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Clinical Significance of De Novo and Inherited Copy-Number Variation

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ABSTRACT: Copy-number variations (CNVs) are a common cause of intellectual disability and/or multiple congenital anomalies (ID/MCA). However, the clinical interpretation of CNVs remains challenging, especially for inherited CNVs. Well-phenotyped patients (5,531) with ID/MCA were screened for rare CNVs using a 250K single-nucleotide polymorphism array platform in order to improve the understanding of the contribution of CNVs to a patients phenotype. We detected 1,663 rare CNVs in 1,388 patients (25.1%; range 0–5 per patient) of which 437 occurred de novo and 638 were inherited. The detected CNVs were analyzed for various characteristics, gene content, and genotype–phenotype correlations. Patients with severe phenotypes, including organ malformations, had more de novo CNVs ($P < 0.001$), whereas patient groups with milder phenotypes, such as facial dysmorphisms, were enriched for both de novo and inherited CNVs ($P < 0.001$), indicating that not only de novo but also inherited CNVs can be associated with a clinically relevant phenotype. Moreover, patients with multiple CNVs presented with a more severe phenotype than patients with a single CNV ($P < 0.001$), pointing to a combinatorial effect of the additional CNVs. In addition, we identified 20 de novo single-gene CNVs that directly indicate novel genes for ID/MCA, including *ZFHX4*, *ANKH*, *DLG2*, *MPP7*, *CEP89*, *TRIO*, *ASTN2*, and *PIK3C3*.

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KEY WORDS: copy number variation; CNV; SNP; genotype-phenotype; human phenotype ontology

Introduction

Copy-number variations (CNVs) are a common type of genomic variation, which can be detected routinely using genomic microarrays [Feuk et al., 2006]. These microarrays have now become first-tier tests in the clinical evaluation of patients with intellectual disability/developmental delay (ID/DD) and/or multiple congenital anomalies (MCA) [Cooper et al., 2011; Koolen et al., 2009; Miller et al., 2010]. The diagnostic yield of microarray analysis in patients with ID/MCA is estimated between 10% and 20% [Cooper et al., 2011; Hochstenbach et al., 2009; Miller et al., 2010; Sagoo et al., 2009], depending on a number of factors, such as the resolution and probe distribution of the microarray platform [Hehir-Kwa et al., 2007], the selection of the patient population, the pretesting performed, and the CNV interpretation criteria used. These criteria usually include:

(1) the frequency and overlap of CNVs present in healthy control individuals [Iafrate et al., 2004; Pinto et al., 2007; Shaikh et al., 2009];
(2) the inheritance pattern of the CNV. Especially for severe phenotypes with reduced fecundity such as ID/MCA, de novo CNVs are often considered likely causal, whereas CNVs inherited from a healthy parent are more likely considered benign variants;
(3) the presence of overlapping aberrations in patients with similar phenotypes. This requires reliable and detailed phenotype data of patients to allow an accurate comparison. Databases that collect detailed chromosomal and phenotype data of large patient cohorts such as ECARUCA (http://www.ecaruca.net) and DECIPHER (http://decipher.sanger.ac.uk) are important to reliably determine the pathogenicity, especially for nonrecurrent CNVs [Feenstra et al., 2006; Firth et al., 2009];
(4) the size, copy-number state (gain or loss), and gene content of the CNV (especially regarding dosage sensitivity, function, expression pattern, and known disease association) can be helpful in determining the pathogenicity of the CNV.

However, the clinical interpretation of CNVs identified in patients with ID/MCA still remains challenging as many pathogenic CNVs are rare and nonrecurrent and CNVs also commonly occur in the normal population, resulting in variability in the interpretation and reporting of CNVs from different clinical laboratories [Tsuchiya et al., 2009]. In particular, rare CNVs that are inherited from healthy parents pose a challenge, as these may still be clinically

Additional Supporting Information may be found in the online version of this article.

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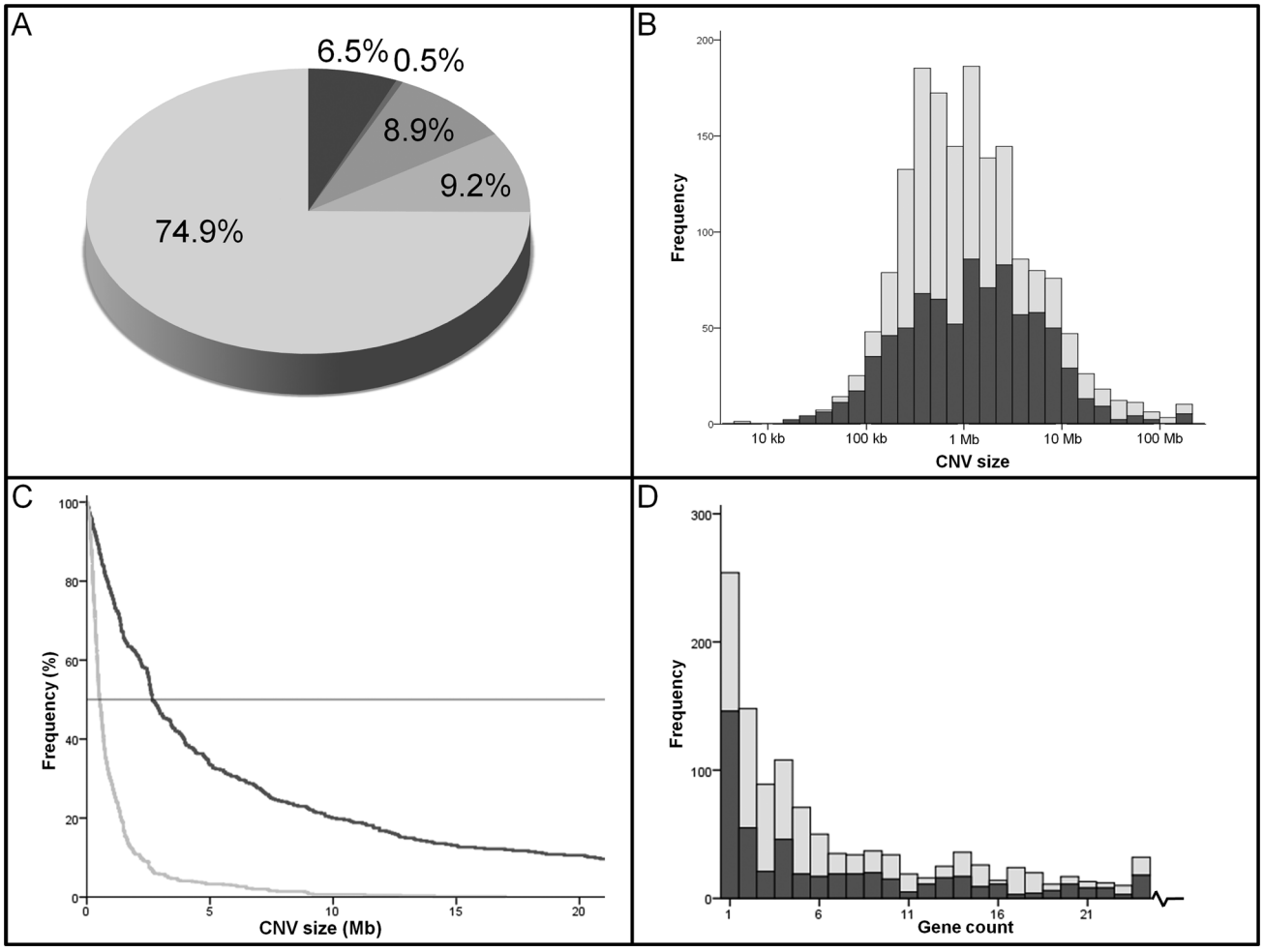

Figure 1. CNV yield of microarray analysis in a cohort of 5,531 patients with ID/MCA. **A:** Results of SNP array analysis in 5,531 patients: a de novo aberration was identified in 6.5% of patients, 0.5% had a inherited X-chromosomal CNV in a male, 8.9% had a inherited CNV, 9.2% had a CNV of which the inheritance pattern could not be determined, and 74.9% had no rare CNVs. **B:** Size distribution of all 1,663 CNVs on logarithmic scale. Dark gray represents losses; light gray represents gains. **C:** Size distribution of de novo (dark gray, $N = 437$) versus inherited (light gray, $N = 638$) CNVs. **D:** Distribution of gene content of CNVs up to 24 genes. Dark gray represents losses; light gray represents gains.

relevant through, for example, variable expressivity and decreased penetrance.

To improve the understanding of the contribution of de novo and inherited CNVs to the patients' phenotype, we performed detailed genotype–phenotype correlation studies in a longitudinal cohort of 5,531 patients with ID/MCA. In addition, we studied the role of multiple CNVs observed in individual patients and examined the gene content of the CNVs, suggesting several novel candidate genes that could be implicated in ID and/or MCA.

Materials and Methods

Study Population

This study consisted of a cohort of 5,531 consecutive patients whose DNA was tested by microarray using the Affymetrix 250K *NspI* single-nucleotide polymorphism (SNP) array platform (Affymetrix, Inc., Santa Clara, CA) in a diagnostic setting in the Department of Human Genetics in Nijmegen, The Netherlands, between 2006 and 2011.

Microarray Analysis

DNA of all patients was analyzed on the Affymetrix 250K *NspI* SNP array platform containing 262,264 SNPs (Affymetrix, Inc.), which covers the whole genome (except the Y chromosome) with a resolution of 200 kb [Hehir-Kwa et al., 2007]. DNA was obtained from peripheral blood in the vast majority of cases, but also DNA from buccal swabs and fibroblasts was used. The microarray analysis was performed as described elsewhere [de Leeuw et al., 2011]. CNVs were called when there were at least five or seven consecutive aberrant SNPs for losses and gains, respectively. The CNV coordinates were mapped to NCBI Genome Build 36/hg18 using the UCSC LiftOver tool (http://genome.ucsc.edu). Genes were ascertained to the identified CNVs using the list of ENSEMBL genes (http://www.ensembl.org) [Hubbard et al., 2002]. We mapped all genes that had at least one protein-coding exon included within the minimal affected region of the CNV.

Identified CNVs were compared with CNVs from healthy control individuals. CNVs that were present with a frequency of 1% or more in the Database of Genomic Variants (http://projects.tcag.ca/variation/project.html) [Iafrate et al., 2004] or in our in-house database containing data from over 800 healthy controls were

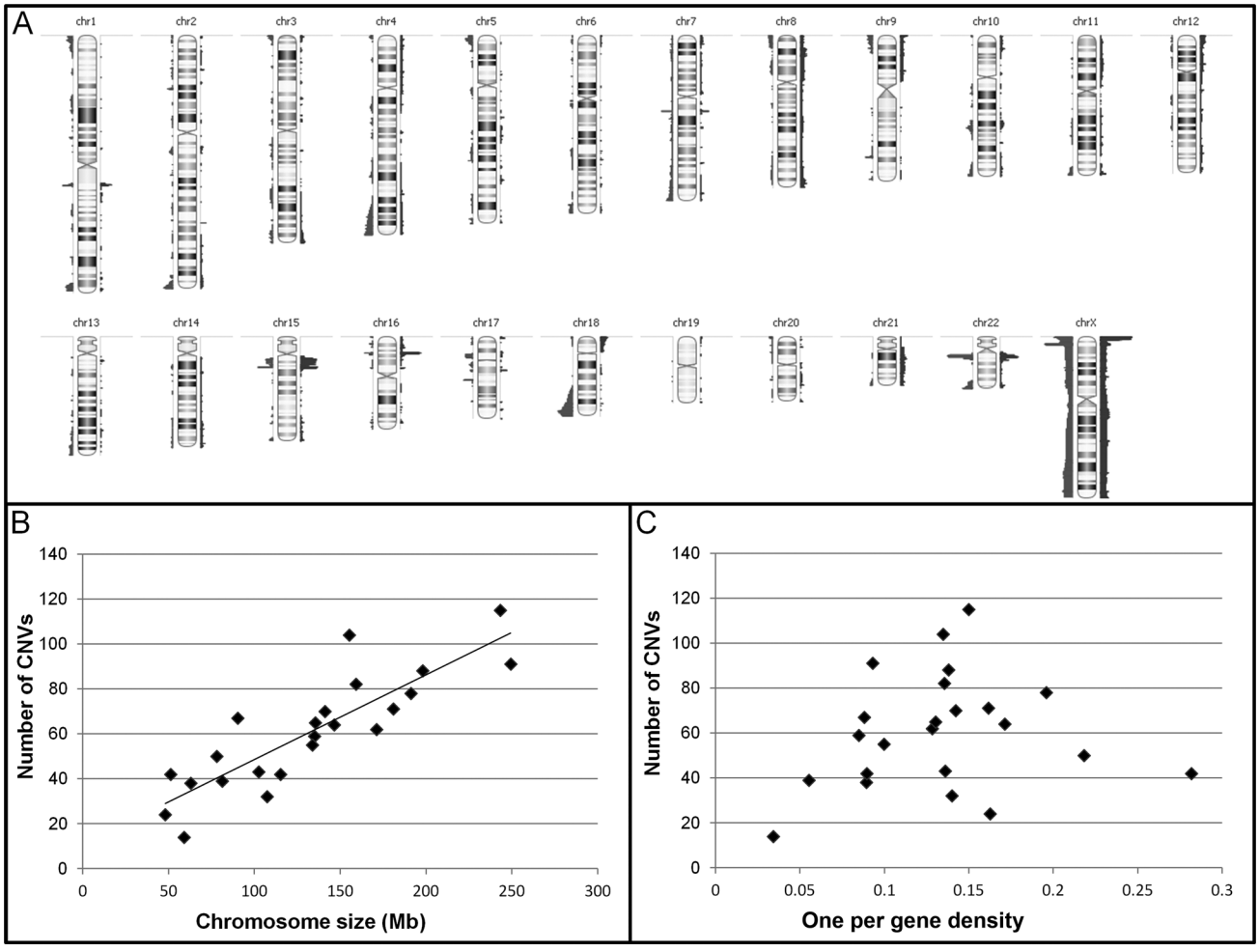

Figure 2. Distribution per chromosome of CNVs identified in 5,531 patients. **A:** Distribution of all CNVs across the genome (left represents losses; right represents gains). **B:** The number of CNVs per chromosome correlates with the chromosome size after exclusion of known LCR-mediated genomic disorders and CNVs in the pseudoautosomal region on the X chromosome ($r = 0.874$). **C:** Correlation between number of CNVs per chromosome (corrected for chromosome size) and inverse of gene density. There is no clear correlation between the gene density of a chromosome and the number of CNVs.

discarded [Franke et al., 2010]. All rare CNVs identified in this study were submitted to dbVAR (http://www.ncbi.nlm.nih.gov/dbvar, Study ID nstd85). Upon detection of a rare CNV, parental samples were requested to determine the inheritance pattern of the CNV.

Objective Classification of CNVs

GeCCO (Genomic Classification of CNVs Objectively), a free online available prediction tool, was used to aid distinguish ID-associated CNVs from nonpathogenic CNVs [Hehir-Kwa et al., 2010]. GeCCO gives a probability that a CNV belongs to the ID-associated CNV class based on 13 genomic features. A probability ≥0.5 is considered pathogenic, whereas a probability <0.5 is considered benign. Validation on a set of 1,203 CNVs showed an accuracy of 94% with a sensitivity of 88% and a specificity of 94%.

Phenotype Data

Clinical data were collected from all patients prior to SNP array analysis using a uniform clinical form and standardized using the Human Phenotype Ontology (HPO) [Robinson et al., 2008]. Each patient was scored according to a modified version of the 5-item De Vries score, which includes ID, growth abnormalities (prenatal and postnatal), facial dysmorphisms, and congenital anomalies allowing a score from zero to 10 (Supp. Table S1) [Baralle, 2001; de Vries et al., 2001; Feenstra et al., 2011]. Genotype–phenotype comparisons were performed for specific malformations that were divided into mild and severe (Supp. Table S2). MCA were defined as major organ malformations affecting the central nervous system, heart, urogenitalia, anus, skeleton, and/or orofacial clefting. The De Vries score was used as a measure of the general severity of the phenotype.

Enrichment Analysis for Haploinsufficient Genes

Enrichments of CNV gene sets for haploinsufficient genes were determined by comparing against a background of randomly generated CNVs controlling for gene number and adjacency. Sets of random CNVs were generated by randomly placing an equivalent number of CNVs within the genome and extending the boundaries until they contained the same number of genes as the original set. Enrichments were determined as the increase in haploinsufficient genes overlapped in the real data set as compared with the median

Table 1. De Novo Single-Gene CNVs

Gene name	Copy number	Pat ID	Phenotype	Other genetic aberrations	Protein function	Disease association	Reference
CRYL1	0	5,493	Mild ID, SS, HL	–	Glucose metabolism	–	–
ISPD	0	5,078	Walker–Warburg syndrome	–	Glycosylation of alpha-dystroglycan	Walker–Warburg syndrome	[Roscioli et al., 2012]
ANKH	0	4,500; 5,527	Sibs with ID, HL, joint pain, band keratopathy	–	Pyrophosphate regulation	Previous report same phenotype	–
CEP89	0	68	Severe ID, SS, ataxia, B, cataract, contractures, HL	–	Mitochondrial metabolism	–	[van Bon et al., 2013]
ASTN2	1	3,338	ID, E	–	Neuronal migration	Associated with B	–
CHL1	1	364	Severe ID, atrial septum defect, E, tetraplegia, cryptorchidism, D	23 Mb dn dup Xpterp22	Nervous system development	In 3p deletion syndrome	–
DLG2	1	1,339	Mild ID, A, E	5 Mb dn dup 15q11q13	Synapse formation	–	–
DPYD	1	1,452	Mild ID, A, O, D	–	Pyrimidine metabolism	Dihydropyrimidine dehydrogenase deficiency, miR-137 deletions same phenotype	[Willemsen et al., 2011]
EYS	1	2,622	–	–	Retinal formation	AR retinitis pigmentosa	–
SOGA2	1	3,620	–	430 kb inh del 16p13	?	–	–
NCKAP5	1	3,221	E, hypomyelinization	–	?	–	–
NRXN1	1	221	ID, A, tall stature	900 kb inh dup 12q24	Neuronal cell adhesion	Associated with ID	–
PIK3C3	1	3,746	Mild ID, agenesis left cerebellum	730 kb inh del 4q35	Endocytic trafficking, neurodegeneration	–	–
SMARCA2	1	2,424	ID, SS, D	–	Chromatin remodeling	Nicolaides–Baraitser syndrome	–
SNTG1	1	1,229	Mild ID, CP, HL, D	1.1 Mb dn del 8q11	Subcellular localization organization	–	[Ockeloen et al., 2010]
TCF4	1	388	Severe ID, D	–	Transcription regulation	Pitt–Hopkins syndrome	–
TRIO	1	1,748	Mild ID, B, D	–	Rho–GEF, axon guidance	Mutations in ID	–
ZFHX4	1	1,791	DD, macrocephaly, ventriculomegaly, hypermetropia, recurrent infections, D	–	Transcription regulation	8q21.1 deletion syndrome	–
MELK	3	4,386	Mild ID, CL, skeletal anomalies, D	–	Cell cycle regulation	–	–
MPP7	4[a]	1,798	Moderate ID, E, B, O, micropenis, D	150 kb inh hmz dup 7p15	Synapse formation	–	–

[a]Shown by fluorescence in situ hybridization not to be due to an insertion elsewhere in the genome.
A, autism; AR, autosomal recessive; B, behavioral problems; CL/P, cleft lip/palate; D, dysmorphisms; del, deletion; dn, de novo; dup, duplication; E, epilepsy; HL, hearing loss; hmz, homozygous; inh, inherited; O, obesity; SS, short stature.

obtained from 10,000 randomizations. The investigated haploinsufficient gene set included 299 known human haploinsufficient genes [Dang et al., 2008].

Statistical Analyses

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY). CNV sizes and gene content were described with medians and interquartile ranges (IQR). Survival curves were created to study CNV size. Other CNV characteristics were given as percentages of the total number of CNVs or patients. Phenotype data were described as percentages of the total number of patients for whom phenotype data were available. As the clinical significance of X chromosomal CNVs is gender specific and thereby different from that of autosomal CNVs, these were excluded for all further analyses. The Mann–Whitney *U* test was used to compare CNV sizes and gene content. Fisher's exact test was performed to study the relation between the genotype and the phenotype, as well as to compare CNV characteristics. Spearman's correlation analysis was used to study the association between the chromosome size and the number of CNVs per chromosomes, as well as the association between the CNV size, gene content, and CNV number with the severity of the phenotype. We repeated all analyses after exclusion of low copy repeat (LCR)-mediated genomic disorders. All *P* values were calculated two-sided and to control the false discovery rate at 0.05 the Benjamini–Hochberg method was used for the genotype–phenotype correlations [Benjamini and Hochberg, 1995].

Results

Yield of Microarray Analysis

DNA samples from 5,531 consecutive ID/MCA patients were analyzed with a 250K SNP array. In this cohort, we identified a total of 1,663 rare CNVs in 1,388 patients (25.1% of the cohort) (Fig. 1A and Supp. Table S3). The 1,663 CNVs consisted of 825 copy-number losses (49.6%) and 838 copy-number gains (50.4%) and ranged in size from 5.4 kb to 158.4 Mb with a median size of 1.0 Mb (IQR 0.4–2.7 Mb) (Fig. 1B). We identified 242 patients (17.4%) with more than one CNV (517 CNVs in total, range 2–5 per patient, Supp. Table S4).

Table 2. Cohort Description

Patient characteristics	*N* = 4,297
Age (years): median (IQR)	7 (3–17)
Gender	
Male	2,497 (58.1%)
Female	1,800 (41.9%)
Seen by clinical geneticist	2,827 (65.8%)
Phenotype data	
HPO features	
Total	34,433
Features per patient: median (IQR)	6 (2–11)
Frequencies	
Neurologic	
ID/DD	3,215 (74.8%)
Behavioral problems	1,350 (31.4%)
Epilepsy	605 (14.1%)
Hypotonia	610 (14.2%)
Microcephaly	495 (11.5%)
Macrocephaly	197 (4.6%)
CNS malformations	424 (9.9%)
Craniofacial	
Facial dysmorphisms	1,431 (33.3%)
Cleft lip/palate	168 (3.9%)
Hearing loss	236 (5.5%)
Organ malformations	
Cardiac malformations	359 (8.4%)
Urogenital malformations	483 (11.2%)
Growth	
Prenatal growth retardation	331 (7.7%)
Short stature	688 (16.0%)
Tall stature	108 (2.5%)
Combined criteria	
MCA	1,391 (32.4%)
De Vries score: median (IQR)	2 (1–4)

CNS, central nervous system.

The CNVs showed a nonrandom distribution across chromosomes (Fig. 2A). The CNVs occurred more often near the telomeric regions. In addition, specific regions such as the 1q21.1, 7q11.23, 15q13.11, 16p11.2, and 17q21.31 regions showed recurrent CNVs, which are known to be due to nonallelic homologous recombination between LCRs (Supp. Table S5). After exclusion of these known LCR-mediated genomic disorders ($N = 276$) and aberrations of the pseudoautosomal region on the X chromosome ($N = 25$), the number of CNVs per chromosome was positively correlated with the chromosome size ($P < 0.001$, $r = 0.874$; Spearman's correlation; Fig. 2B). There was no clear correlation between the gene density of a chromosome and the number of CNVs observed on the chromosome (Fig. 2C).

DNA of both parents could be analyzed for 63.6% of the patients, revealing 437 CNVs (40.7% of the CNVs for which parental DNA was available) occurred de novo, 638 CNVs were inherited, and for 588 CNVs, the inheritance pattern could not be determined. We investigated the relationship between the characteristics of autosomal CNVs and their inheritance pattern. De novo CNVs were significantly larger in size (median 2.6 Mb; IQR 1.0–7.3 Mb) than inherited CNVs (median 0.5 Mb; IQR 0.3–1.1 Mb) ($P < 0.001$; Mann–Whitney *U* test) (Fig. 1C). Losses were more frequently de novo (52.5%) than gains (28.7%) ($P < 0.001$, Fisher's exact test), also after the exclusion of known LCR-mediated genomic disorders ($P < 0.001$, Fisher's exact test, Supp. Table S6). The inherited CNVs were more often from maternal (56.5%) than paternal origin (43.5%) ($P = 0.002$, Binomial test). The classifier GeCCO (Hehir-Kwa et al., 2010) classified 510 (30.7%) of all CNVs as benign and 1,153 (69.3%) as pathogenic (Supp. Table S3 and Supp. Fig. S1). Comparison by inheritance pattern revealed that 17.6% of the de novo CNVs were classified as benign, whereas 42.6% of the inherited CNVs were predicted to be benign ($P < 0.001$, Fisher's exact test).

Gene Content

The total set of 1,663 CNVs contained a median of seven ENSEMBLE genes (IQR 2–27; Fig. 1D). De novo CNVs contained more genes (median 24; IQR 9–58) than inherited CNVs (median 3; IQR 1–8) ($P < 0.001$; Mann–Whitney *U* test) as expected due to their larger sizes. However, the gene density of de novo CNVs (median 8.0 genes/Mb; IQR 4.6–14.7) was also higher than inherited CNVs (median 6.6 genes/Mb; IQR 2.6–12.3) ($P < 0.001$; Mann–Whitney *U* test). We examined the gene content of de novo and inherited CNVs for the presence of known haploinsufficient genes [Dang et al., 2008]. Genes within de novo losses were enriched for known human haploinsufficient genes (+41.3%; $P < 0.001$) (Supp. Fig. S2). However, this enrichment was no longer present when known LCR-mediated genomic disorders were removed from the analysis, indicating that especially these LCR-mediated CNVs were enriched for known human haploinsufficient genes (Supp. Table S6). We observed no enrichment in haploinsufficient genes for inherited CNVs or de novo gain CNVs (Supp. Fig. S2).

De novo or homozygously inherited single-gene CNVs were identified in 20 families (four homozygous losses, 14 heterozygous losses, one heterozygous gain, and one homozygous gain) (Table 1). These include three deletions of genes previously associated with ID syndromes, namely *TCF4* (MIM #602272; http://www.omim.org) implicated in Pitt–Hopkins syndrome (MIM #610954) [Amiel et al., 2007; Zweier et al., 2007], *SMARCA2* (MIM #600014) implicated in Nicolaides–Baraitser (MIM #601358) [Van Houdt et al., 2012], and *ISPD* (MIM #614631) implicated in Walker–Warburg syndrome (MIM #614643) [Roscioli et al., 2012], in patients with phenotype features overlapping these syndromes. Two genes were previously indicated as candidate genes for ID/MCA, namely *ANKH* (MIM #605145) [Morava et al., 2011] and *ZFHX4* (MIM #606940) [Palomares et al., 2011].

Genotype–Phenotype Correlations

Detailed clinical information was available for 4,297 patients (77.7% of the entire cohort). These patients had a median age of 7 years (IQR 3–17 years), and 2,497 (58.1%) were male (Table 2). In total, we registered 34,433 HPO features (median 6 per patient, IQR 2–11). We summarized the most important features of all patients in Table 2 and Supp. Table S7. The patients had a median De Vries score of 2 (IQR 1–4) (Fig. 3A and Supp. Table S7).

The cohort was divided into three subsets to compare differences in phenotypes, (1) patients with de novo CNVs, (2) patients with only inherited CNVs, and (3) patients in whom no CNVs were detected (Fig. 4). Eight of the 10 phenotype categories tested were significantly more prevalent in patients with de novo CNVs as compared with patients without CNVs, namely ID/DD (corrected $P = 0.020$; Fisher's exact test), MCA ($P < 0.001$), facial dysmorphisms ($P < 0.001$), abnormal head circumference ($P = 0.005$), central nervous system anomalies ($P = 0.001$), heart anomalies ($P = 0.003$), urogenital anomalies ($P = 0.001$), and De Vries score ≥3 ($P < 0.001$; see also Fig. 3C). No significant differences were seen for stature and convulsions. Five of these phenotype categories were also significantly more frequent in patients with de novo CNVs in comparison to patients with only inherited CNVs. Similarly, a trend for increased urogenital and heart anomalies was observed ($P = 0.102$ and $P = 0.066$, respectively). Interestingly, patients with inherited CNVs

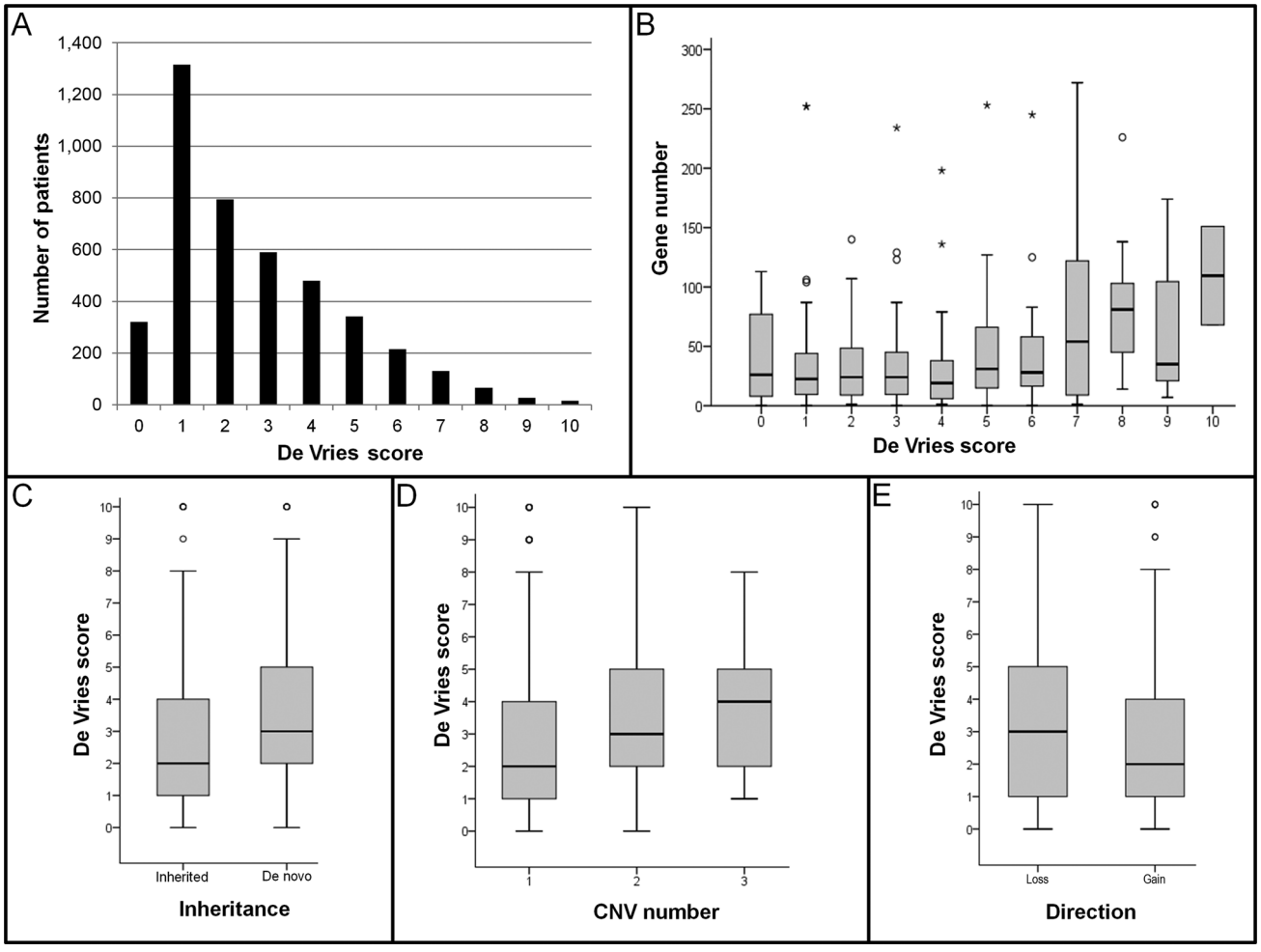

Figure 3. Clinical severity, defined by the De Vries score, and CNV characteristics. **A:** Distribution of the De Vries scores in the total study population (median 2; IQR 1–4). **B:** Comparison of the De Vries scores with number of affected genes in de novo CNVs. CNVs containing an increasing amount of genes are associated with a higher De Vries score ($P = 0.030$, $r = 0.134$; Spearman's correlation). See Supp. Figure S5 for the same comparison for all CNVs and inherited CNVs, and the comparisons with CNV size. **C:** De Vries scores in patients with inherited versus patients with de novo CNVs. Patients with de novo CNVs have a higher De Vries score (median 3; IQR 2–5) than patients with only inherited CNVs (median 2; IQR 1–4) ($P < 0.001$, Mann–Whitney U test). See also Supp. Figure S4A. **D:** De Vries scores in patients per number of CNVs identified. Patients with multiple CNVs have a higher De Vries score (median 3; IQR 2–5) than patients with a single CNV (median 2; IQR 1–4) ($P = 0.001$, Mann–Whitney U test, $P = 0.001$, $r = 0.106$; Spearman's correlation). See also Supp. Figure S4B. **E:** De Vries scores in patients with copy-number losses versus patients with copy-number gains. Patients with losses have a higher De Vries score (median 3; IQR 1–5) than patients with gains (median 2; IQR 1–4) ($P = 0.012$ Mann–Whitney U test). See also Supp. Figure S4C.

showed a higher De Vries score ($P = 0.010$) and significantly more facial dysmorphisms ($P < 0.001$) than patients without CNVs. A similar trend could also be observed for abnormal height ($P = 0.075$). Similar patterns for all phenotypes were observed after exclusion of known LCR-mediated genomic disorders (Supp. Fig. S3).

We then investigated whether there was a significant correlation between various CNV characteristics and the severity of the phenotype. The De Vries score was first compared with the direction of the CNV (gain or loss). We observed a higher De Vries score in patients with losses (median 3; IQR 1–5) compared with gains (median 2; IQR 1–4) ($P = 0.007$; Mann–Whitney U test; Fig. 3E and Supp. Fig. S4C). The CNV size, and especially the number of genes affected by the CNVs, showed a positive correlation with the severity of the patients phenotype ($P = 0.014$, $r = 0.077$; Spearman's correlation; Supp. Fig. S5A and B). This effect was mediated by de novo CNVs ($P = 0.030$, $r = 0.134$, Spearman's correlation; Fig. 3B), as no correlation was seen for the inherited CNVs ($P = 0.815$, $r = -0.011$; Spearman's correlation; Supp. Fig. S5C and D). In addition, we observed a higher De Vries score (median 3.5; IQR 2–5) in patients with multiple CNVs compared with patients with a single CNV (median 2; IQR 1–4) ($P = 0.001$; Mann–Whitney U test/$P = 0.001$, $r = 0.106$; Spearman's correlation; Fig. 3D and Supp. Fig. S4B).

Discussion

In this large, well-phenotyped cohort of 5,531 patients with ID/MCA, we identified rare CNVs in 25.1% of the individuals. Segregation analysis in the parents revealed that 40.7% of the tested CNVs occurred de novo. We showed that severe phenotypes, including abnormal head circumference and organ malformations, were enriched in patients with de novo CNVs, both in comparison to patients without CNVs as well as patients with inherited CNVs. Remarkably, patients with inherited CNVs had significantly more

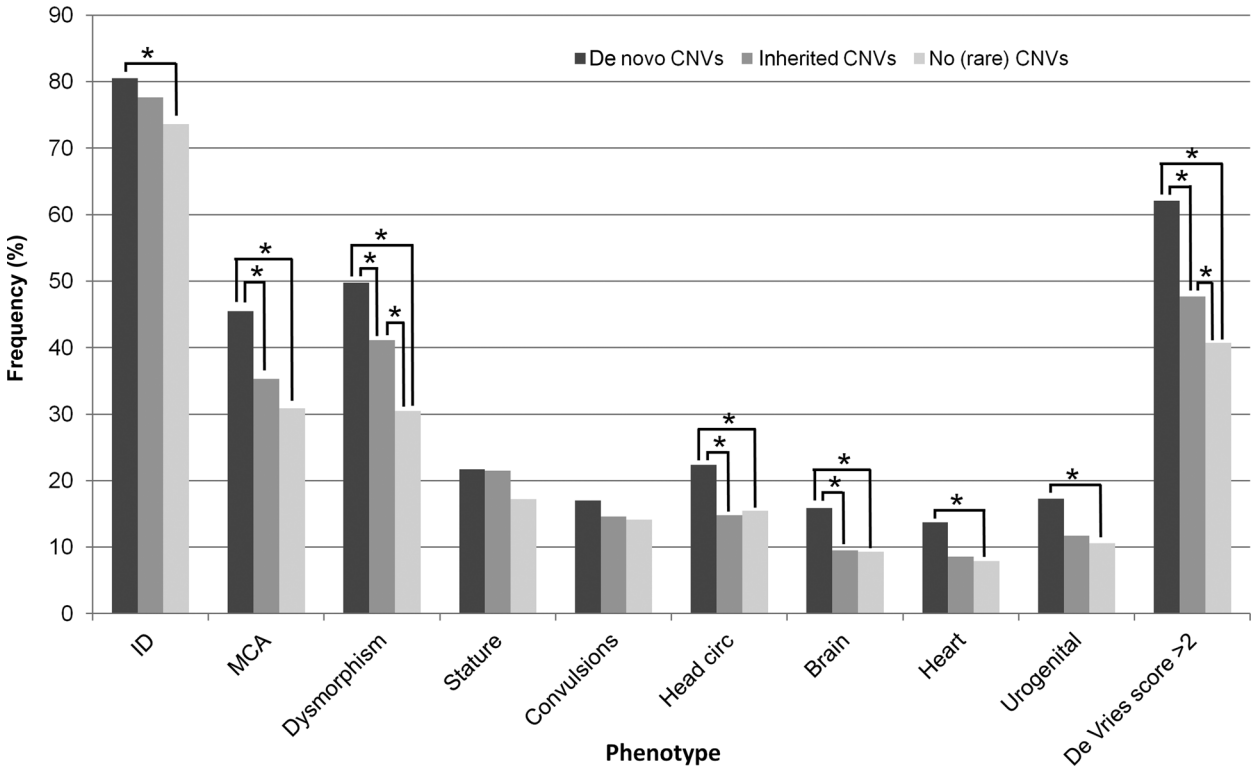

Figure 4. Genotype–phenotype correlations. Frequency of phenotype features in patients with de novo (dark gray), inherited (middle gray), and without CNVs (light gray). Frequencies were compared by Fisher's exact test. $^{*}P < 0.05$, corrected for multiple testing using the method by Benjamini and Hochberg [Benjamini and Hochberg, 1995]. ID, intellectual disability; MCA, multiple congenital anomalies; Head circ, head circumference.

facial dysmorphisms and a higher De Vries score compared with patients without CNVs. Abnormal height also tended to be more prevalent in patients with inherited CNVs, whereas abnormal head circumference, structural brain anomalies, heart anomalies, and urogenital anomalies occurred similarly in patients with inherited CNVs and patients without CNVs. This suggests that the milder clinical features can also be related to the presence of inherited CNVs. Furthermore, computational classification of the inherited CNVs using GeCCO, predicted 56.7% of the inherited CNVs as pathogenic. It has previously been reported that some recurrent LCR-mediated CNVs (e.g., 15q13.3 and 16p11.2) may cause a phenotype, despite their presence in a carrying parent [van Bon et al., 2009; Zufferey et al., 2012]. However, for non-LCR-mediated CNVs, this remains difficult to establish in clinical practice and therefore they are usually considered benign variants or variants of unknown significance [Buysse et al., 2009; Filges et al., 2012]. Our results also indicate that nonrecurrent inherited CNVs may be associated with a clinically significant phenotype.

Our result confirms that severe phenotypes are significantly influenced by the presence of de novo CNVs. It has been shown previously that congenital anomalies, particularly heart defects, were significantly more frequent in patients with clinically significant CNVs as compared with patients with a normal microarray result, although this was based on a study of only 342 patients [Shoukier et al., 2013]. Furthermore, we show that de novo CNVs, especially de novo losses, are significantly enriched for haploinsufficient genes and the classifier GeCCO predicts de novo CNVs more often as pathogenic than inherited CNVs.

The De Vries score was used to ascertain the severity of the phenotype (Supp. Table S1) [Baralle, 2001; de Vries et al., 2001; Feenstra et al., 2011]. The patients in this study had a median De Vries score of 2 (IQR 1–4) (Fig. 3A and Supp. Table S7), indicating that they were milder affected than those tested by microarray analysis in previous studies [de Vries et al., 2005; Fan et al., 2007; Hoyer et al., 2007; Koolen et al., 2009; Newman et al., 2007; Rauch et al., 2006; Schoumans et al., 2005]. Using the De Vries score as an overall measure for the severity of the phenotype, we confirmed that losses were more likely to result in severe phenotypes than gains and that the CNV size was correlated with the severity of the phenotype [Brewer et al., 1998]. In addition, we show that the number of CNVs identified in a patient was correlated with the severity of the phenotype, indicating a combinatorial effect of the additional CNVs. A similar effect was previously observed for a number of genomic disorders, wherein the presence of a second-site variant was associated with a more severe phenotype [Girirajan et al., 2010; Girirajan et al., 2012].

Once a likely pathogenic CNV is detected, the genes contributing to the various phenotypes can be determined. This gene identification process is simplified when the CNV is disrupting only a single gene. In our cohort of 5,531 patients with ID/MCA, we identified 20 de novo single-gene CNVs of which three contained known ID genes (*TCF4*, *SMARCA2*, and *ISPD*) and two previously postulated candidate genes (*ANKH* and *ZFHX4*). The homozygous deletion of *ANKH* is the first confirmation of this gene in autosomal-recessive ID, in combination with deafness, joint abnormalities, and hypophosphatemia. Previously, a homozygous missense mutation in this gene was described in a consanguineous family with a similar phenotype, and also *ank* mouse mutants show joint abnormalities, abnormal bone metabolism, and hearing loss [Morava et al., 2011]. The deletion that disrupted the last seven exons of *ZFHX4* is situated within the critical region of the 8q21.11 microdeletion syndrome, which has been associated with ID and a characteristic facial phenotype [Palomares et al., 2011]. Clinical comparison of this patient

with the previously described patients showed striking overlap in clinical features (Table 1), including typical facial dysmorphisms (high forehead, downslanting palpebral fissures, ptosis, and prominent low-set ears), underscoring that *ZFHX4* is the underlying gene for this microdeletion syndrome.

The other 15 single-gene CNVs contain six other potential candidate genes for ID based on their role in neuronal development and function. *DLG2* (MIM #603583), also known as *PSD93*, is deleted in a patient with ID, an autism spectrum disorder and epilepsy. DLG2 is a member of the membrane-associated guanylate kinase (MAGUK) protein family, which plays an important role in synapse formation and trafficking of glutamate receptors, especially N-methyl-D-aspartate (NMDA) receptors [Oliva et al., 2012]. Interestingly, *DLG3* (MIM #300189) [Tarpey et al., 2004] and two of the NMDA receptors, *GRIN2A* (MIM #138253) and *GRIN2B* (MIM #138252) [Endele et al., 2010], have already been implicated in ID. A double duplication in *MPP7* (MIM #610973), identified in a patient with mild ID, an autism spectrum disorder and epilepsy, would disrupt the open reading frame of *MPP7*, another member of the MAGUK protein family, shown to interact with DLG1 and LIN7 [Bohl et al., 2007]. A homozygous loss of *CEP89* was found in a patient with a complex phenotype including mitochondrial dysfunction. Knockdown of this gene in *Drosophila* supported the role of *CEP89* in mitochondrial metabolism and neuronal functioning [van Bon et al., 2013]. The *TRIO* gene (MIM #601893), partially included in the deletion in a patient with DD and facial dysmorphisms, is a Rho–guanine exchange factor that regulates axon guidance during nervous system [van Rijssel and van Buul, 2012]. Two de novo missense mutations in *TRIO* were previously identified in patients with ID. However, both patients also carried a mutation in another known ID gene (*TCF4* and *PDHA1*, respectively) [de Ligt et al., 2012]. A de novo deletion of *ASTN2* (MIM #612856) was found in a patient with ID and seizures. This gene has been associated with attention deficit hyperactivity disorder, autism spectrum disorders, and schizophrenia in various studies [Glessner et al., 2009; Lesch et al., 2008; Vrijenhoek et al., 2008] and functions together with ASTN1 (MIM #600904) in glial-guided neuronal migration [Wilson et al., 2010]. *PIK3C3* (MIM #602609), deleted in a patient with ID and cerebellum agenesis, is involved in endocytic trafficking and disruption in mice leads to neuronal degeneration and gliosis [Wang et al., 2011].

Conclusion

We show in a unique cohort of 5,531 phenotypically well-characterized patients that not only de novo but also inherited CNVs can be associated with a clinically relevant phenotype. Description of additional cohorts with detailed phenotype information of both patients and their parents will further elucidate the role of inherited CNVs in disease. In addition, we show that multiple CNVs within a single patient were associated with a more severe phenotype pointing to a combinatorial effect of the additional CNVs and we propose several novel candidate ID genes.

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