**An imaging, genomic and transcriptomic basis for disturbed iron homeostasis in Gilles de la Tourette syndrome**

**Materials and Methods**

**Design**

The overarching goal of this work is to investigate the role of iron in GTS pathophysiology and the downstream effects of its abnormality. We leveraged data available from diverse measurement techniques to identify disease relevant perturbations at multiple-scales to provide biological information that is both shared and distinct across modalities. First, we implemented Quantitative Susceptibility Mapping (QSM) as an in-vivo proxy measure of brain iron levels within well-defined loci of pathophysiology in a sample of N=54 individuals. This data was complemented by a comprehensive clinical assessment battery to assess heterogeneity in symptomatology and measurement of serum ferritin as a proxy measure of global iron. QSM data from an independent sample of N=100 healthy controls were also available for supplementary analyses. Second, we implemented a human genetics approach to assess the genetic correlation between GTS and iron-related proteins leveraging Genome Wide Association studies (GWAS) data from the Tourette Syndrome Association and the Genetics of Iron Status Consortia. Shared genetic structure between GTS and iron-related proteins was estimated using LD-score regression of GWAS summary statistics in GTS (N=4,819 patients, N=9488) and Ferritin/Transferrin (N=23,986) (Yu, 2019; Benyamin, et al., 2014, Bulik-Sullivan, 2015). Third, given the conserved and highly stereotyped pattern of gene expression in the brain and its ﻿heterogeneity within nuclei subdivisions, we employed a spatial transcriptomic approach using microarray gene expression data from the Allen Human Brain Atlas (AHBA) to assess associations between patterns of gene expression and case-control QSM statistical maps within subdivisions of the striatum. The rationale here is that the spatial variation in the expression of genes tracks variations in an image-derived phenotype. As proteins form the backbone of cellular machinery, we finally analyzed functional protein-protein interactions networks of genes exhibiting maximum co-variance with striatal QSM statistical maps by uncovering latent variables via Partial Least Squares (PLS) regression.

**Neuroimaging**

*T*1- and susceptibility-weighted data were available from 28 GTS patients (5 female, 18-65 years) and 26 healthy controls (8 female, 18-65 years) from a multi-parametric investigation focused on GTS parts of which have been published elsewhere (Kanaan, 2017, Forde, 2016, Gerasch, 2016). Data available from an independent sample of N=100 healthy controls were used for supplementary correlational investigations (Babayan, 2019). To maximize the usability of the data, care was employed to limit the effects of (a) pharmacological agents, (b) psychoactive substances, and (c) variations in circadian rhythm and sleep cycles. 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. Phase maps were reconstructed from multi-channel complex signals using an automated, data-driven coil combination method (Bilgic,2016). Susceptibility maps were reconstructed via the superfast dipole inversion approach (Schweser et al. 2013) and 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 the deep grey matter nuclei were generated via (a) automated segmentation of hybrid-contrast MP2RAGE-QSM images for the basal ganglia (FSL-first ref) and (b) non-linear transformation of atlas-based masks for the brainstem (ATAK ref). Outlier data as a result of possible motion artifacts were detected via a multivariate outlier-detection approach implemented on spatial image quality and susceptibility indices (Korkmaz, Goksuluk, and Zararsiz 2014).

All patients underwent a thorough clinical assessment battery indexing *(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). Whenever possible, a 10ml blood sample was collected from the subjects (*N*=37 GTS focused sample, N=100 independent sample) for the quantitative determination of serum ferritin (Supplementary Information).

As susceptibility data tended to exhibit non-parametric distributions (Kolmogorov-Smirnov test), group differences of median Δχ values for each region of interest (ROI) were assessed using Mann-Whitney-Wilcoxon rank sum tests and corrected using fasle discovery rate (FDR). Group differences in serum ferritin values were assessed using Welche’s test given the inhomogeneous variance (Leven’s test). Given the dimensionality and complementarity of the clinical data, we decomposed them into a set of low-dimensional scores using principal component analysis (PCA) to examine relationships with surrogate iron measures. The correlation matrix between all clinical variables revealed sufficient complementarity for data reduction and multi-collinearity was alleviated by implementing an orthogonal Varimax rotation. A multiple linear-regression model accounting for age, gender and two indices of spatial image quality was used to inspect correlations between iron measures and behavioral principal components (PCs). The variance inflation factor was used to assess multi-collinearity between predictor variables.

**Genomics**

As recent GWAS studies implementing novel heritability estimation methods have demonstrated that individual common risk variants exhibit overlaps across boundaries of neuropsychiatric disorders and cognitive-behavioral phenotypes, we assessed the heritability between GTS and iron-related traits using similar techniques (Antilla, Science, 2019). Specifically, we implemented linkage disequilibrium score (LDSC) regression to calculate heritability estimates and correlations, while assessing statistical significance from block jackknife-based standard errors (Bulik-Sullivan et al., 2015). LDSC regression utilizes summary statistics of GWAS data from two traits to estimate (a) common variant heritability, defined as the proportion of phenotypic variance in the population that could theoretically be explained by an optimal linear predictor formed using the additive effects of all common autosomal single-nucleotide polymorphisms (SNPs); and (b) the genetic correlation, defined as the correlation between the traits optimal genetic predictors (Antilla, Science, 2019). GTS summary statistics were derived from the most recent GWAS including N=4918 European ancestry patients and N= 9488 population-matched controls (Yu, et al., 2019). GWAS summary statistics of the two iron-related traits, ferritin and transferrin, were derived from a study performed on N=23,986 European ancestry samples collected by the Genetics of Iron status Consortium (Benyamin, et al., 2014). Evaluations were conducted on LDHub, a web interface for LD score regression analysis and Bonferroni correction was applied for multiple testing (Zheng, et al., 2017).

**Transcriptomics**

The canonical genetic signature of the human brain exhibits a highly conserved architecture with differential transcriptional patterns observed between and within major structures driving functionally relevant circuitry (Hawrylycz, 2012,2015). To investigate the relationships between the spatial patterning of default transcriptional architecture and variations in magnetic susceptibility as a result of GTS pathophysiology, we employed a novel validated spatial transcriptomic approach utilizing gene expression values from the Allen Human Brain Atlas (AHBA) (Hawrylycz, 2012, ﻿Arnatkevicute,2019, Fornito, 2019). The AHBA is a high-resolution gene expression atlas with whole-brain coverage containing tissue samples extracted from N=3702 spatially distinct coordinates mapped to MNI standard space and obtained from N=6 neurotypical individuals postmortem (2 female, Age = XXX). From each tissue sample, transcriptome data were analyzed using Agilent microarrays using N=58,692 probes resulting in transcriptional levels from 20,737 unique annotated genes.

Our overarching aim here was to uncover the major genetic classes of variation that maximum co-variance with iron-related differences in GTS as indexed via magnetic susceptibility. We focused on estimating iron-related variations within the striatum, a major locus of pathophysiology in GTS by computing voxel-wise magnetic susceptibility differences within the motor, executive and limbic subdivisions. T-statistical maps were calculated via non-parametric permutation testing with 10,000 permutations using FSL randomize (REF). Care was employed in deriving the well-defined striatal subdivision masks with no overlap, by transforming the Harvard-Oxford striatal atlas to an average study before binning to 0.5 and erosion by one. Tissue sample seed masks (1mm3) were created based on AHBA coordinates within each subdivision yielding N=48 seeds for the motor and executive subdivisions and N=40 seeds for the limbic subdivision. Ontological annotations as defined by the AHBA were inspected to ensure that the seeds fall within the striatum and seeds outside of the striatum were removed.

To uncover latent associations between magnetic susceptibility differences as a result of GTS pathophysiology and patterns of gene expression, we implemented Partial Least Squares regression. For each functional domain within the striatum, we constructed matrices exhibiting gene expression data and case-control susceptibility t-statistical values from the MRI defined tissue coordinates that lie within the confines of the motor, and limbic subdivisions. Microarray gene expression matrices (e.g. 48x20737 for motor division) were used to predict regional magnetic susceptibility variations as defined by case-control t-statistical map (e.g. 48x1 for motor division) within each subdivision via PLS regression. In other words**,** PLS regression was used to identify the linear combination of genes that best predicted the case-control magnetic susceptibility t-statistical differences as the response variable. Statistical significance of the goodness of fit for the PLS principal components was tested with a two-tailed test at a=0.05 by permuation of the response variable 1000 times. The error in estimating each genes weight in the PLS principal components was assessed by bootstrapping. The ratio of the weight of each gene to its bootstrap error was used to calculate Z-scores and rank the genes according to their contributions to each PLS component. PPI networks were constructed using STRING (stringdb) for downregulated (PLS–) and upregulated (PLS+) genes for each component, which were defined as genes outside the range the 5% and 95% percentiles, respectively.

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**

* Reduced magnetic susceptibility in subcortical nuclei
* Reduced Ferritin associated with magnetic susceptibility
* 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).

***Demographic and clinical characteristics***

Following quality control, the remaining samples included 23 patients (5 female, 36.7±10.8 years) and 26 controls (8 female, 37.9±11.8 years). Both groups were comparable in terms of age (*t*47=0.38, *P*=0.70), gender (Fischer’s odds ratio = 0.52, *P*=0.36) and handedness (all right handed). Control subjects significantly differed from patients on scales measuring obsessions/compulsions, attentional-deficits/hyperactivity and depressive symptoms. Reliable diagnosis of psychiatric comorbidities was achieved using the combination of self-reported and physician-rated clinical scales (Gerasch et al. 2016; Kanaan et al. 2017). Fourteen patients were classified into the tics-only category, whereas the remaining patients exhibited additional OCB/D (*N*=3), ADHD (*N*=5), or the combination of both (*N*=1). Group comparisons of the clinical measures are summarized in Table 1.

***Differences in iron measures***

Group comparison of Δχ revealed significant reductions in bilateral brainstem (*U*47=184, *P*=0.01), basal ganglia (*U*42=167, *P*=0.004) and the combination of all subcortical nuclei (*U*42=172, *P*=0.005) in patients (Supplementary Information). These effects were mainly driven by reductions in the striatum (*U*47=180, *P*=0.009), pallidum (*U*47=197, *P*=0.021), STN (*U*47=159, *P*=0.0026), SN (*U*47=206, *P*=0.0032), DN (*U*47=191, *P*=0.016) and trends for reduction in the RN (*U*47=232, *P*=0.091) (Fig. 1). Further inspection using FDR correction revealed the striatum, pallidum, STN, SN and DN (Cohen’s D effect sizes between 0.5 and 0.83 indicating practical significance). Comparison of serum ferritin levels in controls (196±151 ng/ml) and patients (89±49 ng/ml) revealed similar findings with significant reductions in GTS (*t*33=2.84, *P*=0.0097). Multivariate linear regression revealed significant correlations between serum ferritin levels and susceptibility in the basal ganglia (*r*=0.55, *P*=0.03). This result was replicated in a larger independent sample (*N*=100) of healthy controls (*r*=0.5, *P*=5×10−5) acquired in a separate study (Babayan et al. 2019)  (Fig. 2C).

***Association between iron measures and clinical scores***

To inspect correlations between surrogate measures of iron and clinical variables, the dimensionality of the clinical data was reduced via PCA, which yielded four PCs explaining 77% of the variance. Based on the weights of component loadings, the PCs were interpreted as representative scores of *(i)* depression/anxiety; *(ii)* motor-tics, *(iii)* obsessions/compulsions, *(iv)* attention-deficits/hyperactivity (Fig. 3). Regression analysis between the motor-tic score and surrogate measures of iron revealed a trend with serum ferritin levels (*r*=0.51, *P*=0.091) and a significant negative association with striatal susceptibility (*r*=0.54, *P*=0.027) (Fig. 3D). All correlations exhibited an approximate variance inflation factor of 1.5 indicating little to no multi-collinearity between predictor variables.

***GTS exhibits shared genetic heritability (hg2) with ferritin and transferrin***

In order to investigate whether the observed inverse (linear) relationship between serum ferritin levels and tic severity is indicative of shared pathophysiology, we tested whether TS and iron-related traits have overlapping genetic risk by evaluating the genetic correlation between GWAS summary statistics of ferritin, transferrin, and TS using LDSC regression (Bulik-Sullivan et al., 2015). We found a significant negative genetic correlation between ferritin and TS (rg = -0.34; SE = 0.13; P = 0.0087), indicating that shared genetic variants contribute both to increased TS risk and decreased ferritin levels. A similar genetic correlation between higher TS risk and higher transferrin levels (rg = 0.28; SE = 0.13; P = 0.036), though the finding did not surpass a Bonferroni correction threshold (P = 0.025).

***Association between susceptibility differences and iron-related gene expression***

Driven by these results, iron-related gene expression profiles were extracted within three functionally distinct sub-territories of the striatum (motor, associative, limbic) and cross-correlated with statistical maps of Δχ reductions at the same coordinates (Fig. 4). Permutation-based inference revealed significant positive associations between striatal-motor susceptibility and the PCs of iron-related gene sets (Fig. 5). Inspection of associations between the mean expression profile of the gene sets and Δχ statistical values revealed similar findings (Fig. 5).

**﻿Gene Expression associated with Magnetic Susceptibility**

﻿MORGAN: Gene Expression Related to Morphometric Similarity. We used PLS regression to identify patterns of gene expression that were cor- related with the anatomical distribution of case–control morpho- metric similarity differences. The first PLS component explained 13% of the variance in the case–control morphometric similarity differences combining data from all three studies, significantly more than expected by chance (permutation test, P<0.001). PLS1 gene expression weights were positively correlated with case–control morphometric similarity differences in the Dublin study (r =0.49, P<0.001) and the Cobre study (r =0.37, P< 0.001) (Fig. 2A) but not in the Maastricht study (r =0.006, P=0.94). These positive correlations mean that genes positively weighted on PLS1 are overexpressed in regions where mor- phometric similarity was increased in patients, while negatively weighted genes are overexpressed in regions where morphome- tric similarity was decreased in patients (Fig. 2D). Hence, genes that are positively (or negatively) weighted on PLS1 were related to increased (or decreased) morphometric similarity in cases compared with controls.

WHITAKER: The top two PLS components explained 28% of the variance in the MRI response variables (permutation test; P < 0.001). The first partial least squares component (PLS1) (Fig. 3A) represented a significant association between a rostrocaudally patterned gene expression ﻿expression profile and baseline measures of CT and MT at 14 y old (Fig. 3 B and C and SI Appendix,Fig.S5). The second independent partial least squares component (PLS2) (Fig. 3D) represented a significant association between a dorsoventrally patterned gene expression profile and measures of adolescent cortical shrinkage and myelination (Fig. 3 E and F). Dataset S1 has a full list of sig- nificantly over- or underexpressed genes represented by the first two PLS components. Focusing on the gene expression profile defined by PLS2, because it was specifically associated with adolescent cortical shrink- age and intracortical myelination, we found that this transcriptional signature was significantly enriched in genes relating to synaptic transmission (P < 0.001), regulation of glutamatergic signaling (P < 0.001), and potassium ion channels (P < 0.001) (36) (SI Appendix, Fig. S3G). We also found that the transcriptional profile associated with association cortical consolidation was significantly enriched for an oligodendroglial gene set (P < 0.001) (37) (SI Appendix,Fig. S3F) as well as a set of genes robustly associated with risk for schizophrenia, a neurodevelopmental disorder (P < 0.001) (38) (SI Appendix,Fig. S3G).

**PLS**

1. **Motor**
   1. **Explained variance**: 82.2% p=0.003
      1. PLS1=48.2%,
      2. PLS2=17.1%
      3. PLS3=16.1%
   2. **Correlation:**
      1. PLS1 R= 0.696, p= 4.0x10-8
      2. PLS2 R= 0.41, p=3.7x10-3
      3. PLS3 R=0.41, p=3.8x10-3
2. **EXEC**
   1. **Explained variance**: 74%, p=0.0001
      1. PLS1=52.2%,
      2. PLS2=23.0%
   2. **Correlation:**
      1. PLS1 R=0.72 , p = 9.2 x10-9)
      2. PLS2 R=0.48, p=5.6 x10-4
3. **LIMBIC**
   1. **Explained variance**: R=73.1%, p=0.02
      1. PLS1=49.1%
      2. PLS2=23.9%
   2. **Correlation:**
      1. PLS1 R=0.70, p= 4.7 x10-7
      2. PLS2 R=0.48, p=1.3 x10-3