibility of performing manual segmentation of multiple nuclei in many subjects, we utilized an atlas-based registration approach to achieve accurate delineations of brainstem/cerebellar nuclei. Specifically, the diffeomorphic greedy-SyN ANTs non-linear transformation model (http://github.com/stnava/ANTs) was used to compute a nonlinear transformation warp between MP2RAGE and MNI space, which was used to map each subject’s QSM data into standard space for subsequent calculation of a population-specific average image. The standardized QSM template exhibited high contrast in brainstem/cerebellar regions and was used to carefully delineate masks of the STN, SN, RN and DN (Fig. 1). All masks were delineated by the same operator and were subsequently warped back into native QSM space. The same atlas-based registration procedure was applied to obtain subject-specific masks of the lateral ventricles, which were used for referencing the QSM data to cerebrospinal fluid (CSF; median values). All masks were thresholded at 0.5 to ensure maximal inclusion of GM tissue while limiting partial-volume effects. Following visual inspection of all the masks for quality, median susceptibility values from all ROIs were computed for further analysis.

**Quality Control**

To account for potential differences in the severity of motion-related image artifacts, we used a step-wise, multivariate outlier-detection approach implementing a robust Mahalanobis distance framework (15) to remove low-quality data based on *(a)* structural image quality indices calculated on the magnitude structural images and *(b)* susceptibility values extracted from subcortical nuclei. In general, Mahalanobis distance calculates how far each observation is to the center of a joint distribution, which can be thought of as the centroid in multivariate space. Robust distances are estimated from minimum covariance determinant estimators rather than the sample covariance. Data were regarded as outliers if the robust Mahalanobis distance was greater than the 97.5% quantile of the chi-square distribution (Supplementary Figure S1). In the first step, multivariate outliers were detected based on *(a)* the Shannon entropy focus criterion (EFC), which is an index for image ghosting and blurring (21); *(b)* the quality index 1 (QI1), which is an index for image degradation resulting from bulk motion, residual magnetization, incomplete spoiling and ghosting (22); and *(c)* the smoothness of voxels calculated as the full width at half maximum (FWHM) of the spatial distribution of image intensity values in voxel units (https://github.com/preprocessed-connectomes-project). This step was implemented on the whole sample and identified one severely affected dataset, which was marked for removal (Supplementary Figure S1A). To ensure that the remaining datasets did not contain further outliers, multivariate robust squared Mahalanobis distance outlier detection was additionally performed on vectors of median Δχ values extracted from the subcortical masks for each sample separately. This procedure identified four outlier datasets within the patient sample, which were marked for removal (Supplementary Figure S1B). Following quality control, group comparisons of magnitude image quality metrics (signal-to-noise ratio; SNR; contrast-to-noise ratio, CNR; foreground-to-background ratio, FBER; voxel smoothness; EFC; QI1) revealed no significant differences between patients and controls (Supplementary Table S1).

**Genetic Heritability**

Genetic correlation between TS and iron-related traits were evaluated by LD score (LDSC) regression analysis (Bulik-Sullivan et al., 2015). LDSC regression analysis uses the summary statistics from genome-wide association studies (GWAS) of two traits of interest to estimate the heritability and genetic correlation of these traits. TS summary statistics were derived from the most recent TS GWAS of 4819 European ancestry TS cases and 9488 population-matched controls (Yu, et al., 2019). GWAS summary statistics for two iron-related traits, ferritin and transferrin, are available at LD Hub, a web interface for LD score regression analysis (<http://ldsc.broadinstitute.org/ldhub/>) (Zheng, et al., 2017). GWAS studies of both iron-related traits were performed on 23,986 European ancestry samples collected by the Genetics of Iron Status Consortium (GISC) (Benyamin, et al., 2014). The genetic correlations between ferritin, transferrin, and TS were evaluated at LD Hub, and Bonferroni correction was applied for multiple testing.

**Transcriptomics**

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The structure and function of the human brain are highly stereotyped, implying a conserved molecular program responsible for its development, cellular structure and function. We applied a correlation-based metric called differential stability to assess reproducibility of gene expression patterning across 132 structures in six individual brains, revealing mesoscale genetic organization. The genes with the highest differential stability are highly biologically relevant, with enrichment for brain-related annotations, disease associations, drug targets and literature citations. Using genes with high differential stability, we identified 32 anatomically diverse and reproducible gene expression signatures, which represent distinct cell types, intracellular components and/or associations with neurodevelopmental and neurodegenerative disorders. Genes in neuron-associated compared to non-neuronal networks showed higher preservation between human and mouse; however, many diversely patterned genes displayed marked shifts in regulation between species. Finally, highly consistent transcriptional architecture in neocortex is correlated with resting state functional connectivity, suggesting a link between conserved gene expression and functionally relevant circuitry.

The

To investigate the molecular mechanisms that may drive abnormalities in iron levels, we employed a cross-correlation approach to examine the relationship between Δχ differences with the spatial gene transcriptional levels of iron-related genes extracted from the AHBA (Arnatkevic̆iūtė, Fulcher, and Fornito 2019). As transcriptional profiles are known to exhibit distinct expression patterns across and within brain regions, relationships between regional expression patterns in subcortical GM and statistical maps of Δχ differences were investigated to glean further insights on underlying pathophysiological mechanisms of iron-related changes.

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Motivated by previous work implicating the striatum as a major locus of GTS pathophysiology, voxel-wise Δχ difference statistical maps within three functionally distinct striatal sub-territories (motor, associative, limbic) were calculated via nonparametric permutation testing (FSL-randomise; 10,000 permutations). Transcriptional levels of genes incorporated within four iron-related gene sets were extracted at coordinates sampled in the AHBA and contained in the striatal subdivisions. They included: *(a)* a manually curated *iron-homeostasis* gene set, *(b)* an experimentally determined gene set representing *iron-deficiency* (Clardy et al. 2006), *(c)* an *iron transport and uptake* gene set generated via biochemical modeling (Hower et al. 2009), and *(d)* an *iron-storage* set of all iron sequestrating genes (Supplementary Information). For each gene set, average values and PC scores were cross-correlated to statistical values of striatal Δχ differences at each coordinate sampled in the AHBA in the same space (MNI-152). To statistically evaluate the correlations, a permutation-based approach was implemented, in which the null distribution was constructed using a re-sampling approach with 10,000 permutations. For each permutation, the Pearson correlation between Δχ differences and the average gene-expression value of random sets of genes was calculated with an equal size to the gene set of interest. The null hypothesis was rejected if the observed correlation exceeded an alpha level of 0.05 (FDR).

**PLS1 Gene list**

The attached file was generated as described below (In our case, the background gene list is 20737.. All genes as annotated in AHBA).  The header is as follows:

"Gene Name", "Entrez ID", 'Z-score"

Morgan SE,  et al. **Cortical patterning of abnormal morphometric similarity in psychosis is associated with brain expression of schizophrenia-related genes.**Proc Natl Acad Sci 2019: 201820754.

* *"We used PLS to relate the regional morphometric similarity case–control differences (t statistics from the 152 cortical regions in the left hemisphere calculated from intrahemispheric edges only) to the post mortem gene expression measurements for all 20, 647 genes. PLS uses the gene expression measurements (the predictor variables) to predict the regional morphome- tric similarity case-control t statistics from all three datasets (the response variables). The first PLS component (PLS1) is the linear combination of the weighted gene expression scores that have a cortical expression map that is most strongly correlated with the map of case–control morphometric similarity differences. The statistical significance of the variance explained by PLS1 was tested by permuting the response variables 1,000 times. The error in estimating each gene’s PLS1 weight was assessed by bootstrap- ping (resampling with replacement of the 308 cortical regions), and the ratio of the weight of each gene to its bootstrap SE was used to calculate the Z scores and, hence, rank the genes according to their contribution to PLS1 (6).*
* We constructed PPI networks from the genes with PLS1 weights Z>3 and Z<−3 (all FDR<0.05) using STRING version 10.5 (14). Our key results were robust to changing these thresholds to Z>4 and Z<−4 (all FDR<0.01) (SI Appendix, section S8.3). We used DAVID (39, 40) to calculate enrichments of KEGG pathways and GO enrichments of biological processes for genes with Z>3 or Z<−3 using a background gene list of 15,745 brain-expressed genes (SI Appendix, section S8.3) (38).*"*

Results

**Results**

* Reduced QSM in major subcortical nuclei
* Reduced Ferritin
* Ferritin associated with striatal QSM in small and big sample
* Ferritin associated with Motor principal component
* GTS exhibits shared genetic heritability (hg2) with ferritin and transferrin
* Motor striatal Magnetic susceptibility enriched for neurotransmitter and cytoskeletal genetic pathways (which are affected in iron deficiency).

**Discussion**

**Conclusions**

1. **Exploratory examination of the association between iron-related gene-expression and ∆χ.**
   * A machine learning partial least squares approach to link a ranked gene list to susceptivity differences as done by the Bullmore group [PNAS paper](https://www.pnas.org/content/113/32/9105) (using their code).
   * The STRING: functional protein association networks (<https://string-db.org/>) was used to do gene-set enrichment and look at protein-protein networks.
   * Kindly see results in pdf.
2. **LD-Score regression to compare genomewide results of the TS GWAS (~5000) with a GWAS of ferritin levels and a GWAS of transferrin levels in ~ 20,000 cases.**
   * Neither of the two iron-related GWASes demonstrated that much common-variant heritability (9% of variance for ferritin and 16% for transferrin),
   * but the overlapping (bivariate) heritability between ferritin levels and TS case-control status was significant, with a negative correlation (higher TS polygenic risk was associated with polygenic risk for lower ferritin levels with a genetic correlation (rg) =-0.34
   * that this is the same direction of effect as you found in your MRI study (lower ferritin = higher tic severity)

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **trait1** | **trait2** | **PMID** | **ethnicity** | **rg** | **se** | **z** | **p** | **h2\_obs** | **h2\_obs\_se** | **h2\_int** | **h2\_int\_se** | **gcov\_int** | **gcov\_int\_se** |
| TS | Ferritin | 25352340 | European | **-0.3411** | 0.13 | -2.6239 | **0.0087** | 0.0928 | 0.0271 | 1.0268 | 0.0096 | 0.0156 | 0.0063 |
| TS | Transferrin | 25352340 | European | **0.2828** | 0.1348 | 2.0975 | **0.0359** | 0.1609 | 0.0755 | 1.0631 | 0.025 | -0.0163 | 0.0069 |