Dissecting the cross-trait effects of the *FOXP2* GWAS hit on clinical and brain phenotypes in adults with ADHD

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Abstract

The Forkhead box P2 (*FOXP2*) encodes for a transcription factor with a broad role in embryonic development. It is especially represented among GWAS hits for neurodevelopmental disorders and related traits, including attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder, neuroticism, and risk-taking behaviors. While several functional studies are underway to understand the consequences of *FOXP2* variation, this study aims to expand previous findings to clinically and genetically related phenotypes and neuroanatomical features among subjects with ADHD. The sample included 407 adults with ADHD and 463 controls. Genotyping was performed on the Infinium PsychArray-24 BeadChip, and the *FOXP2* gene region was extracted. A gene-wide approach was adopted to evaluate the combined effects of *FOXP2* variants (n=311) on ADHD status, severity, comorbidities, and personality traits. Independent risk variants presenting potential functional effects were further tested for association with cortical surface areas in a subsample of cases (n=87). The gene-wide analyses within the ADHD sample showed a significant association of the *FOXP2* gene with harm avoidance (P=0.001; P_{FDR}=0.015) and nominal associations with hyperactivity symptoms (P=0.026; P_{FDR}=0.130) and antisocial personality disorder (P=0.026; P_{FDR}=0.130). An insertion/deletion variant (rs79622555) located downstream of *FOXP2* was associated with the three outcomes and nominally with the surface area of superior parietal and anterior cingulate cortices. Our results extend and refine previous GWAS findings pointing to a role of *FOXP2* in several neurodevelopment-related phenotypes, mainly those involving underlying symptomatic domains of self-regulation and inhibitory control. Taken together, the available evidence may constitute promising insights into the puzzle of the *FOXP2*-related pathophysiology.

Keywords: ADHD, *FOXP2*, psychiatric disorders, structural neuroimaging, personality traits.

Introduction

Genome-wide associated risk loci for attention-deficit/hyperactivity disorder (ADHD) include a hit near the Forkhead box P2 *(FOXP2)* gene region [1]. This gene encodes a member of the forkhead/winged-helix family of transcription factors that modulates the embryonic expression of hundreds of genes involved mainly in neuronal growth, neural development, and synaptic plasticity [2–4]. These molecular functions take part in key mechanisms related to learning, memory, and cognitive functions, which are central processes in the pathophysiology of ADHD and several other psychiatric disorders [5, 6].

FOXP2 is highly expressed in the cortex, basal ganglia, striatum, and cerebellum [7–9], regions known to be involved in motor learning and control [10]. In fact, cortical-subcortical circuitry is also crucial to cognitive performance. It regulates several symptomatic domains often impaired in the neuropsychiatric conditions associated with *FOXP2*, such as self-regulation, decision making, and attention [11–18]. In line with this, neuroimaging studies have reported associations between these brain regions and neurodevelopmental disorders, including ADHD and autism spectrum disorder, for which cognitive and impulse control impairments are observed [19–21]. As cognitive processes are also involved in the sequential learning and working memory required for social communication, it is not surprising that *FOXP2*, which has long been studied due to its causal role in speech and language development [22–24], also has a role in underlying mechanisms involved in psychiatric disorders.

In this sense, *FOXP2* has been associated by GWAS with a wide variety of psychiatric-related phenotypes, that besides ADHD, includes autism spectrum disorder [25], neuroticism (especially the domains of irritability and tension) [26], sleep disturbances such as insomnia [27, 28] and risk-taking/risk tolerance behaviors [29–31]. Taken together, such evidence suggests that a potentially shared component underlying these associations could be related to emotional or impulse control. Dysregulation in these symptom domains has shown to be a common factor among psychiatric disorders and is relevant to many aspects of cognitive functioning and learning [32].

While the consistent pattern of associations in behavior has spurred *FOXP2* research on evolutionary [33], cell biology [2, 34–36], animal models [23, 37, 38], and other promising approaches, there is also a need for in-depth dissection of its effects on psychiatric clinical aspects. Thus, this study aims to extend previous genome-wide associations involving *FOXP2* variants to ADHD clinically and genetically related (endo)

phenotypes, including symptom severity, comorbidities, personality traits, and neuroanatomical measures. An integrated and thorough approach involving gene-wide analyses followed by functional characterization of *FOXP2* signals to prioritize potential causal variants was performed in this comprehensive evaluation.

Material and Methods

Subjects

The sample was composed of 407 adults with ADHD from the adult division of the ADHD Outpatient Program (ProDAH-A) from *Hospital de Clínicas de Porto Alegre* (HCPA) and 463 blood donor controls from the same hospital. After the study was publicized in the local media, the patients self-referred to the ProDAH-A, where an initial screening interview was performed to confirm ADHD diagnosis before their inclusion in the study. All subjects are white Brazilians of predominantly European descent aged 18 years or older. The first step to defining individuals as white Brazilians was based on self-classification and the psychiatrist's perception of skin color, followed by their confirmation that their parents or grandparents were of European origin. Besides, it is important to notice that most individuals (94%) from Southern Brazil are of European descent, according to reported estimates of interethnic admixture [39–42]. The second step considered genomic information as described in the "Genomic analysis" section below. The exclusion criteria included evidence of a clinically significant neurological disease that might affect cognition (such as a history of head trauma and/or epilepsy or dementia) and an estimated intelligence quotient score (IQ) below 70.

The diagnosis of ADHD followed the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria [43] from 2001 to 2012 and DSM-5 from 2013 onwards [44, 45]. ADHD and oppositional defiant disorder (ODD) diagnoses were performed according to the Portuguese version of the Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS-E) [46], adapted for adults [45]. As expected for a sample of adults, patients with the combined presentation were the most common in our sample (53,3%; n = 217), followed by the inattentive (42,3%; n = 172) and hyperactive/impulsive (4,4%; n = 18) presentations. Antisocial personality disorder (ASPD) diagnosis was assessed through the Brazilian version of the Mini-International Neuropsychiatric Interview (MINI) [47, 48]. All other psychiatric comorbidities were evaluated by trained psychiatrists using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) [49] from 2001 to 2012, an adapted version of SCID from 2012 to 2015, and the SCID-

5 from 2015 onwards [50]. The severity of ADHD symptoms was measured using the Swanson, Nolan, and Pelham Rating Scale, version 4 (SNAP-IV) [51]. Temperament/personality dimension scores were assessed by the Temperament and Character Inventory (TCI version 9) [52].

The control sample had a negative screening for ADHD, as assessed by the 6-item Adult ADHD Self-Rated Scale Screener (ASRS) [53]. In this sample, psychiatric disorders were evaluated through SCID-I screening module [49] from 2001 to 2015 and SCID-5 [50] after 2015, including psychosis and anxiety, mood, substance use, and eating disorders.

All participants were fully informed of all study procedures and provided signed informed consent, approved by the institutional review board of HCPA (IRB 0000921). This work was carried out following the Declaration of Helsinki.

Genomic analysis

Genotyping was performed on the Infinium PsychArray-24 BeadChip (Illumina, San Diego, CA, USA). Pre imputation quality control (QC), principal components (PC) analysis to exclude ancestry outliers and related individuals, and genotype imputation procedures were implemented using the Ricopili pipeline following default parameters (//sites.google.com/a/broadinstitute.org/ricopili/home). The European population of the 1000 Genomes Project Phase 1 was used as the reference panel mapped to the GRCh37 build. Post imputation QC was performed using the following settings for inclusion of variants or individuals: info score > 0.8, minor allele frequency > 10%, SNP and individual call rate > 95%, and Hardy-Weinberg equilibrium test with p-value > 1e-06. The *FOXP2* gene region plus 35 kb upstream and 10 kb downstream window was extracted to include regulatory elements [54], and 311 variants were retained in the final dataset.

Magnetic Resonance Imaging (MRI) acquisition data

The brain images were acquired for a subsample (n = 87) of individuals with ADHD during a follow-up reassessment 13 years after the initial diagnosis. MRI acquisition was performed in a 3.0 T Siemens SPECTRA scanner with a 16-channel head coil. A high-resolution structural MRI volume was acquired using T1-weighted 3D MPRAGE sequence with 192 slices, flip angle = 7°, TE = 2.55 ms, TR = 2530 ms, TI = 1100 ms, matrix size = 256 × 256, isotropic resolution of 1 mm, and a GRAPPA factor of 2. Our

MPRAGE T1-weighted structural images were visually inspected before segmentation at the Freesurfer software v. 5.3 [55]. The quality control of images followed Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) protocol [56], using as input the data generated after preprocessing at Freesurfer.

Statistical analyses

A gene-wide approach was performed using PLINK software, version 1.9, with the --*set-based* command followed by 10,000 permutations [57]. The set-based test provides a joint association value for the combined effects of all included variants within the gene region (n = 311; gene-wide association). It also retrieves individual association values per variant (single variant association), indicating the associated independent variants that are, by default, the ones with the lowest p-values. We tested the *FOXP2* gene-wide association with ADHD case-control status, as well as with ADHD symptom severity, personality dimensions, and comorbidities within the ADHD sample. Apart from the 10,000 permutations performed for each outcome, FDR correction was applied to the gene-wide analyses considering the number of outcomes tested (ADHD status plus 14 outcomes tested in the ADHD sample).

Besides the independent variants retrieved from the analyses, all single variants presenting p-values < 0.05 for traits that were at least nominally associated in the gene-wide analyses were submitted to an exploratory search using *in silico* tools (see *Functional prediction in silico search* below). Among them, representative variants of each linkage disequilibrium block with the highest potential to present a functional regulatory role based on *in silico* tools and evidence of an association by previous GWAS were selected to further test their association with neuroimaging measures within the ADHD sample. The neuroimaging measures evaluated were cortical surface areas of 30 brain regions previously associated with ADHD [20]. FDR correction was applied considering the variants and the brain regions tested. This strategy was adopted to search for evidence of effects in neurogenesis and/or neurodevelopmental risk, considering that variants associated with psychiatric-related traits and with evidence of regulatory features probably affect gene expression and function in the brain.

All analyses were based on an additive genetic model. Sex, age, and the ten first principal components were included as covariates. The analyses of neuroimaging measures also included average surface area as a covariate.

Functional prediction in silico search

The *in silico* tools used to assess the potential regulatory features of the variants selected from the single variant analyses mentioned above were:

(1) RegulomeDB [58], a tool that annotates SNPs with known and predicted regulatory elements in the intergenic regions of the human genome. It provides probability scores that integrate functional genomics features with ChIP-seq, DNase-seq, and other sources, ranging from 0 to 1, with 1 being most likely to be a regulatory variant. Since there is no exact cut-off definition to classify functionality, we considered probability scores higher than 0.6 as a reasonable threshold for a moderate to a high probability of functionality for our purposes.

(2) CADD (Combined Annotation Dependent Depletion) [59], a tool that provides a score of deleteriousness predictions of a single nucleotide (a scaled CADD score - PHRED - greater than 10 indicates that these are predicted to be the 10% most deleterious substitutions in the human genome, while a score greater than 20 points the 1% most deleterious and so on).

(3) HaploReg v4.1 [60], a tool that examines annotations of the noncoding genome at variants on haplotype blocks at disease-associated loci, including information on chromatin state, protein binding annotation, sequence conservation, the effects of variants on regulatory motifs, and expression from eQTL studies.

(4) Variant Effect Prediction (VEP) [61], a tool for predicting functional consequences of known and unknown variants.

(5) Variant Annotation Integrator (VAI) [62], a tool for associating annotations from the UCSC database to predict functional effects of variants on transcripts.

(6) MirSNP [63], a collection of human SNPs in predicted miRNA-mRNA binding sites that promote post-transcriptional effects.

The set of variants was also evaluated for associations by GWAS considering the psychiatric and cognitive domains of the GWAS atlas website [64]. Moreover, a heatmap of pairwise linkage disequilibrium statistics using the CEU population as the reference was obtained with the LDmatrix tool [65].

Results

The characteristics and the comorbidity profile of the total sample of individuals with ADHD and controls are presented in **Table 1**.

In the gene-wide analyses, there was no significant association between the *FOXP2* gene and ADHD case-control status (P = 0.654) (**Table 2**). The analyses within the ADHD sample showed a significant association of *FOXP2* gene with harm avoidance (HA; P = 0.001; P_{FDR} = 0.015), and nominal associations with hyperactivity symptoms (P = 0.026; P_{FDR} = 0.130) and ASPD (P = 0.026; P_{FDR} = 0.130) (**Table 2**). The single variant associations comprising the 311 variants in the *FOXP2* region for these traits are depicted in the region plots generated from LocusZoom [66] in **Supplementary Figures 1, 2, and 3**, respectively.

Summarized single variant association results are presented in **Table 2**, where one variant with p-value < 0.05 was observed for HA, 42 (3 independent) for hyperactivity symptom severity, and 24 (3 independent) for ASPD. **Supplementary Tables 1 and 2** present the summary statistics of the association results between each of these variants and hyperactivity severity and ASPD, respectively, the LD relationship between each independent variant and those comprising the block, the *in silico* prediction of functionality, and previous GWAS findings**.** A linkage disequilibrium matrix for all pairwise comparisons of these variants is shown in **Supplementary Figure 4** (missing data for rs79622555). This data was used for the selection of variants to test in the subsequent neuroimaging analyses.

The only variant associated with HA (rs79622555) was a shared association with hyperactivity and ASPD. It is an insertion/deletion variant located at chr7:114340504, and the Insertion allele was associated with lower HA scores, higher hyperactivity severity, and increased risk for ASPD (**Table 3**). As presented in **Supplementary Tables 1 and 2**, a potential regulatory feature is suggested for this variant since it is located at a DNAse hypersensitivity area in the brain germinal matrix tissue and presents a relatively high probability score of functionality (probability score = 0.67) according to RegulomeDB. Also, no LD pairs were identified for rs79622555. Based on this evidence, this variant was among those selected for subsequent analyses with neuroimaging measures. Although we did not observe a significant association after FDR correction of rs79622555 with cortical surface areas in any tested brain region, nominal associations were found for caudal anterior cingulate and superior parietal cortices (**Table 3**). We additionally tested the dominant model considering the low frequency of homozygotes for the minor allele (genotype frequencies in the ADHD sample: Del/Del, n = 327; Del/Ins, n = 68, Ins/Ins, n = 9) and similar results were obtained.

Besides rs796622555, five additional independent variants (rs2244419, rs1852638, rs1668333, rs4730637, and rs12705984) with the highest potential to be causal were selected to be tested for association with neuroimaging measures considering the integrated set of information presented in **Supplementary Tables 1 and 2**. The association analyses between cortical surface areas of 30 brain regions and the selected variants are depicted in **Supplementary Table 3**, and no significant results were found after FDR correction.

Discussion

Our study extends and refines previous GWAS findings pointing to a role of *FOXP2* in neurodevelopmental-related phenotypes, mainly those involving underlying domains of impulse control and decision making. By adopting a gene-wide approach, we found suggestive evidence that the personality trait of HA, hyperactivity symptom severity, and ASPD might be influenced by the combined effects of all measured variation in the *FOXP2* gene region in adults with ADHD. Notably, a specific insertion/deletion variant located downstream of *FOXP2* (rs79622555) was a common finding among the phenotypes.

Consistent with our findings, genome-wide significant hits for *FOXP2* include neuroticism [26] and risk-taking behavior [64]. Neuroticism is a personality trait positively correlated to HA, and both characteristics are related to decision-making and can interfere in risk-taking behavior [67]. Risk-taking is a common behavioral trait of neuropsychiatric disorders characterized by difficulties in impulse control, such as substance use and ADHD. Such behavior refers to the individuals' propensity to select a high-gain/high-risk over a low-gain/low-risk alternative even when associated with a disadvantageous long-term outcome [68]. The previously reported association between polygenic risk scores for ADHD with neuroticism, tobacco and alcohol use, and risk-taking behavior [69] reinforces the correlation among these traits. It suggests a shared neurobiological mechanism that may be related to emotional and impulse control. In this sense, *FOXP2*, as a shared genetic component, might exert its effects by modulating such intermediate phenotypes that underlie the neurobiology of a variety of psychiatric disorders.

Indeed, cortical *FOXP2* deletion in mice impaired behavioral flexibility [36], a cognitive function that involves problem-solving and the capacity to learn the reversal of a task that has been well trained and is considered a strategy to inhibit impulsivity and

compulsive drug-seeking behavior [70, 71]. Interestingly, impaired cognitive flexibility and poor impulse control are also characteristic of antisocial behaviors [72, 73], including ASPD, a frequent comorbidity of ADHD. Our suggestive findings of an association between ASPD and *FOXP2* corroborate the hypothesis of a potential role of this gene in endophenotypes related to impulse control. Multiple component processes likely encompass self-regulation and might explain its broad relevance for personality traits and several aspects of psychopathology.

Considering that brain structure and functioning are influenced by genetic factors, especially in the context of neuropsychiatric conditions, we tested whether *FOXP2* variation associated with the ADHD-related traits in our sample would also affect cortical structure in specific regions previously identified as relevant for ADHD [20]. Although these analyses did not support a robust role of *FOXP2* common variation in brain structure, which might reflect the reduced sample included in the neuroimaging analyses, the nominal associations of the rs79622555 with caudal anterior cingulate cortex and superior parietal cortex should be highlighted. Both regions have been implicated in cognitive functions [74, 75], and the anterior cingulate cortex, one of the key structures of the cortical-basal ganglia network, is specifically involved in reward-based learning, decision-making, response inhibition [75], and behavioral shift after error [76]. Also, its caudal region has an important role in negative emotion control and processing [77, 78], which manifests as individual differences in personality that relate to reward sensitivity and persistence [79]. Interestingly, this region is activated during inhibition tasks in healthy adults [80–85] and children with disorders characterized by lack of impulse control such as ADHD [86], autism [87], and disruptive behavior disorders [88], supporting a connection between this brain region, self-regulation and neuropsychiatric disorders.

As mentioned above, rs79622555 was a common association among the evaluated clinical traits (see Table 2). The suggestive evidence of its influence in the cortical surface area also indicates that brain structural alterations might be on the pathway of the genetic effects of *FOXP2* on psychiatric-related traits. The *in silico* search showed that rs79622555 is located 6.7 kb 3' of *FOXP2* at a DNAse hypersensitivity site. It is also noteworthy that we did not find any LD pairs for this specific variant, possibly reflecting either its location near a recombination hotspot (see **Supplementary Figures 1-3**) or a drawback of the tools available. Although there is limited information on the functionality of this specific variant, the 3'UTR region of *FOXP2* is known to be highly targeted by

miRNAs [89] that control gene expression at a post-transcriptional level. Moreover, loss of function in this region implicates ectopic expression, delays in neurite outgrowth, and altered cellular migration [89, 90], suggesting a potential regulatory mechanism of this region that might impact brain structure or functioning and susceptibility to psychiatric disorders.

Our results should be interpreted considering some limitations. Although gene-wide approaches, as the one applied here, based on previously reported GWAS hits, have substantially higher statistical power than genome-wide single variant associations, the relatively small sample size may have precluded the identification of more robust *FOXP2* effects. Even though we did not specifically replicate the association of *FOXP2* with ADHD status, our findings reinforce a pattern of associations with ADHD-related traits that might represent underlying mechanisms involved in the disorder's pathophysiology. The sample size limitation is even more critical in the subgroup with neuroimaging measures and might explain the lack of association in these analyses. In addition, unfortunately, we do not have information on language-related phenotypes to investigate a possible common mechanism of *FOXP2* effects on psychiatric-related traits and language skills. Finally, although refined *in silico* analyses were performed, which integrated information from several bioinformatics tools, there is scarce information on the functionality of the associated variants, especially for the insertion/deletion one. This limitation impairs more refined inferences on regulatory mechanisms underlying the observed associations and whether it might involve brain structure alterations.

Conclusion

In summary, the present findings evaluating a sample of adults with ADHD are consistent with previous studies implicating the *FOXP2* gene in several psychiatric-related phenotypes. Our results, combined with previous *FOXP2* GWAS associations and the reported evidence of a genetic correlation between ADHD and neuroticism, substance use, and risk-taking behavior, might suggest an underlying common neurobiological mechanism related to self-regulation and inhibitory control that might be mediated, at least in part, by *FOXP2* effects. Fine mapping and characterization of noncoding risk variants by integrating genomic/transcriptomic associations over a wide range of common neuropsychiatric phenotypes, molecular data, bioinformatics, and other sources should be further pursued by future studies to prioritize the most likely causal variants before proceeding with biological follow-up experiments.

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Declaration of conflicting interests: LAR has received honoraria, has been on the speakers' bureau/advisory board, and/or has acted as a consultant for Medicine, Novartis/Sandoz, and Shire/Takeda in the last two years; and receives authorship royalties from OxfordPress and ArtMed. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by him have received unrestricted educational and research support from the following pharmaceutical companies in the last three years: Janssen-Cilag, Novartis/Sandoz, and Shire/Takeda. EHG has served as a speakers' bureau/advisory board for Novartis and Shire Pharmaceuticals in the past three years; and has received travel awards from Novartis and Shire for taking part in psychiatric meetings. The other authors declare no conflicts of interest.

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Authors' contributions: GPM and BSS designed the study, performed the data analysis, and prepared the first drafts of the manuscript. CEB, MEAT, and RBC collected and processed the neuroimaging data, providing essential contributions to these analyses. EPO, DM, and DBK provided substantial contributions to data acquisition, analyses, and interpretation of the results. SPT and ESV were responsible for the clinical assessment of

the patients and helped with the evaluation of the clinical outcomes. DLR, LAR, and EHG provided critical discussion and insights into the intellectual content of the manuscript. CHDB contributed to the conception and design of the study and participated in all its stages of its preparation. All authors carefully revised and approved the final version of this manuscript.

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