An integrative approach to investigate the respective roles of single-nucleotide variants and copy-number variants in Attention-Deficit/Hyperactivity Disorder

Leandro de Araújo Lima1,2, Ana Cecília Feio-dos-Santos3, Sintia Iole Belangero5,6, Ary Gadelha4,6, Rodrigo Affonseca Bressan4,6, Giovanni Abrahão Salum4,5, Pedro Mario Pan5,6, Tais Silveira Moriyama3,4,6, Ana Soledade Graeff-Martins3,4, Ana Carina Tamanaha4,6, Pedro Alvarenga3,4, Fernanda Valle Krieger3, Bacy Fleitlich-Bilyk3,4, Andrea Parolin Jackowski4,6, Elisa Brietzke4,6, João Ricardo Sato4,7, Guilherme Vanoni Polanczyk3,4, Jair de Jesus Mari4,6, Gisele Gus Manfr3,5, Maria Conceição do Rosário1,9, Eurípedes Constantino Miguel3,4, Renato David Puga8, Ana Carolina Tahira3, Viviane Neri Souza3, Thais Chile3, Gisele Rodrigues Gouveia3, Sérgio Nery Simões3,9, Xiao Chang2, Renata Pellegrino2, Lifeng Tian2, Joseph T. Glessner2, Ronaldo Fumio Hashimoto1,10, Luis Augusto Rohde3,4,5, Patrick M.A. Sleiman2,11, Hakon Hakonarson2,11 & Helena Brentani3,4

Many studies have attempted to investigate the genetic susceptibility of Attention-Deficit/Hyperactivity Disorder (ADHD), but without much success. The present study aimed to analyze both single-nucleotide and copy-number variants contributing to the genetic architecture of ADHD. We generated exome data from 30 Brazilian trios with sporadic ADHD. We also analyzed a Brazilian sample of 503 children/adolescent controls from a High Risk Cohort Study for the Development of Childhood Psychiatric Disorders, and also previously published results of five CNV studies and one GWAS meta-analysis of ADHD involving children/adolescents. The results from the Brazilian trios showed that cases with de novo SNVs tend not to have de novo CNVs and vice-versa. Although the sample size is small, we could also see that various comorbidities are more frequent in cases with only inherited variants. Moreover, using only genes expressed in brain, we constructed two “in silico” protein-protein interaction networks, one with genes from any analysis, and other with genes with hits in two analyses. Topological and functional analyses of genes in this network uncovered genes related to synapse, cell adhesion, glutamatergic and serotonergic pathways, both confirming findings of previous studies and capturing new genes and genetic variants in these pathways.

1Inter-institutional Grad Program on Bioinformatics, University of São Paulo, São Paulo, SP, Brazil. 2Center for Applied Genomics, The Children’s Hospital of Philadelphia, Philadelphia, PA, USA. 3Department & Institute of Psychiatry, University of São Paulo Medical School, São Paulo, SP, Brazil. 4National Institute of Developmental Psychiatry for Children and Adolescents (INCT-CNPq), São Paulo, SP, Brazil. 5Department of Psychiatry, Hospital de Clínicas de Porto Alegre, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. 6Department of Psychiatry, Federal University of São Paulo, São Paulo, SP, Brazil. 7Center of Mathematics, Computation and Cognition. Universidade Federal do ABC, Santo André, Brazil. 8Hospital Israelita Albert Einstein, Clinical Research, São Paulo, SP, Brazil. 9Federal Institute of Espírito Santo, Serra, ES, Brazil. 10Inter-institutional Grad Program on Bioinformatics, University of São Paulo, São Paulo, SP, Brazil. 11Department of Pediatrics, The Perelman School of Medicine, University of Pennsylvania Philadelphia, PA, USA. Correspondence and requests for materials should be addressed to H.B. (email: helena.brentani@gmail.com)
In the last decade, multiple studies have investigated the genetic susceptibility for ADHD, most notably including assessment of enrichment of copy number variation (CNV). Genome-wide association studies (GWAS) of single nucleotide variants (SNVs) in ADHD have also been executed and a few variants of small or moderate effect sizes have been reported. Despite these promising results, genes identified by GWAS (capturing CNVs and SNVs) still only contribute for a small percentage of the heritability of this complex trait and replication across these studies has been inconsistent. This is likely attributed to the combination of modest effect sizes of causative mutations on disease susceptibility and underpowered studies due to small sample sizes. Meta-analyses in ADHD, while theoretically more powerful, has not succeeded in discovering novel loci. However, copy-number variation (CNV) analysis has been more successful than SNV analysis.

Extensive literature has also emerged from the integration of different databases to prioritize variants of biological interest involving complex diseases such as psychiatric disorders. Poelmans et al. used PPI data to investigate networks with genes overrepresented in CNVs affecting multiple cohorts. Elia and colleagues used GWAS data and PPI networks have been published with focus on psychiatric disorders, including bipolar disorder, schizophrenia, autism, as well as ADHD. Elia and colleagues reported that CNVs in glutamate receptor family genes and in genes interacting with them were enriched in approximately 10% of cases, showing for the first time association of glutamate receptor genes with ADHD.

With the development of new genomic techniques to investigate changes in DNA, it has been possible to discover the genetic causes for a larger range of conditions and phenotypes than ever before. For complex disorders, it has been necessary to apply methods to analyze genome-wide variants with SNP arrays in conjunction with sequencing data to combine both common and rare variants. Analysis of high throughput sequencing data has been successful in covering genomic regions with low frequency mutations, which also have impact in complex diseases. Moreover different types of very rare variants, such as de novo variants, compound heterozygosity and even heritable heterozygous missense variations have been shown to contribute to neurodevelopmental disorders.

In other complex psychiatric disorders, such as autism and schizophrenia, the role of common or inherited variants has been shown to be important. Small CNVs have also been reported. These studies show that the genetic landscape of neurodevelopmental psychiatric disorders can be composed by the combination of different types of variants.

According to Hawi et al., “As with other psychiatric disorders, the monogenic concept of ADHD has now been supplanted by a more plausible polygenic hypothesis where multiple risk genes (each of minor/modest effect) contribute to the etiology of the disorder. As detailed previously, ADHD-associated genes (and those showing trends towards association) are scattered through the genome but tend to be enriched within specific functional categories. This suggests that the emphasis on any individual candidate gene should be shifted to consider a broader network view of biological pathways involving ADHD-implicated genes.”

As previously proposed by Giriajaran and Eichler, we have used in this present study both de novo and inherited variants to integrate our model of ADHD genetic architecture using an exome analysis of 30 sporadic ADHD Brazilian trios and SNP-array from 503 typically Brazilian developed children. To validate our findings we integrated our results with public ADHD genetic resources, gene expression data bases and PPI databases exploring the ADHD network in accordance to Hawi et al. and the hypothesis of network medicine described by Barabasi et al., that genes related to the same disease tend to interact.

Methods

In all analyses we only accepted variations in genes expressed in brain based on data from the Brain Span Atlas of the Developing Human Brain (http://brainspan.org), Brain Atlas (http://human.brain-map.org) and GTEx Portal (http://gtexportal.org). The analyses were conducted in 3 steps:

1. we used only data from Brazilian samples, searching for de novo and inherited SNVs and CNVs comparing ADHD trios and control samples to better explore the genomic architecture of our ADHD samples.
2. we constructed a protein-protein interaction network (PPI) based on all genes with SNVs and CNVs findings by our work and studies with samples of children and adolescents reported by ADHDgene database (http://adhdpsych.ac.cn) and explore functional annotation of the network as well as topological properties of ADHD genes in the PPI network.
3. to achieve a highly reproducible ADHD gene set we selected only genes with two evidences: presented in our SNV and CNV analyses in different families or presented in our analyses and also in a previous GWAS or CNV public ADHD analyses. We used this gene set as seeds to growth a PPI.

A summary of steps 1 and 2 can be found in Fig. 1.

Brain expression data. The samples from Brain Span Atlas of the Developing Human Brain (http://brainspan.org), Allen Human Brain Atlas (http://humanbrain-map.org) and GTEx Portal (http://www.gtexportal.org) were used to understand what genes are expressed in brain. Genes with 1 reads per kilobase per million reads

| 1. we used only data from Brazilian samples, searching for de novo and inherited SNVs and CNVs comparing ADHD trios and control samples to better explore the genomic architecture of our ADHD samples. |
| 2. we constructed a protein-protein interaction network (PPI) based on all genes with SNVs and CNVs findings by our work and studies with samples of children and adolescents reported by ADHDgene database (http://adhdpsych.ac.cn) and explore functional annotation of the network as well as topological properties of ADHD genes in the PPI network. |
| 3. to achieve a highly reproducible ADHD gene set we selected only genes with two evidences: presented in our SNV and CNV analyses in different families or presented in our analyses and also in a previous GWAS or CNV public ADHD analyses. We used this gene set as seeds to growth a PPI. |

A summary of steps 1 and 2 can be found in Fig. 1.

Brain expression data. The samples from Brain Span Atlas of the Developing Human Brain (http://brainspan.org), Allen Human Brain Atlas (http://humanbrain-map.org) and GTEx Portal (http://www.gtexportal.org) were used to understand what genes are expressed in brain. Genes with 1 reads per kilobase per million reads
(RPKM) in any area of the brain in at least one sample were considered expressed. After evaluation, our list contained 54,141 transcripts.

STEP 1 - Brazilian samples
The sample is part of the High Risk Cohort Study for Psychiatric Disorders in Childhood, a two-stage large community study of children aged 6 to 12 years (at screening) from 57 schools in two Brazilian cities - Porto Alegre (n = 22) and São Paulo (n = 35). The ethics committee of the University of São Paulo approved the study and written consent from parents of all participants were collected. Detailed data could be found in Salum et al. Briefly, during the screening phase at their school registry day, 9,937 informants were interviewed. Two subgroups using a random-selection (n = 958) and high-risk group (n = 1,514) selection procedure that consisted of selecting individuals with high family loading of symptoms and ongoing psychiatric symptoms, were recruited resulting in a total sample of 2,512. Saliva was collected from all subjects and their parents. To establish child psychiatric diagnoses, the parent-reports of the Brazilian Portuguese version of the Development and Well-Being Assessment – DAWBA was performed by trained psychiatrists and to assess parent psychiatric diagnoses the Mini International Psychiatric Interview (MINI) and MINI Plus were used. Out of 2,512 subjects 240 met DAWBA criteria for any ADHD, being 66 from the randomly selected group and 240 from the high-risk group. For the exome analysis, we selected 35 trios, in which the child was affected by ADHD and the parents were healthy as well as no familial history of ADHD was reported in the screening interview. A sub-sample of 724 subjects out of the 2,512 was selected to collect blood and perform a functional magnetic resonance imaging (fMRI) study. We used 503 subjects from this sub-sample, in which the child was not affected by any DAWBA diagnosis and has available genotypes after quality control criteria using an SNP-array platform assay.

Exome data processing of Brazilian samples. The Nextera® Exome Enrichment Kit (Illumina Inc., San Diego, USA) was utilized to construct the libraries, which were prepared over a target region of 62 Mb, 20,794 genes, 201,121 exons. The sequencing was performed using two technologies. In eight trios, Illumina HiScan SQ™ (Illumina, Inc.) was utilized. In 27 other trios, HiSeq 2500™ (Illumina, Inc.) was utilized. The reads were sequenced with the paired-end technique, producing in average 24 million reads per sample with length of 100bp, resulting in 2.4Gb sequenced per sample. The reads were mapped to National Center for Biotechnology Information (NCBI) reference human genome build 37 (GRCh37/hg19), using the software Burrows-Wheeler Aligner, or BWA (http://bio-bwa.sourceforge.net). These mapped reads were the input for Picard (http://broadinstitute.github.io/picard) and GATK Unified Genotyper (https://www.broadinstitute.org/gatk) to find single-nucleotide variants through the best practices pipeline (https://www.broadinstitute.org/gatk/guide/best-practices) and calculate the exons coverage to run the program to identify CNVs. We used the software KING (http://people.virginia.edu/~wc9c/KING) to check kinships between parents and children. It is expected that the value of the kinship between one parent and one child must be between 0.17 and 0.35. If the kinship is much lower than this value, it means that there is no relation of parenthood or that the samples might be contaminated. Trios with at least one kinship relation below 0.19 were removed from our analyses.
Single-nucleotide variant (SNV) analysis in exome of Brazilian cases. Variants were annotated using AnnoVAR (http://annovar.openbioinformatics.org) and SnpEff (http://snpeff.sourceforge.net), and databases dbSNP build 137 (http://www.ncbi.nlm.nih.gov/SNP/). We called ‘common’ the variants with maximum value minor allele frequency (MAF) greater than 5% in the ExAC database (http://exac.broadinstitute.org). Variants with at maximum MAF between 1% and 5% were called ‘rare’, ‘very rare’ if the maximum MAF was less than 1% and ‘novel’ if the variant was not found in the databases. After the annotation, we looked for three types of SNVs that could contribute to the disease: 1. de novo mutations with moderate and high impact (missense, nonsense or splice site); 2. rare, moderate impact (missense) homozygous mutations for loci where both parents are heterozygous; and 3. very rare, high impact (nonsense and splice site) heterozygous mutations.

Copy-number variant (CNV) analysis in exome array data of Brazilian healthy children. The samples were genotyped in HumanCore-12 v1.0 BeadChip (Illumina, Inc.), with 298,930 SNPs. We used PennCNV to detect the CNVs in Brazilian control samples.

Copy-number variant (CNV) analysis in exome of Brazilian cases. We used XHMM (http://atgu.mgh.harvard.edu/xhmm) to find copy-number variants in exome sequencing data. We selected only CNVs with at least 3 exons, minimum of 1kb, phred-scaled qualities of some CNV event in the interval and the quality of the region not being diploid were at least 90 and that were not present in Brazilian controls (SNP arrays data). We considered false positive and removed from the analysis CNVs present in more than three healthy parents, deletions with heterozygous SNVs rate above 10%, and duplications with average B allele frequency between 0.4 and 0.6.

Single-nucleotide polymorphisms in public data. We used the set of genes related to the 50 resulting SNPs with P-value ≤ 10^{-3} from Neale et al. meta-analysis. This meta-analysis included only studies with children/adolescents and with the same categorical diagnostic variables, which decreases chances of phenotypic heterogeneity. All genes expressed in brain inside the CNV regions were considered.

Copy-number variants in public data. We also used the CNV studies from predominantly Caucasian samples of children/adolescents reported in the ADHGene database. They are part of the following publications: Williams et al. (57 CNVs reported), Lesch et al. (17 CNVs reported), Lionel et al. (23 CNVs reported), Elia et al. (19 CNVs reported) and Jarick et al. (1 CNV reported). From the regions reported by these studies, we searched for genes that had an overlap with these regions, using package biomaRt (http://bioconductor.org/packages/biomaRt). We converted all the regions to hg19 (version 19 of the human genome reference) positions using the package rtracklayer (http://bioconductor.org/packages/rtracklayer) of R language, which uses the same algorithm of UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgLiftOver). All genes expressed in brain inside the CNV regions were considered.

Network measures. In order to analyze the nodes and topological features, centrality measures (brokerking centrality and betweenness centrality) and clustering coefficients were calculated. The degree is the number of links of a node with other nodes (called neighbors) in the network. Nodes with very high degree are called hubs since they are connected to many neighbors. The removal of such nodes has great impact on the network topology. It has been shown that biological networks tend to be robust against random perturbations, but disruption of hubs often leads to system failure. In this work, a measure called brokerking centrality was used to identify hubs whose neighbors do not connect each other (or they connect less than expected). This measure uses the degree and the clustering coefficient to be calculated. The betweenness centrality is the measure of how a node is in the center of the network, and it shows important nodes that lie on a high proportion of paths between other nodes in the network. Proteins with high betweenness centrality have been termed “bottlenecks”, for their role as key connectors of proteins with essential functional and dynamic properties. For a review of formulas of all these measures, see Cai et al. To compute nodes/genes measures, it was used the Python library NetworkX (http://networkx.github.io). We calculated such measures for all the nodes in the network and classified the top 5% ranked for each measure as, respectively, brokers or bottlenecks.

Analysis of overrepresented pathways, biological functions, cytogenetic bands and diseases associated to the different sets of genes. To perform the enrichment analyses, the tool WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt) was utilized. In this program, the set of genes in the human genome was used as reference, the corrections were made using Benjamini and Hochberg method, and only the top 10 terms with an adjusted P-value less than 0.05 were considered significant for our analysis. For KEGG analysis, we
used the program STRING (http://string.embl.de), because the version was more updated (2014) than WebGestalt for KEGG (2011). For STRING, the Bonferroni correction was utilized in the adjustment.

STEP 3 – To achieve a highly reproducible ADHD gene set we selected only genes harboring probably deleterious variations presented by at least two evidences: presented in Brazilian SNV and CNV analyses of two different families or presented in our analyses and also in a previous public analyses. Using only directed interaction to growth the seed genes considered highly reproducible we use the iRefIndex database (http://irefindex.org), to construct a PPI network and explore functional ontology of the network as already described above.

Results
Psychiatric disorders are considered polygenic and multifactorial, so today it is clear that both rare and common variation contribute to the genetic architecture of such diseases. In addition, it is also known that individuals from different families could have different variations for the same diseases42. For these reasons, stringent analysis criteria have been used, as comparison with public databases in the gene level, not in the variant level. On the other hand, various disorders have the same genes, and one gene could participate in different processes, which leads us to believe that we should look at such diseases in a systemic view. One way to investigate it is by searching for biological networks and its topological properties, like clusters of genes and their biological functions, which could better explain genetic mechanisms of predisposition for a range of diseases or even for a specific disease. Following this idea, to prioritize genes and networks related to ADHD we present our results in three steps:

1. Analysis of individual variations in a specific population (SNV and CNV of Brazilian samples);
2. Integration of our data with public data in a gene level and analysis of such data in a systems biology approach, using protein-protein interaction networks of genes expressed in brain (Integrated PPI ADHD network);
3. Finding a highly reproducible ADHD gene set and the ADHD network.

To have a high reliability in each of these steps, the following criteria detailed in the method section were used:

In the first one we describe our exome results of 30 trios using high stringent criteria to variant call: target coverage of 45× (average) and 50% of target regions with at least 20× of coverage. We removed trios with bad overall mapping quality and low values of kinship. Regarding overall single-nucleotide variants (SNVs), we removed variants with less than 10 read depth or genes not expressed in brain. More specifically for de novo SNVs, we selected only moderate to high impact (nonsense, missense or splice site) SNVs. For copy-number variants (CNVs), we used an independent sample of healthy Brazilian children/adolescents and removed any CNVs also found in this sample. Besides applying the best practices of XHMM (a reliable software for CNV calling in exoma data)43, we developed a new method for removing false positive CNVs taking into account single-nucleotide variants (SNVs) for both deletions and duplications. Our method, inspired by PennCNV™ algorithm, performs a post-processing analysis checking the allele frequencies of SNVs in each CNV to remove those with a large difference of expected proportions.

In the second part we used data from public GWAS data exactly to find more confident results. Up to date, none of the previous GWAS studies performed in children and adolescents with ADHD was able to reach statistical significance in genomic level. Although no variant reached significance, some findings show that common and rare variants could contribute to the disease. Given that, we decided to integrate our exome data results and public datasets of main genetic studies – including 50 SNPs from Neale et al.7 meta-analysis with P-value ≤ 10−5, and four studies that reported validated CNVs1,2,4,5 – all performed with children/adolescents having ADHD. Genes with no expression in brain, according to GTEx database, were removed.

In the third step, to achieve a highly reproducible ADHD gene set we selected only genes harboring probably deleterious variations presented by at least two evidences: presented in Brazilian SNV and CNV analyses of two different families or presented in our analyses and also in a previous public analyses. Using only directed interaction to growth the seed genes considered highly reproducible we use the iRefIndex database (http://irefindex.org), to construct a PPI network and explore functional ontology of the network as already described above.

1. Analysis of individual variations in a specific population (SNV and CNV in Brazilian samples).

Single-nucleotide variants (SNV) analysis in the exome of Brazilian samples. Five trios were excluded from the analysis: one because the father sample did not pass the quality criteria of the sequencing pipeline, 3 trios because at least one kinship factor was less than 0.17 and one trio because both kinship factors were exactly at 0.17. Thirty trios remained in the analysis. We looked for three types of single-nucleotide variants that can be contributing to the disease:

(i) De novo mutations with moderate and high impact (missense, nonsense or splice site): 26 variants were found in 25 genes, and all of them are expressed in brain (Table 1). In the gene VWD1 two variants were found in the same family. One cytogenetic band presented hits in three different families: 19p13 (size: 20 Mb).

(ii) Very rare, high impact (nonsense or splice site) heterozygous mutations (Sup. Table 2): 134 different variants were found in 134 genes expressed in brain. Six out of these 134 genes presented the same variant in two families: ACOXL, ANKRD42, CFHFP2, MSR1, NPSR1, OBSL1. The other 128 genes presented only one variant. Five cytogenetic bands presented hits in at least 4families: 3q13 (size: 89.7 Mb), 3q33 (size: 10.1 Mb), 11p15 (size: 21.7 Mb), and 19p13 (size: 20 Mb); and 19q13 (size: 26.7 Mb) presented hits in 10 families.

(iii) Rare, moderate impact (missense) homozygous mutations for loci where both parents are heterozygous: 127 different variants were found in 120 genes expressed in brain. Five genes (ACSMI, GIMAP6, IRLD1, MUC6, RGS12) presented two variants in the same family, one gene (IFLTD1) presented three variants in the same family and one gene presented (DNAH3) the same variant in two different families. The other 113 genes
After mapping the reads to the Human genome (GRCh37/hg19), we used the XHMM software to call copy-number variations (CNVs). We found 13 occurrences of putative CNVs in the Brazilian children, three being de novo CNVs, seven inherited exclusively from father and three inherited exclusively from mother. In these regions, 22 genes were found to be de novo expressed in brain. The Sup. Table 1 has all CNVs found in the 30 trios. The gene TRIM48 (11q11) was found in exclusively from father and three inherited exclusively from mother. The genes found were distributed in the Brazilian children, we merged inherited SNVs and CNVs and compared it with the Human genome (GRCh37/hg19), we used the XHMM software to call copy-number variations (CNVs).

Table 1. The 26 de novo single-nucleotide variants found in the trios exome sequencing data.

| chr | pos | rsID | band | gene       | impact               | family | frequency |
|-----|-----|------|------|-----------|----------------------|--------|-----------|
| 1   | 2494330 | rs2234167 | 1p36.32 | TNFRSF14 | NON SYNONYMOUS CODING | NN2624 | common    |
| 2   | 41296828 | rs34287852 | 1p34.2 | KCNQ4     | NON SYNONYMOUS CODING | NN2901 | common    |
| 2   | 233537125 | rs11506997 | 2q37.1 | EFHD1     | NON SYNONYMOUS CODING | NN3752 | common    |
| 3   | 193380726 | rs200412464 | 3q29   | OPAL      | NON SYNONYMOUS CODING | NN3506 | rare      |
| 4   | 169195114 | .      | 4q23.2 | DDX60     | NON SYNONYMOUS CODING | NN3506 | novel     |
| 5   | 825280  | rs1809008 | 5p15.33 | ZDHHC11   | NON SYNONYMOUS CODING | NN1075 | common    |
| 7   | 12409263 | rs17165906 | 7p21.3 | WWDE      | NON SYNONYMOUS CODING | NN2624 | common    |
| 7   | 12409327 | rs17165910 | 7p21.3 | WWDE      | NON SYNONYMOUS CODING | NN2624 | common    |
| 7   | 73969541 | rs2361805 | 7q11.23 | GTF3R1D1  | NON SYNONYMOUS CODING | 1659F  | common    |
| 8   | 87338590 | .      | 9q21.31 | NTRK2     | NON SYNONYMOUS CODING | NN3506 | novel     |
| 9   | 101831995 | rs10519 | 9q22.33 | COL15A1   | NON SYNONYMOUS CODING | 1013F  | common    |
| 9   | 133577672 | .      | 9q34.12 | EXOSC2    | NON SYNONYMOUS CODING | NN3506 | novel     |
| 9   | 139694569 | rs7859194 | 9q34.3 | KIAA1984  | NON SYNONYMOUS CODING | 1350F  | common    |
| 12  | 52544805 | rs1801244 | 13q14.3 | ATP7B     | NON SYNONYMOUS CODING | NN2624 | common    |
| 13  | 20249176 | rs2815960 | 14q11.2 | OR4M1     | NON SYNONYMOUS CODING | 1802F  | common    |
| 13  | 24629768 | .      | 14q12  | RNF31     | NON SYNONYMOUS CODING | 1013F  | novel     |
| 14  | 25043951 | rs16737120 | 14q12  | CTSG      | NON SYNONYMOUS CODING | 691F   | rare      |
| 14  | 102391577 | rs3742424 | 14q32.31 | PPP2R5C   | NON SYNONYMOUS CODING | 1732F  | common    |
| 15  | 90818469 | rs12595409 | 15q26.1 | NCRN      | NON SYNONYMOUS CODING | NN3506 | common    |
| 17  | 7329310 | .      | 17p13.1 | C17orf74  | SPLICE SITE ACCEPTOR | 1372F  | novel     |
| 17  | 11784614 | .      | 17p12  | DNAH9     | NON SYNONYMOUS CODING | 1659F  | common    |
| 17  | 61323012 | rs3180227 | 18q21.33 | SERPINB3  | NON SYNONYMOUS CODING | 894F   | common    |
| 18  | 61570529 | rs6104  | 18q21.33 | SERPINB2  | NON SYNONYMOUS CODING | NN3506 | common    |
| 19  | 5691424  | .      | 19p13.3 | RPL36     | NON SYNONYMOUS CODING | NN3538 | novel     |
| 19  | 8191184  | rs35025963 | 19p13.2 | FBXN3     | NON SYNONYMOUS CODING | NN1075 | common    |
| 19  | 17660300 | rs11666267 | 19p13.11 | FAM129C   | NON SYNONYMOUS CODING | 1659F  | common    |

Presented only one variant. Two cytogenetic bands presented hits in 4 different families: 1p36 (size: 28 Mb) and 19q13 (size: 26.7 Mb). The other results are detailed in Sup. Table 3.

Copy-number variant (CNV) analysis of the exome of Brazilian samples. After mapping the reads to the Human genome (GRCh37/hg19), we used the XHMM software to call copy-number variations (CNVs). We found 13 occurrences of putative CNVs in the Brazilian children, three being de novo CNVs, seven inherited exclusively from father and three inherited exclusively from mother. In these regions, 22 genes were found to be expressed in brain. The Sup. Table 1 has all CNVs found in the 30 trios. The gene TRIM44 (11q11) was found in a de novo and in an inherited CNV in two different children. We used the software BEDTools (http://bedtools.readthedocs.org) to find the overlap and calculate the distances between CNV regions.

Analysis of SNVs and CNVs in the Brazilian trios. To investigate how inherited and de novo variants were distributed in the Brazilian children, we merged inherited SNVs and CNVs and compared it with de novo SNVs and de novo CNVs (Fig. 2). It is interesting to note that although our sample size is small we could observe that in average, children with only inherited variants, when compared to children with de novo variants, have two times more comorbidities. This is shown with more details in Sup. Table 4.

2. Integration of our data with public data in a gene level and analysis of such data in a systems biology approach, using protein-protein interaction networks of genes expressed in brain (Integrated PPI ADHD network). Taking into account all the genes reported with SNP or in CNV in public databases or genes with SNV or in CNV in the Brazilian data, we ended up with a set of 1128 genes expressed in brain. The GO analysis of these genes showed significant results for molecular function “lignand-gated ion channel activity” (GO:0015276), with 18 genes and adjusted P-value of 0.0093. The KEGG analysis showed significant results for “Neuroactive ligand-receptor interaction” (22 genes, adj. P-value = 9.63e−05), “Metabolic pathways” (50 genes, adj. P-value = 0.0015), “Calcium signaling pathway” (14 genes, adj. P-value = 0.0025), “Tight junction” (12 genes, adj. P-value = 0.0025), “Ubiquitin mediated proteolysis” (12 genes, adj. P-value = 0.0025), “Adherens junction” (8 genes, adj. P-value = 0.0056), “Cytokine-cytokine receptor interaction” (15 genes, adj. P-value = 0.0238). The cytogenetic bands significantly associated with ADHD were 15q11 (88 genes, adj. P-value = 1.30e−87, size = 6.7 Mb), 16p11 (70 genes, adj. P-value = 6.82e−12, size = 4.2 Mb), 4p15 (15 genes, adj. P-value = 3.86e−08, size = 2.8 Mb).
P-value $= 6.02e^{-08}$, size $= 24.5$ Mb), 15q12 (20 genes, adj. P-value $= 2.69e^{-07}$, size $= 2.4$ Mb), 20p13 (15 genes, adj. P-value $= 3.22e^{-07}$, size $= 5.1$ Mb) and 8q21 (16 genes, adj. P-value $= 4.08e^{-07}$, size $= 49.8$ Mb).

To study how the genes were relatively located in the network space, we plotted a network with the genes related to ADHD and their direct interactions using protein-protein interaction databases. This network is composed only of genes expressed in brain. Out of 1128 genes, only 557 were mapped in the interactome. Besides the 557 seed genes, 2240 more genes were added to the network. The network is shown in Fig. 3.

3. Finding a highly reproducible ADHD gene set and the ADHD network.

To obtain a more robust view of the genes with SNVs and/or in CNVs, we selected genes with variants found in both the Brazilian exome data and public ADHD CNV data. Table 2 includes these 30 of the most significant and informative genes and which type of analyses they were derived from. All the other genes resulted from a single analysis.

Using only these 30 genes as seeds, we built a subnetwork adding the direct interactions to analyze which biological functions were associated with these genes. Out of 30 genes, only 15 were in the interactome. The final network ended up having 101 genes (Fig. 4). Using WebGestalt, we found gene ontology categories and protein modules over-represented in this network. Regarding cellular components, there are 13 genes related to neuron projection (adj. P-value $= 6.00e^{-04}$), 5 genes related to cell-substrate adherens junction (adj. P-value $= 3.00e^{-03}$), 34 genes related to membrane-enclosed lumen (adj. P-value $= 1.60e^{-03}$) and 14 genes related to synapse (adj. P-value $= 7.80e^{-05}$). Moreover, the central nervous system genes are significantly connected in the network, according to WebGestalt analysis of protein modules (adj. P-value $= 3.02e^{-05}$). Using STRING, we performed the enrichment analysis for KEGG and obtained the more specific synapse results: glutamatergic synapse (7 genes, adj. P-value $= 3.28e^{-04}$) and serotoninergic synapse (6 genes, adj. P-value $= 4.81e^{-03}$). This is shown in Table 3.

### Table 2. Genes with hits in at least two categories of variants found from Brazilian samples exome and from public data.

| Gene                      | public CNVs | public SNPs | exome CNVs | exome SNVs | band     | reference |
|---------------------------|-------------|-------------|------------|------------|----------|-----------|
| ACOXL (acyl-CoA oxidase-like) | no          | no          | no         | sv2c       | 2q13     | 59        |
| ACSM1 (acyl-CoA synthetase medium-chain family member 1) | no          | no          | no         | 2vsc       | 16p12.3  | 60        |
| ANKRD42 (ankyrin repeat domain 42) | no          | no          | no         | sv2c       | 11q14.1  |           |
| CNGB3 (cyclic nucleotide gated channel beta 3) | yes         | no          | no         | yes        | 8q21.3   |           |
| CFIT2 (cytoplasmic FMR1 interacting protein 2) | no          | no          | no         | sv2c       | 3q33.3   | 61        |
| CYP19A1 (cytochrome P450, family 19, subfamily A, polypeptide 1) | yes         | no          | no         | yes        | 15q21.2  |           |
| DDX60 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 60) | yes         | no          | no         | yes        | 4q32.3   |           |
| DNAH3 (dynein, axonemal, heavy chain 3) | no          | no          | no         | sv2c       | 16p12.3  |           |
| DNHD1 (dynein heavy chain domain 1) | no          | no          | yes        | no         | 11p15.4  |           |
| EMP2 (epithelial membrane protein 2) | yes         | yes         | no         | no         | 16p13.13 | 62        |
| GMAP6 (GT-Pase, IMAP family member 6) | yes         | no          | no         | no         | 7q36.1   |           |
| GOLGA8DP (golgin A8 family, member D, pseudogene) | yes         | no          | yes        | no         | 15q12.2  |           |
| HEATR1 (HEAT repeat containing 1) | yes         | yes         | no         | no         | 1q43     |           |
| IFITD1 (lamin tail domain containing 1) | no          | no          | no         | 3vsc       | 12p12.1  |           |
| ILDR1 (immunoglobulin-like domain containing receptor 1) | yes         | no          | no         | no         | 3q13.33  |           |
| TGF3 (integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)) | yes         | yes         | no         | yes        | 17q21.33 |           |
| MS1 (macrophage scavenger receptor 1) | no          | no          | no         | sv2c       | 8p22     |           |
| MUC6 (mucin 6, oligomeric mucus/gel-forming) | no          | no          | no         | 2vsc       | 11p15.5  |           |
| NPSR1 (neuropeptide S receptor 1) | no          | no          | no         | sv2c       | 7p14.3   |           |
| OBSL1 (obscurin-like 1) | no          | no          | no         | sv2c       | 2q35     |           |
| OR8K3 (olfactory receptor, family 8, subfamily K, member 3) | yes         | no          | no         | yes        | 11q12.1  |           |
| PP6R3 (protein phosphatase 6, regulatory subunit 3) | yes         | no          | yes        | no         | 11q13.2  |           |
| RGS12 (regulator of G-protein signaling 12) | yes         | no          | no         | 2vsc       | 4p16.3   | 63        |
| SOD1 (sidekick cell adhesion molecule 1) | yes         | no          | no         | yes        | 7p22.2   |           |
| SMYD3 (SET and MYND domain containing 3) | yes         | no          | yes        | no         | 1q44     |           |
| TRIM22 (tripartite motif containing 22) | yes         | no          | no         | yes        | 11p15.4  |           |
| TRIM48 (tripartite motif containing 48) | no          | no          | sv2c       | no         | 11q11    |           |
| TRIML2 (tripartite motif family-like 2) | yes         | no          | yes        | yes        | 4q35.2   |           |
| UQRC2 (ubiquinol-cytochrome c reductase core protein II) | yes         | yes         | no         | yes        | 16p12.2  | 56        |
| VDVE (von Willebrand factor D and EGF domains) | yes         | no          | no         | 2vsc       | 7p21.3   |           |

P-value $= 6.02e^{-08}$, size $= 24.5$ Mb), 15q12 (20 genes, adj. P-value $= 2.69e^{-07}$, size $= 2.4$ Mb), 20p13 (15 genes, adj. P-value $= 3.22e^{-07}$, size $= 5.1$ Mb) and 8q21 (16 genes, adj. P-value $= 4.08e^{-07}$, size $= 49.8$ Mb).

To study how the genes were relatively located in the network space, we plotted a network with the genes related to ADHD and their direct interactions using protein-protein interaction databases. This network is composed only of genes expressed in brain. Out of 1128 genes, only 557 were mapped in the interactome. Besides the 557 seed genes, 2240 more genes were added to the network. The network is shown in Fig. 3.

3. Finding a highly reproducible ADHD gene set and the ADHD network.

To obtain a more robust view of the genes with SNVs and/or in CNVs, we selected genes with variants found in both the Brazilian exome data and public ADHD CNV data. Table 2 includes these 30 of the most significant and informative genes and which type of analyses they were derived from. All the other genes resulted from a single analysis.

Using only these 30 genes as seeds, we built a subnetwork adding the direct interactions to analyze which biological functions were associated with these genes. Out of 30 genes, only 15 were in the interactome. The final network ended up having 101 genes (Fig. 4). Using WebGestalt, we found gene ontology categories and protein modules over-represented in this network. Regarding cellular components, there are 13 genes related to neuron projection (adj. P-value $= 6.00e^{-04}$), 5 genes related to cell-substrate adherens junction (adj. P-value $= 3.00e^{-03}$), 34 genes related to membrane-enclosed lumen (adj. P-value $= 1.60e^{-03}$) and 14 genes related to synapse (adj. P-value $= 7.80e^{-05}$). Moreover, the central nervous system genes are significantly connected in the network, according to WebGestalt analysis of protein modules (adj. P-value $= 3.02e^{-05}$). Using STRING, we performed the enrichment analysis for KEGG and obtained the more specific synapse results: glutamatergic synapse (7 genes, adj. P-value $= 3.28e^{-04}$) and serotoninergic synapse (6 genes, adj. P-value $= 4.81e^{-03}$). This is shown in Table 3.
Discussion

Despite a 70% to 80% heritability estimate\(^4^4\), finding gene variants associated with the ADHD phenotype has been extremely challenging. Some genes seem to be more related to the susceptibility of any psychiatric neurodevelopmental disorder but the complexity of combinations of non-specific variations with more individual ones and the environment will bring the colors of a specific disorder\(^4^5\). With this idea, we explored different types of genetic variations using ADHD trios exome data. An intuitive method to find variations related to a disorder is to look for de novo variants in trios where the parents do not have ADHD and the children show the phenotype. Such variants have been shown as potential causes of several psychiatric diseases\(^1^2,1^3,3^4,4^6\). Here we found 26 de novo SNVs with moderate and high impact in 25 brain-expressed genes in 14 different families and three de novo CNVs in different families. Although combining exome and GWAS studies has some technical limitations, studies in the literature have demonstrated the effectiveness of using combined analysis methods\(^4^7\). To search CNVs related to ADHD, it is important to mention that we compared in this present study the copy-number variants found in the Brazilian trios using whole-exome data combined with CNV regions found in 503 Brazilian controls using SNP-array data. The intention was to remove any CNVs that are present in controls from the analysis.

However, it also has been shown that rare inherited variants\(^3^5\) play an important role in other neurodevelopmental disorders\(^3^7\). Assuming that ADHD should not be different from other neurodevelopmental disorders, we searched for inherited SNVs and CNVs. Regarding SNVs, we found 127 rare, moderate impact (missense) homozygous mutations for loci where both parents are heterozygous in 120 reported genes expressed in brain, suggesting a possible recessive heritability in ADHD as has been suggested for autism\(^3^8\). Besides that, 134 very rare, high impact (nonsense and splice site) heterozygous mutations were found in 134 genes expressed in brain. Regarding structural variants, we found 10 inherited CNVs that were not present in Brazilian controls. Although we found putative CNVs inherited more from father than from mother, the limitation of our small sample size makes difficult to assert that this is a recurring pattern.

Regarding the variants found only in the Brazilian cases, it was observed that usually it was necessary the combination of exclusively inherited variants or inherited variants with a few de novo variants. Interestingly, the children who presented de novo SNVs did not present de novo CNVs, and vice-versa (only one child is an exception, with one de novo CNV and one de novo SNV). This corroborates what has been shown in other psychiatric diseases\(^3^7\), such as autism\(^2^9–3^1\), schizophrenia\(^1^2,1^3,3^3\), and bipolar disorder\(^4^9\). In these diseases, the studies reported the role of de novo and inherited variants, as well as common and rare variants. Particularly in ADHD, Yang and colleagues\(^1^4\) performed a case-control analysis using SNP-array approach (Affymetrix 6.0) of a large cohort of Chinese subjects aiming to discover both common and rare variants associated with ADHD. Although they

| GO id | Term                                      | no. of genes | p-value     | p-value Bonferroni |
|-------|-------------------------------------------|--------------|-------------|--------------------|
| 4810  | Regulation of actin cytoskeleton          | 11           | 6.59E–09    | 1.89E–06           |
| 4730  | Long-term depression                      | 7            | 1.42E–08    | 4.07E–06           |
| 4915  | Estrogen signaling pathway                | 8            | 1.92E–08    | 5.51E–06           |
| 5016  | Huntington's disease                      | 9            | 2.83E–07    | 8.11E–05           |
| 4724  | Glutamatergic synapse                     | 7            | 1.14E–06    | 3.28E–04           |
| 4540  | Gap junction                              | 6            | 3.46E–06    | 9.93E–04           |
| 4723  | Retrograde endocannabinoid signaling      | 6            | 8.07E–06    | 2.32E–03           |
| 4720  | Long-term potentiation                    | 5            | 1.49E–05    | 4.27E–03           |
| 4726  | Serotonergic synapse                      | 6            | 1.67E–05    | 4.81E–03           |

Table 3. KEGG pathways significant after Bonferroni Correction using STRING.
did not find genome-wide significant results, they could point some biological pathways using network analysis integrating the genes with higher association. More recently, Martin and colleagues 50 used UK-based children samples to test whether children with ADHD with large rare CNVs had different polygenic risk scores (based on SNPs) for ADHD when compared to children with ADHD without these CNVs. In other words, their results implied that comparing children with a large, rare CNV with children without, the former require less of a polygenic burden to develop the disease, when we look at the SNPs in these children. However, our exome data made possible to look at smaller CNVs, and we also found CNVs with less than 100kb that could be playing a role in the disease.

When we combined the variants found in CNVs or SNVs taking into account the findings in our exome data and in the public databases of children with ADHD, the results highlighted interesting biological functions and diseases significantly associated to ADHD. For example, the GO molecular function and KEGG exhibited terms related to activity of cells in brain, as “ligand-gated ion channel activity”, of which some genes have been studied as therapeutic targets for ADHD, Alzheimer’s disease, schizophrenia, depression and tobacco addiction. Other pathways found have already been related to ADHD. The most significant were “Neuroactive ligand-receptor interaction” (the ADHDgene database reports 89 genes), “Calcium signaling pathway” (66 genes on ADHDgene) and “Cytokine-cytokine receptor interaction”. So far genes involved in synaptogenesis and calcium channels have been consistently described in the literature51,52. Some of the chromosome regions overrepresented have been described, but only region 11q25 were in the top regions on ADHDgene database suggesting that our network analysis could be contributing with new candidate genes related to ADHD. More interesting in the network analyses, is the topological properties of ADHD genes. Cai and colleagues41 revealed that genes related to diseases tend to be brokers, and this property keeps robust even with the literature bias present in the interactome. Further studies revealed that gene properties in a network are different in autosomal or complex disorders and also that different kinds of genetic variation are related to specific topological areas of the network22. It is interesting to observe that the nodes related to ADHD SNVs or CNVs are not central or highly connected as expected for a complex disease. Although they are linked to such nodes, they are located in the borders of the network. This fact supports that the impact of such variants, separately, is not severe. In contrast, it is necessary a combination of factors to trigger the development of the disease. Together with their interactions, the genes with SNVs or in CNVs make complexes related to important biological functions.

Promising results came up when only genes recurrent in at least two different analyses and their direct interactions on PPI databases have been used, since the connections in the neighborhood of these genes could show pathways with more confidence. Some of our seed genes have already been associated with other neurodevelopmental disorders such as schizophrenia and autism. It is important to note that Cytoplasmic FMR1-interacting
protein 2 (CYFIP2) was identified as an interaction of FMRP, clearly related to Autism. Importantly, CYFIP2 is a component of WAVE regulatory complex, a key regulator of actin cytoskeleton. Han and colleagues showed that CYFIP2 could be implicated in the dendritic spine regulation in cortical neurons and suggest that misregulation of CYFIP2 function and its mGluR-induced expression contribute to the neurobehavioral phenotypes of FXS. OBSL1 is important for the regulation of a signaling mechanism that orchestrates the morphogenesis of the Golgi apparatus and pattern of dendrites. Interestingly NPSR1 and UQCRC2 seem to be more specific for ADHD. NPSR1 modulated by calcium signaling pathways has been involved in modulation of prefrontal functioning in attention systems and motor impairments and UQCRC2 has been related to methylphenidate treatment.

Yang and colleagues also combined CNVs and SNPs to study ADHD. Their study, which also used networks to investigate the role of different types of variants, reported synapse (15 genes) and neuron projection (16 genes) as GO (Cellular component) terms associated with regulatory regions. Interestingly, although we confirmed these categories, the overlap of genes is very low – only gene CNTN2 is in both analyses. Analyzing the enriched pathways, we found genes significantly related to “glutamatergic synapse”, as well as “Serotonergic synapse”. Elia and colleagues reported networks with genes related to glutamatergic neurotransmission that were affected by CNVs in multiple cohorts. These findings were replicated by Akutagava-Martins and colleagues in Brazilian samples, which supports the role of glutamate in ADHD. Regarding the glutamatergic pathway genes, our approach also discovered genes not reported by these previous studies in ADHD: PLCB1, PLCB3, GNAI2, GNAI3 and CACNA1A.

In addition to the small sample size, as a limitation of our study, admixed population such as the one from Brazil brings some particularities to the analyses. In this scenario we used as much data integration as possible. Albeit our results confirm the disruption of pathways already associated to ADHD, it also confirms the complexity and heterogeneity of the disease, showing that in different patients, families or populations, disruption in different genes can be conducing to the development of the disease.

In conclusion, using an innovative data integration using public data and the first exome analysis performed in sporadic ADHD trios, we found other genes related to biological pathways previously reported in literature. Although our results confirmed findings published by other research groups, further analyses are essential to confirm the hypotheses and biological functions in larger and different populations. The analyses made exclusively with exome data showed a balance in inherited and de novo events, separating in three groups probands without any de novo variant, probands with de novo CNVs and those who had de novo SNVs, and also from children only with inherited variants. This could confirm the role of such variations in ADHD, but further work is necessary to find answers in non-coding regions/regulatory elements in the human genome, as well as the genes in the PPI network affect the processes involved in the disease.
References

1. Elia, J. et al. Genome-wide copy number variation study associates metabotropic glutamate receptor gene networks with attention deficit hyperactivity disorder. Nat Genet 44, 78–84, doi: 10.1038/ng.1013 (2012).

2. Lesch, K. P. et al. Genome-wide copy number variation analysis in attention-deficit/hyperactivity disorder: association with neuropeptide Y gene dosage in an extended pedigree. Mol Psychiatry 16, 491–503, doi: 10.1038/mp.2010.29 (2011).

3. Lesch, K. P. et al. Molecular genetics of adult ADHD: converging evidence from genome-wide association and extended pedigree linkage studies. J Neural Transm 115, 1573–1588 (2008).

4. Lione, A. C. et al. Rare copy number variation discovery and cross-disorder comparisons identify risk genes for ADHD. Science translational medicine 3, 95ra75, doi: 10.1126/scitranslmed.3002464 (2011).

5. Williams, N. M. et al. Rare chromosomal deletions and duplications in attention-deficit hyperactivity disorder: a genome-wide analysis. Lancet 376, 1401–1408, doi: 10.1016/S0140-6736(10)61199-9 (2010).

6. Banaschewski, T., Becker, K., Scherag, S., Franke, B. & Coghill, D. Molecular genetics of attention-deficit/hyperactivity disorder: an overview. Eur Child Adolesc Psychiatry 19, 237–257 (2010).

7. Neale, B. M. et al. Analysis of genome-wide association studies of attention-deficit/hyperactivity disorder. J Am Acad Child Adolesc Psychiatry 49, 884–897 (2010).

8. Neale, B. M. et al. Genome-wide association scan of attention deficit hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet 147B, 1337–1344 (2008).

9. Lantieri, E., Glessner, J. T., Hakonarson, H., Elia, J. & Devoto, M. Analysis of GWAS top hits in ADHD suggests association to two polymorphisms located in genes expressed in the cerebellum. Am J Med Genet B Neuropsychiatr Genet 153B, 1127–1133 (2010).

10. Frazier, K. A., Murray, S. S., Schork, N. J. & Topol, E. J. Human genetic variation and its contribution to complex traits. Nat. Rev. Genet. 10, 241–251 (2009).

11. Mitchell, K. J. & Porteous, D. J. Rethinking the genetic architecture of schizophrenia. Psychol Med 41, 19–32 (2011).

12. Sebat, J. et al. Strong association of de novo copy number mutations with autism. Science 316, 445–449, doi: 10.1126/science.1138659 (2007).

13. Girirajan, S. et al. Relative burden of large CNVs on a range of neurodevelopmental phenotypes. PLoS Genet 7, e1002334, doi: 10.1371/journal.pgen.1002334 (2011).

14. Yang, L. et al. Polygenic transmission and complex neuro developmental network for attention deficit hyperactivity disorder: genome-wide association study of both common and rare variants. Am J Med Genet B Neuropsychiatr Genet 162B, 419–430, doi: 10.1002/ajmg.b.32169 (2013).

15. Jarick, I. et al. Genome-wide analysis of rare copy number variations reveals PARK2 as a candidate gene for attention-deficit/ hyperactivity disorder. Mol Psychiatry 19, 115–121 (2014).

16. Akula, N. et al. A network-based approach to prioritize results from genome-wide association studies. PLoS One 6, e24220 (2011).

17. Na, P. & Zhao, Z. Searching joint association signals in CATIE schizophrenia genome-wide association studies through a refined integrative network approach. BMC Genomics 13 Suppl 6, 515 (2012).

18. Lee, T. L., Raygada, M. J. & Rennert, O. M. Integrative gene network analysis provides novel regulatory relationships, genetic contributions and susceptible targets in autism spectrum disorders. Gene 496, 88–96 (2012).

19. Poelmans, G., Pauls, D. L., Buitelaar, J. K. & Franke, B. Integrated genome-wide association study findings: identification of a neurodevelopmental network for attention deficit hyperactivity disorder. Am J Psychiatry 168, 365–377 (2011).

20. Rossin, E. J. et al. Proteins encoded in genomic regions associated with immune-mediated disease physically interact and suggest underlying biology. PLoS Genet 7, e1002173 (2011).

21. Sun, Y. V. Integration of biological networks and pathways with genetic association studies. Hum Genet 131, 1667–1686 (2012).

22. Barabasi, A. L., Gulbahce, N. & Loscalzo, J. Network medicine: a network-based approach to human disease. Nat. Rev. Genet. 12, 56–68 (2011).

23. Zhao, J., Yang, T. H., Huang, Y. & Holme, P. Ranking candidate disease genes from gene expression and protein interaction: a Katz centrality based approach. PLoS One 6, e24306, doi: 10.1371/journal.pone.0024306 (2011).

24. Lee, S. H. et al. Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. Nat Genet 45, 984–994, doi: 10.1038/ng.2711 (2013).

25. Kavanagh, D. H., Tansey, K. E., O’Donovan, M. C. & Owen, M. J. Schizophrenia genetics: emerging themes for a complex disorder. Mol Psychiatry 17, 909–916, doi: 10.1038/mp.2014.148 (2014).

26. Hamshere, M. L. et al. High Loading of Polygenic Risk for ADHD in Children With Comorbid Aggression. Am J Psychiatry 170, 909–916, doi: 10.1176/appi.ajp.2013.12081129 (2013).

27. Escudero, I. & Johnstone, M. Genetics of schizophrenia. Curr Psychiatry Rep 16, 502, doi: 10.1007/s11920-014-0502-8 (2014).

28. He, X. et al. Integrated model of de novo and inherited genetic variants yields greater power to identify risk genes. PLoS Genet 9, e1003671, doi: 10.1371/journal.pgen.1003671 (2013).

29. Devlin, B. & Scherer, S. W. Genetic architecture in autism spectrum disorder. Curr Opin Genet Dev 22, 229–237, doi: 10.1016/j.ogd.2012.03.002 (2012).

30. State, M. W. & Levitt, P. The conundrums of understanding genetic risks for autism spectrum disorders. Nat Neurosci 14, 1499–1506, doi: 10.1038/nn.2924 (2011).

31. Wang, K. et al. Common genetic variants on 5p14.1 associate with autism spectrum disorders. Nature 459, 528–533, doi: 10.1038/nature07999 (2009).

32. Rodriguez-Murillo, L., Gogos, J. A. & Karayiorgou, M. The genetic architecture of schizophrenia: new mutations and emerging paradigms. Annu Rev Med 63, 63–80, doi: 10.1146/annurev-med-072010-091100 (2012).

33. Schizophrenia Working Group of the Psychiatric Genomics Consortium. Biological insights from 108 schizophrenia-associated genetic loci. Nature 511, 421–427, doi: 10.1038/nature13595 (2014).

34. Krumm, N. et al. Transmission disequilibrium of small CNVs in simplex autism. Am J Hum Genet 93, 595–606, doi: 10.1016/j.ajhg.2013.07.024 (2013).

35. Poulltney, C. S. et al. Identification of small exonic CNV from whole-exome sequence data and application to autism spectrum disorder. Am J Hum Genet 93, 607–619, doi: 10.1016/j.ajhg.2013.09.001 (2013).

36. Hawi, Z. et al. The molecular genetic architecture of attention deficit hyperactivity disorder. Mol Psychiatry 20, 289–297, doi: 10.1038/mp.2014.183 (2015).

37. Girirajan, S. & Eichler, E. E. Phenotypic variability and genetic susceptibility to genomic disorders. Hum Mol Genet 19, R176–187, doi: 10.1093/hmg/ddq366 (2010).

38. Salum, G. A. et al. High risk cohort study for psychiatric disorders in childhood: rationale, design, methods and preliminary results. Int J Methods Psychiatr Res 24, 58–73, doi: 10.1002/mpr.1459 (2015).

39. Sato, J. R. et al. Default mode network: maturation and psychopathology in children and adolescents. J Child Psychol Psychiatry, doi: 10.1111/jcpp.12444 (2015).

40. Wang, K. et al. PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. Genome Res 17, 1665–1674, doi: 10.1101/gr.561907.107 (2007).

41. Cai, J. J., Borenstein, E. & Petrov, D. A. Broker genes in human disease. Genome Biol Evol 2, 815–825 (2010).

42. Lupski, J. R., Belmont, J. W., Boerwinkle, E. & Gibbs, R. A. Clan genomics and the complex architecture of human disease. Cell 147, 32–43, doi: 10.1016/j.cell.2011.09.008 (2011).
Author Contributions
L.A.L. and H.B. designed the experiments. S.I.B., A.G., R.A.B., G.A.S., P.M.P., T.S.M., A.S.G.M., A.C., T.P.A., F.V.K., B.F.B., A.P.J., E.B., J.R.S., G.V.P., J.I.M., G.G.M., M.C.R. and E.C.M. worked in sample collection and characterization. L.A.L., A.C.F.S., R.D.P., A.C.T., V.N.S., T.C., G.R.G., S.N.S., X.C., L.T., J.T.G. and P.M.A.S. performed Bioinformatics analyses. L.A.L., R.P., L.A.R., G.A.S., P.M.A.S., R.F.H., H.H. and H.B. wrote the main manuscript text. All authors reviewed the manuscript.

Additional Information
Competing financial interests: Ary Gadelha, Pedro Mario Pan, and Tais Silveira Moriyama have received continuous medical education support from Astra Zeneca, Eli-Lilly and Janssen-Cilag. Guilherme Vanoni Polanczyk has aided as a speaker and/or consultant to Eli-Lilly, Novartis, and Shire Pharmaceuticals, developed educational material to Janssen-Cilag, and receives unrestricted research support from Novartis. Maria Conceição do Rosário has worked for the last five years as a speaker for the companies Novartis and Shire. Rodrigo A. Bressan was on the speakers’ bureau and/or acted as consultant for Eli-Lilly, Janssen-Cilag, Novartis, Lundbeck and Roche in the last three years. Luis Augusto Roehde was on the speakers’ bureau and/or acted as consultant for Eli-Lilly, Janssen-Cilag, Novartis and Shire in the last three years. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by LAR received unrestricted educational and research support from the following pharmaceutical companies in the last three years: Abbott; Eli-Lilly; Janssen-Cilag; Novartis; Shire.
All other authors declare no potential conflicts of interest.

How to cite this article: de Araújo Lima, L. et al. An integrative approach to investigate the respective roles of single-nucleotide variants and copy-number variants in Attention-Deficit/Hyperactivity Disorder. Sci. Rep. 6, 22851; doi: 10.1038/srep22851 (2016).
Corrigendum: An integrative approach to investigate the respective roles of single-nucleotide variants and copy-number variants in Attention-Deficit/Hyperactivity Disorder

Leandro de Araújo Lima, Ana Cecília Feio-dos-Santos, Sintia Iole Belangero, Ary Gadelha, Rodrigo Affonseca Bressan, Giovanni Abrahão Salum, Pedro Mario Pan, Tais Silveira Moriyama, Ana Soledade Graeff-Martins, Ana Carina Tamanaha, Pedro Alvarenga, Fernanda Valle Krieger, Bacy Fleitlich-Bilyk, Andrea Parolin Jackowski, Elisa Brietzke, João Ricardo Sato, Guilherme Vanoni Polanczyk, Jair de Jesus Mari, Gisele Gus Manfro, Maria Conceição do Rosário, Eurípedes Constantino Miguel, Renato David Puga, Ana Carolina Tahira, Viviane Neri Souza, Thais Chile, Gisele Rodrigues Gouveia, Sérgio Nery Simões, Xiaochang, Renata Pellegrino, Lifeng Tian, Joseph T. Glessner, Ronaldo Fumio Hashimoto, Luis Augusto Rohde, Patrick M. A. Sleiman, Hakon Hakonarson & Helena Brentani

Scientific Reports 6:22851; doi: 10.1038/srep22851; published online 07 March 2016; updated on 24 May 2016

The Acknowledgements section in this Article is incomplete.

“This work was supported by the National Institute of Developmental Psychiatry for Children and Adolescents, a science and technology institute funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; National Council for Scientific and Technological Development; grant number 573974/2008-0) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Research Support Foundation of the State of São Paulo; grant number 2008/57896-8). Leandro de Araújo Lima was supported by CAPES foundation (process 10682/13-9).”

should read:

“This work was supported by the National Institute of Developmental Psychiatry for Children and Adolescents, a science and technology institute funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; National Council for Scientific and Technological Development; grant number 573974/2008-0) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Research Support Foundation of the State of São Paulo; grant numbers 2008/57896-8 and 2015/01587-0). Leandro de Araújo Lima was supported by CAPES foundation (process 10682/13-9).”

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/