Molecular Neurobiology
https://doi.org/10.1007/s12035-023-03894-8

Clinical Characterization and Underlying Genetic Findings in Brazilian Patients with Syndromic Microcephaly Associated with Neurodevelopmental Disorders

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Received: 13 July 2023 / Accepted: 20 December 2023

Abstract

Microcephaly is characterized by an occipitofrontal circumference at least two standard deviations below the mean for age and sex. Neurodevelopmental disorders (NDD) are commonly associated with microcephaly, due to perturbations in brain development and functioning. Given the extensive genetic heterogeneity of microcephaly, managing patients is hindered by the broad spectrum of diagnostic possibilities that exist before conducting molecular testing. We investigated the genetic basis of syndromic microcephaly accompanied by NDD in a Brazilian cohort of 45 individuals and characterized associated clinical features, as well as evaluated the effectiveness of whole-exome sequencing (WES) as a diagnostic tool for this condition. Patients previously negative for pathogenic copy number variants underwent WES, which was performed using a trio approach for isolated index cases ($n = 31$), only the index in isolated cases with parental consanguinity ($n = 8$) or affected siblings in familial cases ($n = 3$). Pathogenic/likely pathogenic variants were identified in 19 families (18 genes) with a diagnostic yield of approximately 45%. Nearly 86% of the individuals had global developmental delay/intellectual disability and 51% presented with behavioral disturbances. Additional frequent clinical features included facial dysmorphisms (80%), brain malformations (67%), musculoskeletal (71%) or cardiovascular (47%) defects, and short stature (54%). Our findings unraveled the underlying genetic basis of microcephaly in half of the patients, demonstrating a high diagnostic yield of WES for microcephaly and reinforcing its genetic heterogeneity. We expanded the phenotypic spectrum associated with the condition and identified a potentially novel gene (*CCDC17*) for congenital microcephaly.

Keywords Microcephaly · Neurodevelopmental disorders · Whole-exome sequencing · Clinical characterization · *CCDC17*

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Published online: 05 January 2024

Abbreviations

AD	Autosomal dominant
AR	Autosomal recessive
CMA	Chromosomal microarray analysis
CNS	Central nervous system
CNV	Copy number variant
GDD	Global developmental delay
ID	Intellectual disability
InDel	Insertion/deletion
LoF	Loss of function
MCPH	Microcephaly primary hereditary
MRI	Magnetic resonance imaging
NDD	Neurodevelopmental disorders
OFC	Occipitofrontal circumference
SD	Standard deviation
SNV	Single nucleotide variant
VUS	Variant of uncertain significance
WES	Whole-exome sequencing
XLD	X-linked dominant
XLR	X-linked recessive

Introduction

The functional complexity of the cerebral cortex depends on its intricate morphogenesis, which starts very early in embryogenesis and later produces appropriate size and cytoarchitecture [1]. Disruptions at any step can lead to brain abnormalities [2]. Microcephaly, considered a defect in cell proliferation and maintenance, is clinically characterized by an occipitofrontal circumference (OFC) at least two standard deviations (SD) below the mean for age and sex [3, 4]. This condition involves a highly heterogeneous group of disorders with a worldwide prevalence around 2–3% [5]. Since the reduction in head size reflects a diminished brain volume that may result in cognitive impairment, microcephaly represents a significant cause of global developmental delay (GDD), intellectual disability (ID), and neuropsychiatric conditions such as epilepsy and autism spectrum disorder [3, 5, 6]. Microcephaly can be classified according to patient's age at onset, clinical presentation, and severity. In the first category, it can be congenital (primary), or it can evolve later in infancy (postnatally). Additionally, if the phenotype is restricted to nervous system abnormalities, microcephaly is considered non-syndromic, as opposed to the concomitant presence of a variety of extracranial anomalies [5]. A distinction has been proposed between mild microcephaly, defined by an OFC between −2 and −3 SD of the mean for age and sex, and severe microcephaly when the OFC is below −3 SD [7].

However, such heterogeneity is not restricted to the clinical aspects, as the etiology of microcephaly encompasses a variety of causative agents. Environmental factors, such as pre- and perinatal infections, hypoxia at birth, and maternal exposure to teratogens, as well as genetic alterations are predisposing factors for microcephaly [3]. Multifactorial occurrences are believed to interact to the pathogenesis, as demonstrated in studies with congenital Zika syndrome discordant twins [8]. From a genetic perspective, 58 copy number variant (CNV) syndromes and 957 genes have been associated with this phenotype [9]. Up to May 2023, 1187 entries that included microcephaly with a known molecular basis were reported in the OMIM database, which comprises CNV syndromes and monogenic disorders. Because of this remarkable diversity, microcephaly represents a challenge for molecular diagnosis: in most cases, it is not possible to provide a clinical hypothesis for the phenotype, a scenario that hampers the management of patients and their families [3, 10].

Neurodevelopmental disorders affect ~ 3% of the worldwide population, mostly children, and are commonly associated with dysfunction in normal brain development that triggers an array of cognitive and behavioral conditions [11, 12]. Next-generation sequencing technologies have made significant contributions to the disentanglement of the molecular basis of rare NDDs [13], including those associated with microcephaly [14–16]. Given the relevance of this phenotype and the relative paucity of data on the genetic basis of microcephaly in the Brazilian population, our main goal was to assess the prevalence of rare pathogenic variants and the spectrum of affected genes. For that, we employed whole-exome sequencing (WES) in a cohort of 45 individuals (42 probands) presenting with microcephaly, NDD, and additional signs, whose previous evaluation by chromosomal microarray analysis (CMA) was negative. In addition, we investigated the range of associated clinical features and assessed the effectiveness of WES for the molecular diagnosis of microcephaly.

Materials and Methods

Participants

A group of 53 individuals (48 families) with syndromic microcephaly associated with NDD were evaluated in a previous study from our group. They had undergone karyotyping, and all of them were primarily referred to CMA. In this previous study, clinically relevant CNVs classified as pathogenic/likely pathogenic were detected in seven cases (including two sibships) [9]. We proceed in the present study with the WES analysis of the families in which the index cases were CMA negative ($n = 34$) or carried VUS ($n = 7$) or risk factors CNV ($n = 1$). Therefore, we evaluated here a group of 45 microcephalic individuals from 42 families. These patients were referred by different physicians, and the

clinical characteristics of most of them were collected at the Instituto da Criança of the Hospital das Clínicas (University of São Paulo Medical School, São Paulo, Brazil). The inclusion criteria consisted of an OFC ≤ −2 SD below the mean at birth and/or the latest clinical assessment, based on the World Health Organization and INTERGROWTH 21st charts for head size, with no obvious environmental etiology, such as history of pre- and perinatal infections. None of the individuals had a previous clinical diagnostic hypothesis.

Peripheral blood samples of the patients and, when available, their parents and siblings, were collected for genomic DNA extraction.

Whole-Exome Sequencing and Bioinformatics Analysis

In consanguineous families with a single affected individual, WES was performed only for the proband ($n = 8$). For isolated cases ($n = 31$), a trio strategy (patient/parents) was applied when samples from both parents were available ($n = 19$), and a patient/mother approach ($n = 3$) was used when paternal DNA was unavailable. For the other nine isolated cases, only the proband was evaluated by WES. In three familial cases (including two consanguineous families), sequencing was performed for the affected siblings. Genomic libraries were constructed using either SureSelect Human All Exon V6 (Agilent Technologies) or xGen Exome Research Panel v1/v2 (Integrated DNA Technologies), and sequenced on the HiSeq or NovaSeq 6000 platforms (Illumina). Sequence alignment was conducted as previously described [17]. Analysis of the VCF and BAM files were carried out through the VarSeq (Golden Helix, Inc) and Varstation (https://varsomics.com/varstation/) platforms, with manual curation.

To prioritize rare variants with potential clinical impact, we selected missense and loss-of-function (LoF) coding nonsynonymous single nucleotide variants (SNVs) and insertions/deletions (indels), and canonical splicing site mutations, with minimum quality/confidence (Phred score ≥ 20; read depth ≥ 20; variant allele frequency ≥ 0.3) and a global minor allele frequency ≤ 1% (1000 Genomes (https://www.internationalgenome.org/), gnomAD exomes and genomes (https://gnomad.broadinstitute.org/), and ABraOM (https://abraom.ib.usp.br/) databases). Variants in hypervariable genes [18] were filtered out. We further applied a phenotype-driven analysis by (a) prioritizing entries classified as pathogenic/likely pathogenic in clinical databases (OMIM (https://www.omim.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), ClinGen (https://clinicalgenome.org/), and HGMD (https://www.hgmd.cf.ac.uk/ac/index.php) databases); (b) evaluating variants mapped to a specific list of genes known to be associated with microcephaly, global developmental delay, and/or ID [9]; and (c) performing an HPO-based analysis [19]. Additional analyses were conducted using the *in silico* pathogenic prediction tools FATHMM, FATHMM-MKL, MutationAssessor, MutationTaster, Polyphen2, and SIFT from the dbNSFP Functional Prediction database (http://database.liulab.science/dbNSFP). Prediction of disruption at splicing sites was carried out through the Human Splicing Finder (https://www.genomnis.com/access-hsf) and scSNV ADA Boost splicing (https://varsome.com/) tools. Classification of clinically relevant variants followed the ACMG guidelines for primary and secondary findings [20, 21].

Additional CNV Analysis Through WES Data

To assess the presence of small CNVs below the resolution of CMA (< 10 kb), we used the XHMM software (*eXome-wide Hidden Markov Model*) [22], which makes use of principal component analysis and a hidden Markov model to normalize sequencing coverage data and detect CNVs. The identified variants were further annotated with the AnnotSV software [23] and manually curated.

Validation and Segregation by Sanger Sequencing

WES variants of interest were validated by Sanger sequencing when visual inspection of BAM files was inconclusive. Additionally, segregation analysis was carried out when parental WES was not performed. After polymerase chain reaction amplification, products were sequenced in both directions on an ABI3730 DNA sequencer (ThermoFisher Scientific) and analyzed through the MEGA software (https://www.megasoftware.net/). Primer pair sequences are available upon request.

Statistical Analyses

The studied group was characterized using the median and interquartile range (IQR) values for the continuous descriptive variables, while categorical variables were expressed as frequencies. Multivariate statistical analyses were conducted using SigmaStat for Windows version 3.5 (SPSS Inc.). Differences between groups were tested by the Mann-Whitney rank sum test and Fisher exact test. Statistical significance was assumed for $P < 0.05$.

Results

Clinical Characterization

We enrolled 45 patients from 42 unrelated Brazilian families, ten of which (23.8%) reported parental consanguinity. Positive exome findings were identified in 21 (50%) of the 42 Brazilian families (19 cases with pathogenic/likely

pathogenic variants and two with variants of uncertain significance (VUS)). The summarized phenotypic presentation of the patients with these clinically relevant exome findings is found in Table 1; the full clinical description of each patient is found in Supplementary Table 1. The sex ratio was 49% males ($n = 22$) and 51% females ($n = 23$) (Fig. 1a). Severe microcephaly (OFC < −3 SD) was identified in 30 individuals (64.4%) (Fig. 1b). Twelve patients (26.7%) presented with congenital microcephaly, and 17 (37.8%) evolved with microcephaly postnatally; for 16 patients (35.5%), parents did not have the OFC at birth recorded to establish the onset of microcephaly (Fig. 1c).

Only six individuals out of the 45 presented with non-syndromic microcephaly, including two siblings. For the syndromic patients ($n = 39$; 86.7%), we evaluated the frequencies of several clinical features according to HPO categories, detailed in the Supplementary Table 1 and represented in Fig. 1d.

Thirty-six of the 45 patients had undergone brain imaging examination; abnormal brain findings were detected in 24 (66.7%). Recurrent brain abnormalities, such as ventriculomegaly, corpus callosum dysplasia/atrophy, white matter abnormalities, cerebellar dysplasia/atrophy, cortical dysplasia, and gyri abnormalities, are presented in Fig. 1e. Additionally, the majority of the patients had records of height/weight at birth ($n = 38$) and at their last evaluation ($n = 37$). We identified 14 individuals (36.8%) who were small for their gestational age, and 20 (54.1%) presenting with short stature (Fig. 1f) [24, 25].

We performed an analysis of logistic regression to evaluate whether quantitative and qualitative features were enriched among individuals with or without positive WES findings (Supplementary Table 2). Cardiovascular malformations (P-value = 0.036), morphological abnormalities of the gastrointestinal system (P-value = 0.023), and height at birth ≤ −2 SD (P-value = 0.007; configurating individuals who were born small for their gestational age) showed statistical significance for WES-negative patients.

Genetic Findings

Clinically relevant exome findings were identified in 21 (50%) of the 42 Brazilian families. In 19 families (21 microcephalic individuals), the detected variants were classified as pathogenic or likely pathogenic, resulting in a diagnostic yield of 45.2%. Details are described in Table 2.

The 19 pathogenic/likely pathogenic variants were disclosed in 18 genes (*ANKRD11*, *ASNS*, *BCKDK*, *BRAT1*, *CASK*, *CCDC47*, *DYNC1H1*, *GATA6*, *KNL1*, *LZTR1*, *MCPH1*, *NDP*, *PPP2CA*, *SLC2A1*, *SRCAP*, *TRIO*, *TUBGCP4*, and *UBE3B*), along with the two VUS (*FGFR1* and *KCNT1*), comprising 20 distinct genetic disorders. Only one *locus* was found to be affected in two unrelated patients (*CASK*), in one of them as mosaicism (P18). Ten cases were associated with autosomal dominant disorders (AD; 47.6%); eight were related to autosomal recessive conditions (AR; 38.1%); three cases were X-linked, two of which were dominant (XLD; 9.5%) and one recessive (XLR; 4.8%) (Fig. 2). Seven out of the eight pathogenic homozygous variants were detected in consanguineous families. Among three individuals (P6–P8) with compound heterozygosity, one (P7—two affected siblings from consanguineous parents) had a pathogenic variant and a VUS in *KNL1*, while in the two other families, each patient (P6—affected sibship and P8—sporadic case) encompassed one pathogenic variant and one likely pathogenic variant in the *BRAT1* and *TUBGCP4* genes, respectively.

Out of the 24 observed rare clinically relevant variants, 12 were LoF (50%; seven nonsenses and five frameshifts); nine were missense (37.5%; one of them an in-frame deletion); two were intronic, predicted to affect splicing (8.3%); and one was synonymous, expected to lead to exon skipping (4.2%) (Supplementary Figure 1). Eleven of these variants (45.8%) had already been reported in the literature and/or deposited in clinical databases (ClinVar and HGMD). All these genes have been previously associated with microcephaly and were considered the primary cause of the patients' clinical features. A secondary finding was detected only in P19: a pathogenic variant in *PKP2* (NM_001005242.3:c.308_309del), one of the 73 actionable ACMG genes [21]. Additionally, in the P28 sibship, a third VUS was disclosed in *CCDC17* (NM_001114938.3:c.214C>T), a potential candidate gene for microcephaly.

The parental origin of the variants was assessed mostly through VCF analysis and visual inspection of BAM files. Sanger sequencing was applied to validate selected variants as well as to establish familial segregation of cases in which WES was not performed for parents. Nine variants were *de novo* (42.8%); five were observed in a homozygous state (23.8%); three were cases of compound heterozygosity (14.3%; six variants); two were maternally inherited (9.5%; one case was an autosomal variant inherited from a mother presenting with mild ID but not microcephaly, and the other was a X-linked variant), and in another one, the variant was inherited from a non-affected father (4.8%). Additionally, in one case (4.8%), it was not possible to determine the origin of the variants because paternal DNA sample was not available.

Comparison of Genetic Findings Among Different Cohorts of Microcephaly

To further explore the genetic findings of microcephalic patients who underwent WES or targeted sequencing, we analyzed published data from seven cohorts [5, 10, 26–30] and compared to ours. We aimed to establish frequencies

Table 1 Clinical presentation of the 23 microcephalic Brazilian patients (21 probands) with positive exome findings and the sibship carrying a VUS in a candidate gene for microcephaly

Patient ID	Sex	Age	OFC at birth cm (*Z*-score)	OFC at last assessment cm (*Z*-score)	Microcephaly onset	Brain imaging	Neurodevelopmental findings	Other clinical features
P1[a]	F	11 y	29 (−4.1SD)	49 (−2.9 SD)	Primary	MRI: ventriculomegaly	Intellectual disability, severe (walked independently at 4 y, spoke at 3 y but only isolated words)	Craniofacial dysmorphisms, clinodactyly of the 5th finger
P2[a]	F	1 y 6 m	27 (−5.8 SD)	N.A.	Primary	MRI: simplified gyral pattern	Global developmental delay (walked independently at 1 y 5 m)	Small for gestational age, absent thumb
P3[a,b]	M	19 y	N.A.	49.5 (−4.6 SD)	Unknown	N.A.	Intellectual disability, severe (unable to stand up and walk, never developed speech); hyperactivity, poor interaction	Craniofacial dysmorphisms, cervical kyphosis, congenital hip dislocation, urinary hesitancy, postaxial polydactyly, distal arthrogryposis
P4[a,b]	F	18 y	N.A.	47.5 (−4.9SD)	Unknown	Normal	Intellectual disability, severe (walked independently at 3 y, spoke at 9–10 y (but only isolated words)), learning disabilities (unable to read or write). Possible diagnosis of autism spectrum disorder (ASD), short attention span, hyperactivity, aggressive behavior, makes noises that sound like chirping. History of seizures in infancy	Short stature, delayed skeletal maturation, long-lined appearance, craniofacial dysmorphisms
P5	F	4 y 10 m	33 (−0.7 SD)	46 (−2.7 SD)	Secondary	MRI: ventriculomegaly. Hydrocephalus at birth	Global developmental delay (does not sit up without support, does not walk, spoke at 13 m but only isolated words). Motor stereotypy, aggressive behavior	Hypotonia, osteopenia, neonatal cholestatic liver disease, rickets due to vitamin-D deficiency secondary to liver disease, craniofacial dysmorphisms, hearing impairment, thoracolumbar kyphosis, lumbar scoliosis, pseudocamptodactyly of 3rd–5th fingers of the right hand, gastrostomy tube feeding
P6a	F	5 y 7 m	32 (−1.6 SD)	45 (−4.6 SD)	Secondary	MRI: cerebellar atrophy	Intellectual disability, severe (does not sit up without support, does not walk, never developed speech). Motor stereotypy, cerebellar ataxia	Short stature, failure to thrive, craniofacial dysmorphisms, facial hypertrichosis, pectus excavatum, scoliosis, broad thumb, clinodactyly of the 5th finger, pes planus, broad hallux, clinodactyly of the 5th toe

Table 1 (continued)

Patient ID	Sex	Age	OFC at birth cm (*Z*-score)	OFC at last assessment cm (*Z*-score)	Microcephaly onset	Brain imaging	Neurodevelopmental findings	Other clinical features
P6b	F	8 y	N.A.	45 (−5.2 SD)	Unknown	MRI: cerebellar hypoplasia, cerebral white matter hypoplasia	Intellectual disability, severe (does not sit up without support, does not walk, never developed speech)	Growth delay, craniofacial dysmorphisms
P7a[a]	M	5 y	29 (−4.3 SD)	40.5 (−6.9 SD)	Primary	MRI: polymicrogyria. Head CT: dysplastic corpus callosum, small cerebral cortex, simplified frontal lobe and temporal poles sulci	Intellectual disability, moderate (walked independently at 2 y, speaks few words, comprehension difficulties). Hyperactivity	---
P7b	F	N.A.	30 (−3.3 SD)	N.A.	Primary	MRI: craniofacial disproportion small cerebral cortex. Head CT: frontal cortical dysplasia, cerebellar vermis hypoplasia	Intellectual disability, moderate, comprehension difficulties. Hyperactivity	---
P8[b]	M	7 y 10 m	28 (−5.1 SD)	40 (−8.7 SD)	Primary	MRI: pachygyria. Head CT: ventriculomegaly	Intellectual disability, moderate, dysarthria, illiterate. Aggressive behavior, hyperactivity	Craniofacial dysmorphisms, strabismus, nystagmus, optic disc pallor, coarctation of aorta, atrial septal defect, clinodactyly, small right kidney, 2–3 toe syndactyly
P9	F	13 y 6 m	32 (preterm)[c]	50.4 (−2.4 SD)	Unknown	N.A.	Intellectual disability, severe (developed speech at 2 y, but only few words), illiterate. Dependent on parents for daily activities. Aggressive behavior, apathy, poor eye contact, seizures, gait disturbance	Short stature, nausea, craniofacial dysmorphisms, left arm spasticity, 2–3 toe syndactyly
P10	F	8 y	32 (−1.6 SD)	47 (−3.7 SD)	Secondary	Normal	Intellectual disability, severe (walked independently at 2 y, never developed speech). Motor stereotypy, screaming	Failure to thrive, craniofacial dysmorphisms, strabismus, reduced subcutaneous adipose tissue
P11	F	5 y	30 (preterm)[c]	46.5 (−2.4 SD)	Unknown	N.A.	Intellectual disability, moderate (walked independently at 2 y, spoke at 2 y 6 m). ASD, motor stereotypy, hyperactivity	---
P12	M	17 y	34 (−0.4 SD)	49 (−4.6 SD)	Secondary	MRI: polymicrogyria, dysplastic corpus callosum, white matter volume loss	Intellectual disability, severe (walked independently at 5 y, spoke at 6 y), illiterate. Dependent on parents for daily activities. Motor stereotypy, hyperactivity, incessant tachylalia, history of seizures	Short stature, neonatal hypotonia, episodic vomiting, craniofacial dysmorphisms, scoliosis, gait ataxia

Table 1 (continued)

Patient ID	Sex	Age	OFC at birth cm (*Z*-score)	OFC at last assessment cm (*Z*-score)	Microcephaly onset	Brain imaging	Neurodevelopmental findings	Other clinical features
P13	M	18 y 8 m	34.5 (0 SD)	49 (−4.9 SD)	Secondary	Normal	Intellectual disability, severe (walked independently at 15 m, never developed speech), illiterate. Totally dependent on parents. ASD, aggressive behavior, seizures	Short stature, delayed skeletal maturation, osteopenia, severe malnutrition, cranial hyperostosis, craniofacial disproportion, craniofacial dysmorphisms, patent ductus arteriosus, hypogonadotropic hypogonadism, micropenis, absence of secondary sex characteristics, coxa valga, abnormality of the right femoral and tibial epiphysis
P14	M	2 y 11 m	N.A.	45 (−3.1 SD)	Unknown	Normal	Global developmental delay (language impairment, does not recognize the alphabet and numbers, able to understand simple commands)	Short stature, hypotonia, recurrent infections, craniosynostosis, craniofacial dysmorphisms, pectus excavatum, patent ductus arteriosus, sacral dimple, bilateral cryptorchidism, pes planus
P15	M	3 y 5 m	34 (−0.4 SD)	45.8 (−2.8 SD)	Secondary	N.A.	History of seizures	Short stature, failure to thrive, mild hearing impairment in left ear, truncus arteriosus
P16	M	1 y 6 m	32 (−1.9 SD)	43 (−2.8 SD)	Secondary	Normal	Global developmental delay (walked independently at 1 y 6 m, spoke at 1 y 6 m)	Short stature, craniofacial dysmorphisms, atrial septal defect, bilateral cryptorchidism, hydrocele testis, clinodactyly of the 5th toe
P17	F	10 m	33 (−0.7 SD)	35.7 (−6.3 SD)	Secondary	MRI: Dandy-Walker malformation. Head CT: small cerebral cortex, cerebellar hypoplasia	---	Craniofacial dysmorphisms, optic disc pallor, hearing impairment
P18	M	2 y 6 m	32 (−1.9 SD)	44.5 (−3.2 SD)	Secondary	MRI: periventricular leukomalacia, enlargement of brain cerebrospinal fluid spaces. Head CT: metopic synostosis	Global developmental delay (abnormal speech and walking). Irritability	---
P19	M	7 y 6 m	N.A.	49.5 (−2.1 SD)	Unknown	N.A.	Normal	Recurrent infections, craniofacial dysmorphisms, bilateral retinal detachment, uveitis, nystagmus, poor visual acuity, hearing impairment, atrial septal defect, cryptorchidism, pseudoepiphyses of the 2nd–5th toes

Table 1 (continued)

Patient ID	Sex	Age	OFC at birth cm (*Z*-score)	OFC at last assessment cm (*Z*-score)	Microcephaly onset	Brain imaging	Neurodevelopmental findings	Other clinical features
P20	M	15 y 5 m	N.A.	51 (−3 SD)	Unknown	Normal	Global developmental delay (walked independently at 3 y, spoke at 2 y)[d]. Hyperactivity in childhood	Failure to thrive, craniofacial dysmorphisms, asymmetry of the thorax, butterfly vertebrae, ovoid lumbar vertebrae, patent foramen ovale, atrial septal defect, hypogonadotropic hypogonadism, micropenis, cryptorchidism, hypospadias
P21	F	15 y	N.A.	50 (−2.9 SD)	Unknown	Normal	Intellectual disability, mild. Attention deficit hyperactivity disorder (ADHD), seizures, dysphagia	Failure to thrive, dysphagia, dolichocephaly, optic disc pallor, marfanoid habitus
P28a[a,e]	M	1 y 6 m	30 (−3.5 SD)	36.5 (−8.2 SD)	Primary	Head CT: small cerebral cortex	Walked independently at 1 y 3 m, started speaking dissyllables. Discreet agitation	---
P29b	F	N.A.	N.A.	N.A.	Unknown	N.A.	N.A.	---

N.A. not available, *ADHD* attention deficit hyperactivity disorder, *ASD* autism spectrum disorder, *CT* computed tomography, *MRI* magnetic resonance imaging, *OFC* occipitofrontal circumference, *SD* standard deviation

[*]Phenotypic terms in accordance with the HPO standardized vocabulary; ages reported at the last clinical assessment. For patients up to 5 years of age, the term "global developmental delay" was used instead of "intellectual disability"

[a]Patients from consanguineous parents

[b]Some traits segregate in the family (Supplementary Table 1)

[c]Gestational weeks not available

[d]He initially had delay in the acquisition of the neurodevelopmental milestones, despite presented later with appropriated neurodevelopment, thus, was classified with "global developmental delay" instead of "intellectual disability"

[e]Siblings carrying a VUS in *CCDC17*, a candidate gene for microcephaly

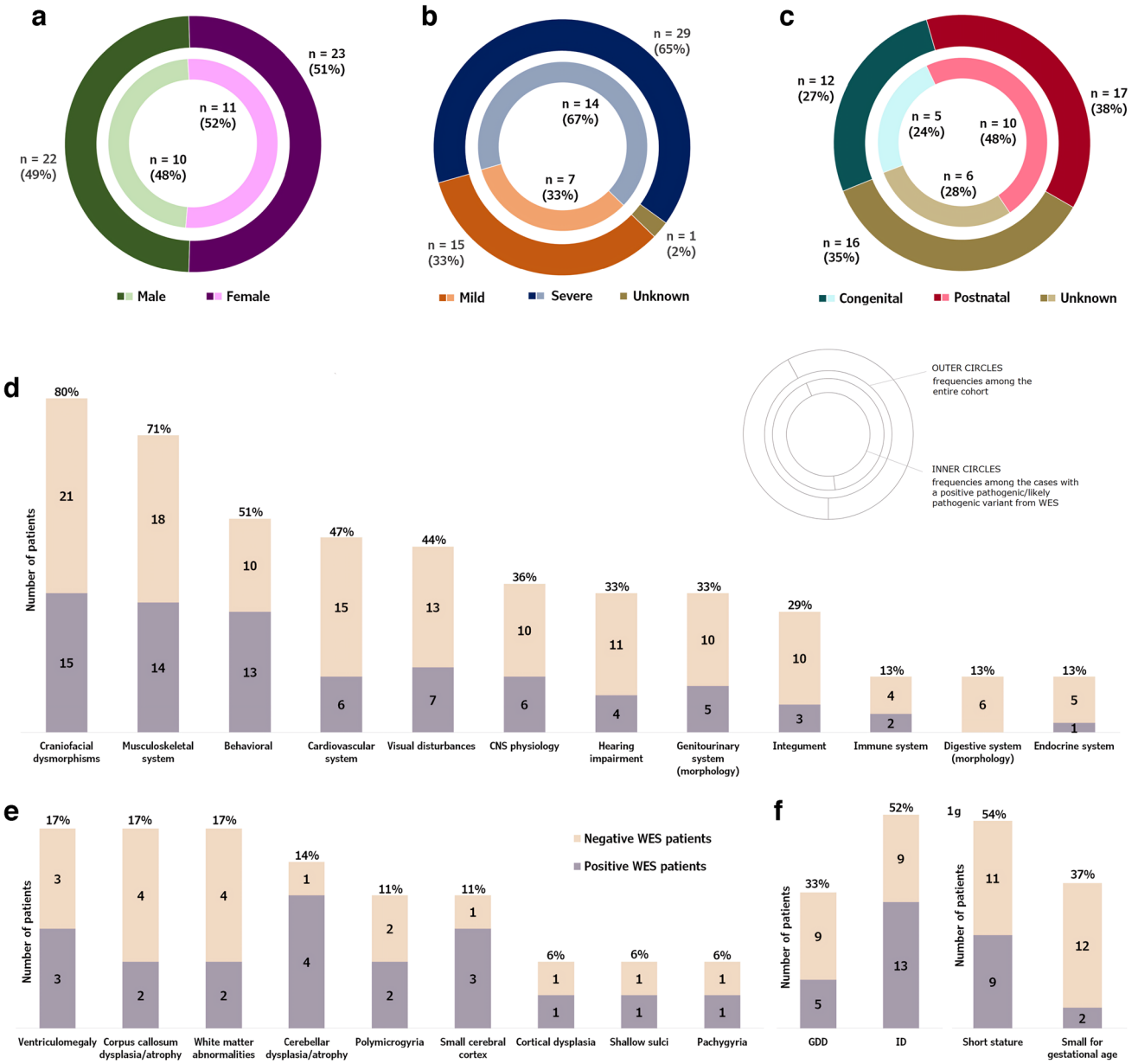

Fig. 1 Clinical characteristics of the Brazilian microcephalic cohort and frequencies of specific phenotypic features in patients. (**a**) Sex ratio, (**b**) severity of microcephaly, and (**c**) age of microcephaly onset—the outer circle represents the frequency of the feature in the total of patients, and the inner circle represents the number of cases with a pathogenic/likely pathogenic variant detected in WES analysis; (**d**) frequencies of the most often observed impaired systems in the group (n = 45), (**e**) frequencies of specific brain malformations (n = 36), (**f**) frequencies of intellectual disability (ID) and global developmental delay (GDD) (n = 41), and (**g**) frequencies of growth abnormalities (small for gestational age (n = 38) and short stature (n = 37)). The two individuals with VUS were categorized as "negative WES patients." CNS: central nervous system

and recurrence of affected genes among different populations and familial structures. In total, families with at least one member with microcephaly from eight distinct cohorts were studied, including ours. In this group, pathogenic/likely pathogenic variants were detected in 154 known genes associated with microcephaly, of which only 52 were disclosed in more than one family (34%). Among the recurrent 52 genes associated with microcephaly, 15 were reported in more than one family from the same study (28.8%), 17 were reported once in different cohorts (32.7%), and 20 were reported in more than one family of the same study and also disclosed in different cohorts (38.5%), leaving 102 genes reported only once among and within cohorts (66%) (Supplementary Table 3).

The most frequently mutated gene, *ASPM*, was reported 27 times, and it was the only gene observed in six out of

Table 2 Exome findings detected in 25 out of 45 Brazilian patients (22 probands) presenting syndromic microcephaly with neurodevelopmental disorders, including a sibship carrying a VUS in a candidate gene for microcephaly

	Variant information (WES)								*Genotype-phenotype correlation*
Patient ID	Sex	Genomic coordinates (hg38)—start position (ref/alt)	ClinVar accession number	Variant	Type	Zygosity (inheritance)	gnomAD exomes	ACMG classification (criteria)[a]	Disorder (inheritance pattern; OMIM #)
P1	F	chr7:97869060 (G/A)	SCV002512193	*ASNS*(NM_183356.4):c.97C>T (p.Arg33Cys)	Missense	Homozygosity (biparental)	0.000004	Pathogenic (PM1/PM2/PP3/PP5)	Asparagine synthetase deficiency (AR; #615574)
P2	F	chr8:6431578 (-/A)	SCV002512194	*MCPH1*(NM_024596.5):c.321dupA (p.Arg108ThrfsTer2)	Frameshift	Homozygosity (biparental)	0.00009	Pathogenic (PVS1/PM2/PP5)	Microcephaly 1, primary, autosomal recessive (AR; #251200)
P3	M	chr12:109516869 (G/-)	SCV002512195	*UBE3B*(NM_183415.3):c.2061delG (p.Ser688ProfsTer19)	Frameshift	Homozygosity (biparental)	---	Pathogenic (PVS1/PM2)	Kaufman oculocerebrofacial syndrome (AR; #244450)
P4	F	chr16:31112185 (C/T)	SCV002522323	*BCKDK*(NM_005881.4):c.1159C>T (p.Gln387Ter)	Nonsense	Homozygosity (biparental)	0.000008	Pathogenic (PVS1/PM2)	Branched-chain keto acid dehydrogenase kinase deficiency (AR; #614923)
P5	F	chr17:63761336 (T/-)	SCV002522325	*CCDC47*(NM_020198.3):c.563delA (p.Asn188ThrfsTer10)	Frameshift	Homozygosity (biparental)	---	Pathogenic (PVS1/PM2)	Trichohepatoneurode-velopmental syndrome (AR; #618268)
P6[b]	F	chr7:2538200 (GG/-); chr7:2544904 (C/T)	SCV002512192; SCV002522319	*BRAT1*(NM_152743.4):c.2334_2335delCC (p.Leu779ArgfsTer99); BRAT1(NM_152743.4):c.430+5G>A	Frameshift; intronic	Compound heterozygosity (paternal/maternal)	---	Pathogenic (PVS1/PM2/PP1); likely pathogenic (PM2/PM3/PP1/PP3)	Neurodevelopmental disorder with cerebellar atrophy and with or without seizures (AR; #618056)
P7[b]	M	chr15:40621340 (G/-); chr15:40654896 (T/G)	SCV002512780; SCV002522320	*KNL1*(NM_144508.5):c.1076del (p.Gly359GlufsTer19); KNL1(NM_144508.5):c.6416-13T>G	Frameshift; intronic	Compound heterozygosity (maternal/paternal)	--- 0.0004	Pathogenic (PVS1/PM2/PP1); VUS (PM3/PP1/BP7)	Microcephaly 4, primary, autosomal recessive (AR; #604321)

Table 2 (continued)

		Variant information (WES)							*Genotype-phenotype correlation*
P8	M	chr15:43401788 (C/T); chr15:43403697 (G/T)	SCV002538639; SCV002538640	*TUBGCP4*(NM_014444.5):c.1669C>T (p.Arg557Ter); *TUBGCP4*(NM_014444.5):c.1746G>T (p.Leu582=)	Nonsense; synonymous	Compound heterozygosity (maternal/not maternal)	--- 0.0003	Pathogenic (PVS1/PM2/PM3); likely pathogenic (PM3/PP5)	Microcephaly and chorioretinopathy, autosomal recessive, 3 (AR; #616335)
P9	F	chr1:42931047 (G/A)	SCV002512189	*SLC2A1*(NM_006516.4):c.274C>T (p.Arg92Trp)	Missense	Heterozygosity (*de novo*)	0.00002	Pathogenic (PS2/PM2/PP2/PP3/PP5)	GLUT1 deficiency syndrome 1 (AD; #606777)/ GLUT1 deficiency syndrome 2 (AD; #612126)
P10	F	chr5:134201981 (T/C)	SCV002512190	*PPP2CA*(NM_002715.4):c.353A>G (p.His118Arg)	Missense	Heterozygosity (*de novo*)	---	Pathogenic (PM2/PS2/PP3)	Neurodevelopmental disorder and language delay with or without structural brain abnormalities (AD; #618354)
P11	F	chr5:14291013 (C/T)	SCV002512191	*TRIO*(NM_007118.4):c.838C>T (p.Gln280Ter)	Nonsense	Heterozygosity (maternal, affected)[c]	---	Pathogenic (PVS1/PM2/PP1)	Intellectual developmental disorder, autosomal dominant 44, with microcephaly (AD; #617061)
P12	M	chr14:102027625 (G/A)	SCV002522321	*DYNC1H1*(NM_001376.5):c.9055G>A (p.Gly3019Ser)	Missense	Heterozygosity (*de novo*)	---	Pathogenic (PM2/PS2/PP3)	Intellectual developmental disorder, autosomal dominant 13 (AD; #614563)
P13	M	chr16:30737370 (C/T)	SCV002522322	*SRCAP*(NM_006662.3):c.7330C>T (p.Arg2444Ter)	Nonsense	Heterozygosity (*de novo*)	---	Pathogenic (PVS1/PM2/PM6/PS4/PP5)	Floating-Harbor syndrome (AD; #136140)
P14	M	chr16:89288528 (C/G)	SCV002522324	*ANKRD11*(NM_013275.6):c.744G>C (p.Lys248Asn)	Missense	Heterozygosity (*de novo*)	---	Likely pathogenic (PM2/PS2/PP3)	KBG syndrome (AD; #148050)

Table 2 (continued)

		Variant information (WES)							*Genotype-phenotype correlation*
P15	M	chr18:22181516 (C/T)	SCV002522326	*GATA6*(NM_005257.6):c.1366C>T (p.Arg456Cys)	Missense	Heterozygosity (*de novo*)	---	Pathogenic (PM1/PM2/PM5/PS2/PP3/PP5)	Pancreatic agenesis and congenital heart defects (AD; #600001)
P16	M	chr22:20990476 (G/A)	SCV002538635	*LZTR1*(NM_006767.4):c.742G>A (p.Gly248Arg)	Missense	Heterozygosity (*de novo*)	---	Pathogenic (PM1/PM2/PM5/PS1/PS2/PS3/PS4/PP3/PP5)	Noonan syndrome 10 (AD; #616564)
P17	F	chrX:41561618 (G/A)	SCV002538636	*CASK*(NM_001126055.2):c.1591C>T (p.Arg531Ter)	Nonsense	Heterozygosity (*de novo*)	---	Pathogenic (PVS1/PM2/PS2/PS4/PP5)	Intellectual developmental disorder and microcephaly with pontine and cerebellar hypoplasia (XLD; #300749)
P18	M	chrX:41660445 (C/T)	SCV002538637	*CASK*(NM_001126055.2):c.825G>A (p.Trp275Ter)	Nonsense	Hemizygosity—mosaicism (*de novo*)	---	Pathogenic (PVS1/PM2/PS2)	
P19	M	chrX:43958537 (G/A)	SCV002538638	*NDP*(NM_000266.4):c.109C>T (p.Arg37Ter)	Nonsense	Hemizygosity (maternal)	---	Pathogenic (PVS1/PM2/PP5)	Norrie disease (XLR; #310600)
P20	M	chr8:38429841 (GCCAGTTGATGCTCT/-)	SCV002538641	*FGFR1*(NM_023110.3):c.185_199del (p.Gln62_Trp66del)	Missense (in-frame deletion)	Heterozygosity (not maternal)	---	VUS (PM2/PM4)	Hypogonadotropic hypogonadism 2 with or without anosmia (AD; #147950)
P21	F	chr9:135786230 (G/A)	SCV003845964	*KCNT1*(NM_020822.3):c.3211G>A (p.Asp1071Asn)	Missense	Heterozygosity (unknown)	---	VUS (PM2)	Developmental and epileptic encephalopathy 14 (AD; #614959)
P28[d]		chr1:45623613 (G/A)	N.A.	*CCDC17*(NM_001114938.3):c.214C>T (p.Gln72Ter)	Nonsense	Homozygosity (biparental)	0.0002	VUS (PM2)	---

Ref/alt reference and alternative alleles, respectively, *N.A.* not available, *AD* autosomal dominant inheritance, *AR* autosomal recessive inheritance, *XLD* X-linked dominant inheritance, *XLR* X-linked recessive inheritance

[a]Based on Richards et al. (19)

[b]These patients have an affected sibling carrying the same variants

[c]The mother is affected, presenting mild intellectual disability, but not microcephaly

[d]Patient carrying a VUS in a candidate gene for microcephaly (his affected sister is also a homozygous carrier)

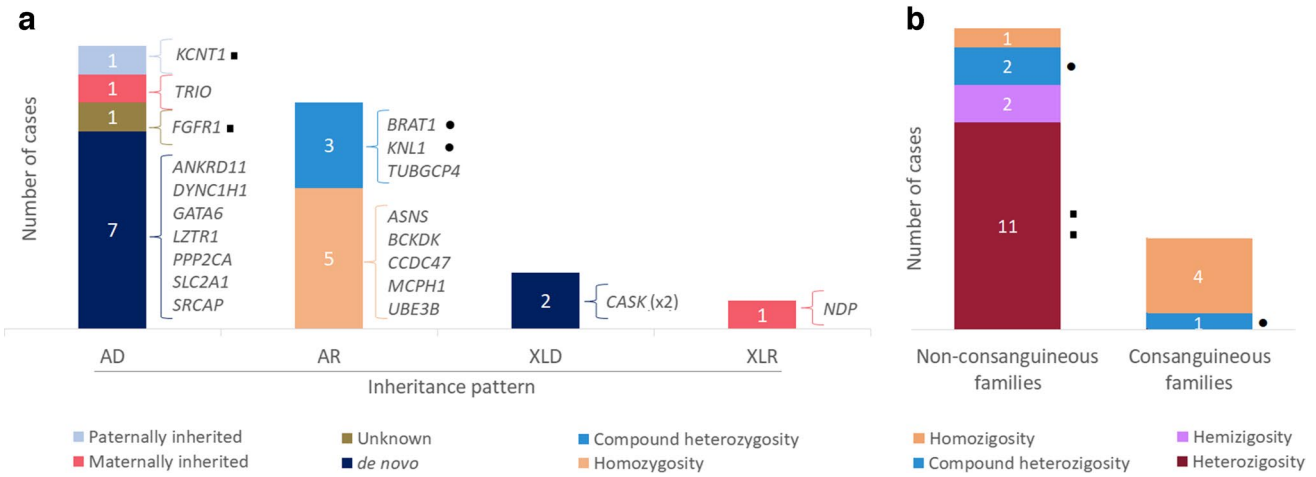

Fig. 2 Genetic findings disclosed by whole-exome analysis in a group of 42 Brazilian families with 45 patients presenting syndromic microcephaly associated with neurodevelopmental disorders. (**a**) Identified genes and their respective inheritance patterns listed at the right side of the vertical bars, which indicates the number of patients with each type of variant (monoallelic *de novo*, inherited, or unknown; biallelic in homozygosity or compound heterozygosity). AD, autosomal dominant; AR, autosomal recessive; XLD, X-linked dominant; XLR, X-linked recessive. (**b**) Differences of the zigosity of the disclosed variants comparing patients born from consanguineous and non-consanguineous parents. A black dot represents the sibships, and a black square represents the cases with VUS

eight studies. Other recurrences were *WDR62* (10×); *ASNS* (9×); *CDK5RAP2*, *PCNT*, and *TUBA1A* (8× each); *CASK*, *KNL1*, and *NDE1* (6× each); *CENPJ*, *DONSON*, *FOXG1*, *MCPH1*, and *TUBB3* (5× each); *CTNNB1*, *CTU2*, *DYRK1A*, *ERCC6*, *PHGDH*, *RNU4ATAC*, *RTTN*, *TSEN54*, and *PNKP* (4× each); *BRCA2*, *CEP152*, *CIT*, *EFTUD2*, *KMT2A*, *PDHA1*, *PLK4*, *MECP2*, *PQBP1*, *TRAPPC9*, and *VPS33B* (3× each); and *DDX11*, *GRIN1*, *MTHFR*, *TUBB2B*, *VRK1*, *WDR4*, *ACTG1*, *COL4A1*, *DHTKD1*, *DYNC1H1*, *ERCC8*, *FBXO11*, *KMT2D*, *SLC25A19*, *TCF4*, *TRIO*, *TRMT10A*, and *UBE3B* (2× each). Fourteen out of the 30 genes related to the subcategory MCPH were identified, with eight of them reported in at least two distinct cohorts (*ASPM*, *CDK5RAP2*, *CENPJ*, *CEP152*, *CIT*, *KNL1*, *MCPH1*, and *WDR62*). All the 14 MCPH genes are associated with AR forms of microcephaly; altogether, they were confirmed as the molecular diagnosis for 73 out of the 321 families (22.8%), of which 48 were consanguineous ($n = 1$ compound heterozygosis; $n = 47$ homozygosis) and 25 reported in non-consanguineous ($n = 21$ compound heterozygosis; $n = 4$ homozygosis) parents.

Additionally, we uploaded the entire set of 154 identified microcephaly genes and the 52 recurrent microcephaly genes on the WebGestalt (http://www.webgestalt.org/) and STRING (https://string-db.org/) websites to investigate pathways and protein-protein interaction (PPI) networks enriched in these sets (Supplementary Figure 2). There was a bias towards mitotic cell cycle regulation, cell cycle phase transition, and microtubule-based processes, and it was observed an enrichment in our PPI network ($P = 3.7e\text{-}8$).

Discussion

The remarkable genetic and clinical heterogeneity of microcephaly poses as a challenge, making it difficult even to establish a clinical diagnosis [3, 10]. We achieved a molecular diagnosis (pathogenic/likely pathogenic SNV/indel variants) for 45.2% of the 42 Brazilian families, a high yield considering that this cohort had been previously screened by karyotyping and CMA. Recent studies from different populations have reported variable diagnostic yields through WES for microcephaly, such as 29% [29], 30% [26], 37.2% [27], 42.9% [5], and 51.7% [10]. All these studies, including ours, corroborated the use of WES as a first-tier approach in subjects with microcephaly and NDD [31]. Our data also reinforced the importance of a genome-wide approach for populations which presents an admixed background throughout the country and striking consanguinity in some regions [32–34], such as the Brazilian.

To refine the interpretation of our data, we analyzed the phenotypic findings of our cohort (Fig. 1). Intellectual disability (ID) is tightly linked to microcephaly [6]. Accordingly, for the 42 patients in whom it was possible to evaluate this feature, 36 (85.7%), or 18 out of the 21 individuals carrying a pathogenic/likely pathogenic variant (85.7%), presented with different levels of ID. Previous studies have described similar frequencies, such as 86.6% [27], 91.7% [5], 93.3% [10], 100% [29], and 100% [26]. Only two of our patients had a positive WES finding without cognitive impairment, displaying mild microcephaly of unknown onset (P15 and P19; Table 1). On the other hand, two of the three patients presenting only learning disabilities who were

WES-negative showed severe microcephaly (P24 and P34; Table 1). The percentage of individuals with severe microcephaly caused by a detected pathogenic/likely pathogenic variant in contrast to those without a molecular diagnosis ($n = 21$) was similar (60.9% against 71.4%). Thus, despite a clear association derived from abnormalities in brain growth and functioning, our findings suggested that it is still controversial the correlation between the degree of microcephaly with intellectual performance [5, 6, 10, 35] and the WES outcome. In our study, the majority of patients for whom birth OFC was available had developed microcephaly after birth (58.6%), similarly to the frequency in Dawidziuk et al. [27] study (58.8%), but distinctly from Boonsawat et al. [5] and Lee et al. [26], who predominantly reported congenital microcephaly. We speculate that dysregulation in biological pathways could be differentially affected by the specifically mutated genes leading to either congenital or postnatal microcephaly in our cohort.

Besides neurological findings, affected individuals can exhibit an array of cranial and extracranial manifestations [3, 36]. The strikingly wide spectrum of clinical features of our patients (Supplementary Table 1) hampered a detailed categorization. We hence applied an HPO-based hierarchization aiming at a comparison with other studies [3, 5, 10, 26, 27, 29]. Our cohort presented similar frequencies of clinical signs, such as for CNS abnormalities (66.7% *vs.* 63–80%), morphological gastrointestinal tract (13.3% *vs.* 9–13%), and genitourinary tract (33.3% *vs.* 13–26%) defects. Conversely, ours was enriched for facial dysmorphisms (80% *vs.* 18–39%) similarly to Lee et al. [26] (70%) and Masih et al. [10] (82.2%), cardiovascular defects (46.7% *vs.* 7–20%), behavioral disturbances (51.1% *vs.* 10–27%), musculoskeletal abnormalities (71.1% *vs.* 10–32%), and hearing impairment (33.3% *vs.* 7–15%). Also, 54.1% cases with height measurement records displayed short stature that can be indicative of proportionate microcephaly (i.e., a decline in height ≤ -2 SD), similar to the frequency found by Lee et al. [26] (65%) and Masih et al. [10] (60%), but discordant to the other cohorts (32–42%) [3, 5, 27, 29]. Interestingly, out of the 14 patients who were born small for their gestational age, only two had clinically relevant WES findings, suggesting that a complex interaction of genetic and environmental factors [37] contributes to a global dysregulation in growth and development.

Severe syndromic cases remained with negative exome results. It is worthy to mention that the negative cases can be divided in two groups: one of them is composed by patients presenting with few clinical signs, likely with an oligogenic/multifactorial etiology, and another includes patients exhibiting severe and multisystemic phenotypes. In the latter group, structural chromosomal alterations, deep intronic SNVs, exonic variants in regions with poor coverage (CG-rich, repetitive sequences), and regulatory or mitochondrial variants could be accountable for a proportion of these cases, going undetected by WES. Some of these possibilities have been demonstrated by comparisons between exome and whole-genome sequencing for rare diseases [38, 39].

Despite not facilitating a diagnosis, brain imaging exams can provide guidelines for the clinical management of patients and help to clarify a genetic result [3]. Brain malformations identified in microcephalic patients are usually unspecific and heterogeneous [3], although they still tend to manifest in the majority of them [3, 5, 10, 27, 29, 30], especially as gyri pattern anomalies, corpus callosum malformations, and decrease of the cortical surface area [3, 40]. We noted elevated frequencies of ventriculomegaly and white matter abnormalities in our cohort, in addition to a variety of alterations that were observed only once (Supplementary Table 1).

Regarding the molecular findings, 24 clinically relevant variants were detected in 18 different genes. It is interesting to note that the only recurrent gene, *CASK* (OMIM *300172), was also consistently found mutated in other microcephalic cohorts [10, 27, 29]. This X-linked gene has been suggested as the second most common cause of microcephaly associated with pontocerebellar hypoplasia and ID (OMIM #300749) in females [41, 42]. A reduced viability or even in utero lethality for hemizygous embryos with LoF *CASK* variants could explain the absence of affected males, with the exception of those carrying hypomorphic or missense variants [43]. One of our cases was a boy (P18) with mosaicism for a *CASK* nonsense variant, as also described by Burglen et al. [41].

Another noteworthy case was a female carrying an *ASNS* homozygous variant (P1). *ASNS* encodes a crucial enzyme for brain development (asparagine synthetase) [44]. To our knowledge, only 26 families with ASNS deficiency (OMIM #615574) have been reported, mostly carrying missense variants at the C-terminal domain of the protein [44–47]. The missense variant disclosed in our patient affects the N-terminal domain, and individuals reported with variants in this domain had more severe clinical presentations [44–47]. Functional studies and new patient reports should be helpful to clarify the impact of N-terminal variants in ASNS deficiency.

Rarer than *CASK* and *ASNS* variants, pathogenic variants were disclosed in the *PPP2CA* (OMIM *176915), *BCKDK* (OMIM *614901), and *CCDC47* (OMIM *618260) genes. *PPP2CA* disruption was only recently linked to NDD (OMIM #618354) [48]. The patient herein reported (P10) displayed some traits of this condition, but she did not have seizures, hypotonia, feeding difficulties, or brain MRI abnormalities, showing marked failure to thrive with reduced subcutaneous adipose tissue, therefore expanding the understanding of the phenotypic expressivity of this disorder.

BCKDK deficiency (OMIM #614923) leads to abnormal decreasing circulating levels of isoleucine, leucine, and valine, and it has been suggested as a treatable metabolic disorder suitable for neonatal screening programs [49–51]. We disclosed a pathogenic homozygous nonsense variant in *BCKDK* in P4. Interestingly, it has been demonstrated in mice that Bckdk is a substrate for the ubiquitin protein ligase E3 encoded by the *UBE3B* gene (OMIM *608047) [52]. Another patient of our cohort (P3) was found to carry a homozygous pathogenic *UBE3B* frameshift variant, associated with Kaufman oculocerebrofacial syndrome (OMIM #244450). Some individuals present with skeletal alterations, such as polydactyly, scoliosis, and arthrogryposis, features observed in P3 [52, 53]. According to Cheon et al. [52] experiments in mice, Ube3b is highly expressed in the brain, liver, and skeletal muscle, which are the major sites of BCAAs metabolism. In *Ube3b* knockout mice, Bckdk levels were elevated in these tissues, and metabolomics profiling of KOS patients displayed similar changes. Ultimately, these results demonstrate that *in silico* analyses of known genes' biological pathways or omics profiling could be compelling strategies to search for new genes spanning rare causative variants in microcephalic NDD patients with negative WES results. In humans, LoF biallelic variants or full duplications encompassing *BCKDK*—thus enhancing its expression similarly to what is seen in *Ube3b*$^{-/-}$ mice—may lead to NDD. This evidence highlights the importance of sustaining proper neuronal homeostasis during development, since for some *loci* both increase and decrease of their encoded proteins can impact the development and functioning of the central nervous system [54].

CCDC47 biallelic variants cause the trichohepatoneurodevelopmental syndrome (OMIM #618268) [55]. Despite P5 carrying a *CCDC47* homozygous variant, parents did not report consanguinity; nevertheless, they come from a small town in the Northeast of Brazil, which could suggest that this is a founder variant in this population. Notably, two *CCDC47* variants disclosed in homozygosis by Morimoto et al. [55] were also considered founder variants in non-consanguineous families.

P8 displayed biallelic variants in *TUBGCP4* (OMIM *609610), OMIM #616335), and multiple cases of compound heterozygosity of p.Leu582= along with a truncating alteration have been reported in *TUBGCP4* [56]. These two cases, P5 (*CCDC47*) and P8 (*TUBGCP4*), are a hint that recessive disorders should not be ignored in isolated cases from non-consanguineous families, as previously suggested [27]. Similarly, synonymous variants are typically disregarded in an initial evaluation given the misleading idea that they will have no effect [57]. It has been demonstrated that the p.Leu582= variant generates a new cryptic splice site that leads to exon 16 skipping [58].

Two other relevant genes disclosed in our cohort were *FGFR1* (OMIM *136350) and *GATA6* (OMIM *601656), both associated with disorders in which microcephaly is not a typical feature. For *FGFR1*, the most suitable condition according to P20 clinical traits was hypogonadotropic hypogonadism 2 with or without anosmia (OMIM #147950), even though microcephaly and GDD are most common in the Hartsfield syndrome (OMIM # 615465) [59–61]. P20 presented a VUS in *FGFR1*, an in-frame deletion of five amino acids (p.Gln62_Trp66del) in the first Ig-like domain of the fibroblast growth factor receptor 1. The five residues are fully conserved in mammals, frog, and chicken. Although there have been no pathogenic variants reported in this domain (NCBI/ClinVar, accessed in April 2023), the variant deletes four of ten residues that encompass one of the domain's eight strands (NP_075598.2). Microcephaly has been reported in pancreatic agenesis and congenital heart defects (OMIM #600001), *GATA6*-matching disorder for P15. The patient's cardiac malformation is the most consistent feature related to this condition [62]. Functional studies could be able to determine if *FGFR1* and *GATA6* variants might cause the neurological signs, expanding the phenotypic spectrum of these disorders. Alternatively, additional yet undisclosed alterations could explain the patients' microcephaly and GDD.

In respect to the individuals with parental consanguinity, we achieved an equal diagnostic yield for non-consanguineous families (50%). As expected, for isolated cases without parental consanguinity, there was an increased rate of *de novo* mutations (60%), as previously reported [5, 27]. Regarding the sibships only, two out of three had positive WES findings, both for recessive disorders, which is aligned to Rump et al. [29] suggestion that for microcephalic patients, AR inheritance is more common than for ID cohorts in general, especially in cases of recurrence in sibs. On this basis, the twice occurrence of compound heterozygosity in our cohort sheds light to the burden for recessive disorders of heterozygous carriers, who may not be aware of their reproductive risk [63]. Furthermore, the third sibship of our cohort (P28), born from consanguineous parents, was found to carry a homozygous nonsense VUS in *CCDC17* (NM_001114938.3:c.214C>T), affecting the protein residue 72 out of 622. *CCDC17* encodes a poorly characterized coiled-coil domain-containing protein, predicted to act at cilium organization (https://www.proteinatlas.org/search/ccdc17). Based on the importance of the coiled-coil motif at the kinetochore for correct chromosome segregation [64], which is one of the core mechanisms of pathophysiological dysregulation in MCPH cases, we hypothesize a role for *CCDC17* at microtubule functioning. Nonetheless, more studies are necessary to corroborate *CCDC17* as a new gene related to microcephaly.

Lastly, our analyses of data from different cohorts and comparison with our findings can validate the preferential use of WES or whole-genome sequencing in microcephalic patients: only 34% of the 154 genes ($n = 52$) was disclosed with pathogenic variants in more than one family among eight distinct cohorts [5, 10, 26–30], highlighting the diverse range of *loci* implicated in microcephaly. Unlike macrocephaly, in which there is a gene enrichment towards the development of the head, skull, and CNS [65], we found for the set of 154 genes a higher prevalence of mitotic regulation and cell cycle phase transition and microtubule-based processes. Our PPI analysis also showed that these proteins had significantly more interactions than expected, suggesting a biological connection. These findings are in accordance with the concept of this condition arising primarily from defects in proliferation, abundance, and cell functioning, especially for cases of congenital microcephaly, and point out to potential strategies to discover new genes [4].

In conclusion, our work was the first to explore the genetic basis of microcephaly in the Brazilian population, besides providing a deeper and more complete clinical characterization of a microcephalic cohort, which helped to delineate more precise genotype-phenotype correlations and enabled the phenotypic expansion of some conditions. Our data also reinforce the underlying genetic heterogeneity of microcephaly in the Brazilian population, corroborating the high yield of WES and shedding light to the importance of comprehensive sequencing strategies when investigating the genomic basis of rare diseases. Besides, we propose *CCDC17* as a candidate gene for microcephaly. However, despite the high diagnostic yield, approximately 50% of the cases remained idiopathic. To be solved from a genomic point of view, more comprehensive and complex genome-wide investigations must be performed, especially for cases with severe phenotypes. These investigations should include whole-genome sequencing, optical genome mapping, RNA-Seq, and methylome studies [66].

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-023-03894-8.

Acknowledgements The authors thank the patients and their families for their collaboration in this study and the funding agencies.

Author Contribution ACVK designed this study. Methodological investigation and data collection and analysis were performed by GCT, GCB, LAH, and CR. Material preparation was performed by SSC. The clinical evaluation of the patients was performed by CFMS, HVDL, WLMF, PAO, AMVM, RSH, GLY, CAK, AALJ, and DRB. Bioinformatic processing was conducted by MOS. Statistical analysis was performed by AALJ. The first draft of the manuscript was written by GCT and ACVK, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by the funding agencies São Paulo Research Foundation ([FAPESP] grant numbers 2013/080828-1 and 2020/15552-2), National Council for the Improvement of Higher Education ([CAPES] 1799650 [to GCT]), and National Council for Scientific and Technological Development ([CNPq] 157816/2018-4 [to GCT], 303375/2019-1 [to DRB], 140271/2020-1 [to GCT], 303294/2020-5 [to AALJ], and 305101/2022-6 [to ACVK]).

Data Availability The pathogenic and likely pathogenic variants reported here are deposited in the ClinVar database (ClinVar accession numbers: SCV002522324 (*ANKRD11*), SCV002512193 (*ASNS*), SCV002522323 (*BCKDK*), SCV002512192 (*BRAT1*), SCV002522319 (*BRAT1*), SCV002538636 (*CASK*), SCV002538637 (*CASK*), SCV002522325 (*CCDC47*), SCV002522321 (*DYNC1H1*), SCV002538641 (*FGFR1*), SCV002522326 (*GATA6*), SCV003845964 (*KCNT1*), SCV002512780 (*KNL1*), SCV002522320 (*KNL1*), SCV002538635 (*LZTR1*), SCV002512194 (*MCPH1*), SCV002538638 (*NDP*), SCV002512190 (*PPP2CA*), SCV002512189 (*SLC2A1*), SCV002522322 (*SRCAP*), SCV002512191 (*TRIO*), SCV002538639 (*TUBGCP4*), SCV002538640 (*TUBGCP4*), SCV002512195 (*UBE3B*)). Data of Patients with positive WES results are deposited in the DECIPHER database (IDs can be found in the Supplementary Table 1). Additional information can be obtained upon request to the corresponding author.

Declarations

Ethics Approval and Consent to Participate This study was conducted with the approval of the Research Ethics Committee of the Institution (protocol CAAE 80921117.5.0000.5464). Signed informed consents to participate and to have their data published were obtained from the patients' parents in accordance with the ethical standards established in the Declaration of Helsinki (1964) and the Resolution 466/2012 of the Brazilian National Health Council.

Competing Interests The authors declare no competing interests.

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