Intragenic and structural variation in the SMN locus and clinical variability in spinal muscular atrophy

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Clinical severity and treatment response vary significantly between patients with spinal muscular atrophy. The approval of therapies and the emergence of neonatal screening programmes urgently require a more detailed understanding of the genetic variants that underlie this clinical heterogeneity. We systematically investigated genetic variation other than SMN2 copy number in the SMN locus. Data were collected through our single-centre, population-based study on spinal muscular atrophy in the Netherlands, including 286 children and adults with spinal muscular atrophy Types 1–4, including 56 patients from 25 families with multiple siblings with spinal muscular atrophy. We combined multiplex ligation-dependent probe amplification, Sanger sequencing, multiplexed targeted resequencing and digital droplet polymerase chain reaction to determine sequence and expression variation in the SMN locus.

SMN1, SMN2 and NAIP gene copy number were determined by multiplex ligation-dependent probe amplification. SMN2 gene variant analysis was performed using Sanger sequencing and RNA expression analysis of SMN by droplet digital polymerase chain reaction. We identified SMN1–SMN2 hybrid genes in 10% of spinal muscular atrophy patients, including partial gene deletions, duplications or conversions within SMN1 and SMN2 genes. This indicates that SMN2 copies can vary structurally between patients, implicating an important novel level of genetic variability in spinal muscular atrophy. Sequence analysis revealed six exonic and four intronic SMN2 variants, which were associated with disease severity in individual cases. There are no indications that NAIP1 gene copy number or sequence variants add value in addition to SMN2 copies in predicting the clinical phenotype in individual patients with spinal muscular atrophy. Importantly, 95% of spinal muscular atrophy siblings in our study had equal SMN2 copy numbers and structural changes (e.g. hybrid genes), but 60% presented with a different spinal muscular atrophy type, indicating the likely presence of further inter- and intragenic variabilities inside as well as outside the SMN locus. SMN2 gene copies can be structurally different, resulting in inter- and intra-individual differences in the composition of SMN1 and SMN2 gene copies. This adds another layer of complexity to the genetics that underlie spinal muscular atrophy and should be considered in current genetic diagnosis and counselling practices.

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Introduction

Proximal hereditary spinal muscular atrophy (SMA) is an important genetic cause of mortality in infants and progressive motor impairment in children and adults (Mercuri et al., 2012; Wadman et al., 2017). It is caused by deficiency of the survival motor neuron (SMN) protein due to the homozygous loss of function of the SMN1 gene (HGNC:11117; OMIM600354). The second SMN gene, SMN2 (HGNC:11118; OMIM601627), differs only at five nucleotide positions from SMN1. One nucleotide substitution in Exon 7 critically influences mRNA splicing, leading to the absence of Exon 7 in the large majority of SMN2 mRNA transcripts (delta7 SMN2) and the production of limited quantities of full-length SMN protein (Lefebvre et al., 1995).

SMA has a striking range of severity with onset from infancy to adulthood. This is reflected in the clinical classification system that distinguishes Types 1–4 (Mercuri et al., 2012). More SMN2 copies are associated with relatively higher SMN protein levels in tissues from patients with SMA and with milder phenotypes. However, variation is only partially explained by copy number variation in SMN2 (Lefebvre et al., 1995). For example, severity in patients with three SMN2 copies ranges from infantile onset with limited motor development (Type 1) to childhood onset with the ability to walk (Type 3). SMA severity-modifying genes outside the SMN locus, including plasin 3 (PLS3) and neurocalcin delta (NCALD), which, when overexpressed, may substitute specific cellular SMN functions, have been identified in specific families but are unlikely to explain clinical variation at the population level (Oprea et al., 2008; Hosseinibarkooie et al., 2016; Riessland et al., 2017; Wadman et al., 2020).

The architecture of the human SMN locus on chromosome 5q is highly complex due to multiple duplications and inversions and has, therefore, not yet been completely elucidated (Burghes, 1997; Wirth, 2000; Rochette et al., 2001; Arkbald et al., 2006; Lunn and Wang, 2008; Thauvin-Robinet et al., 2012). Rare intragenic variants in SMN2 have been described (Prior et al., 2009; Bernal et al., 2010; Wu et al., 2017; Calucho et al., 2018; Ruhno et al., 2019) that modify disease severity, and it has been suggested that variation within the SMN2 locus, such as deletions of the adjacent NAIP1, modifies severity (Burlet et al., 1996; Wathayati et al., 2009; Amara et al., 2012; Ruhno et al., 2019; Vorster et al., 2020). Variation in the sequence of SMN2 and the SMN locus requires further study in large and well-defined patient cohorts.

The relevance of elucidating genetic variability in the SMN locus has further increased with the approval of the first SMN2 splicing modulating therapy and the expectation that more such therapies will become available soon. First experiences with the SMN2-specific antisense oligonucleotide therapy, nusinersen, suggest that not all patients respond equally well to treatment. This could...
partially be explained by currently unidentified genetic variation (Harahap et al., 2015; Wu et al., 2017).

To further improve our understanding of the correlation between genetic and clinical variation, we performed a detailed analysis of the structure, sequence and expression of the SMN locus in 286 SMA patients (Wadman et al., 2017; Wadman et al., 2018). We identified an additional level of genetic heterogeneity of the SMN locus and its association with the clinical phenotype.

Materials and methods

We enrolled patients with SMA Types 1–4 between September 2010 and August 2018 from our single-centre prevalence cohort study in the Netherlands.

The Medical Ethical Committee of the University Medical Center Utrecht approved the study protocol (09-307/NL29692.041.09). This study was registered at the Dutch registry for clinical studies and trials (http://www.ccmo-online.nl). All patients gave written informed consent. Informed consent was obtained from all participants and/or each subject and additionally from their parents if children were younger than 18 years.

The reporting of this study conforms to the Strengthening the Reporting of Observational Studies in Epidemiology statement (von Elm et al., 2007).

Patients

Details of the population-based prevalence cohort study on SMA Types 1–4 in the Netherlands have been described previously (Wadman et al., 2017; Wadman et al., 2018). Inclusion criteria were a clinical diagnosis of SMA Types 1–4 and genetic confirmation of a homozygous deletion of SMN1 or heterozygous deletion with a point mutation on the other allele of SMN1 (HGNC:11117; OMIM600354). There was no age restriction for inclusion. All included patients visited the outpatient clinic for (paediatric) neurology at our centre and were evaluated by one of the medical doctors (R.I.W., C.A.W., M.S.). We interviewed all patients and/or their parents and examined muscle strength using the Hammersmith functional motor scale expanded (Wadman et al., 2017). We used the SMA classification system based on age at onset and the best of two achieved milestones (independent sitting and walking) (Table 1) (Munsat and Davies, 1992; Zerres and Rudnik-Schoneborn, 1995; Zerres et al., 1997; Dubowitz, 1999; Rudnik-Schoneborn et al., 2009; Mercuri et al., 2012; Wadman et al., 2017).

Concordant and discordant patients were defined to analyse the predictive value of SMN2 copy numbers for the clinical phenotype. We used the same model as described previously to define the expected copy number (Ruhno et al., 2019): SMA Type 1 has two copies of SMN2, Type 2 has three SMN2 gene copies, and Type 3 has four SMN2 gene copies. With this model, we selected discordant patients with a milder or more severe phenotype in relation to their SMN2 copy number.

Genetic analysis

Copy number analysis

SMN1, SMN2 and NAIP copy number status was performed at Medical Research Council Holland using SALSA multiplex ligation-dependent probe amplification (MLPA) kit P021 (version B1). All MLPA reactions were carried out according to the manufacturer’s protocol (www.mlpa.com; www.mrcholland.com). A reference sample with two copies of SMN1 and two copies of SMN2 was used in every reaction. The MLPA products were analysed using an ABI Prisma 310 genetic analyser (Applied Biosystems), with LIZ 500 as the internal size standard. Data analysis and interpretation were performed using Coffyalyser.Net software (www.mrcholland.com). Repeated experiments showed good reproducibility of data. Seventy samples were analysed four times, 60 samples were analysed three times and 23 samples were analysed twice in different certified laboratories (i.e. Medical Research Council Holland, Department of Medical Genetics UMC Utrecht, Netherlands, and Department of Medical Genetics UMC Groningen, Netherlands) with various sets of MLPA probe mixes (P021 versions A1 and A2; P060 versions B1 and B2). With regard to inter-experimental differences, a different SMN2 copy number was found in only eight samples out of 286 (3%), all with a borderline of three or four SMN2 copies, a third analysis always confirming one of the previous results.

We used MLPA data to determine SMN2 copy number and other structural variants.

SMN2 copy number was determined using dosage analysis of Exon 7. The recently developed P021 MLPA probe set allows for a detailed interrogation of the structural composition of SMN1 and SMN2 genes (Fig. 1) (Vijzelaar et al., 2019).

A hybrid SMN1–SMN2 gene was suspected in case of a discrepant copy number of Exons 7 and 8. A single hybrid gene consists of one persistent SMN1 Exon 8 copy and a corresponding, inverse downgrade of the copy number of SMN2 Exon 8. A double hybrid consists of two SMN1 Exon 8 copies and a two copies downgrade of SMN2 Exon 8 compared to SMN2 Exon 7. The presence of these hybrid SMN1–SMN2 genes was confirmed with Sanger sequencing. An extra Exon 8 was defined as an increased number of copies of SMN2 Exon 8 compared to SMN2 Exon 7 copy number.

Dosage analysis of SMN2 Exons 1–6 was also performed. A partial SMN2 deletion or duplication was suspected in case of a higher or lower copy number (dosage ≥1 increase compared to the other copies) compared to
the number of Exon 7 copies. Distinction between Exons 1–6 SMN1 or SMN2 was not possible based on homologous region of the two genes.

If no DNA was available for the MLPA experiment, confirmation of SMN1 deletion and SMN2 copy number was retrieved from a previously performed MLPA for diagnosis ($n = 13$).

NAIP1 copy number was detected using the NAIP Exon 5 sequence, as this exon is absent in NAIP2. The copy number was analysed by comparing the signal with the SMN dosage.

### Mutational analysis

SMN2 was analysed by Sanger sequencing of all eight exons and flanking intronic regions as described previously (Koppers et al., 2013). Primers for polymerase chain reaction amplification were designed using ENST00000380743 (SMN2) and ENST00000517649; ENST00000523981 (NAIP) (Ensemble GRCh37) (Supplementary Table 1 and 2), and optimal annealing temperature for each primer set was determined by a temperature gradient polymerase chain reaction. Each identified mutation was confirmed by an independent polymerase chain reaction and sequencing reaction on genomic DNA.

NAIP mutations were determined using multiplexed targeted resequencing, carried out on a MiSeq high-throughput next-generation sequencing platform (Illumina). We used DesignStudio (Illumina) to create a Truseq Custom Amplicon project applying the Standard Truseq Custom Amplicon Library preparation protocol (amplicon library available on request). The amplicons targeting coding, non-coding, and 5' and 3'-untranslated regions covered 96% of the regions of interest with good quality (quality score $>30$). Bar-coded paired-end sequencing libraries with 2 x 250 base pair read length per amplicon were created using prepared Truseq Custom Amplicon Kit (Illumina). Sequencing reads were mapped to the human genome reference build GRCh37 using Burrows Wheeler Aligner (BWA 6.1). Base calling accuracy, measured by the Phred quality score (Q score), was presumed to be ‘good’ from a score of 30. Subsequent depth of coverage, quality filters, variant calling and variant annotation were performed using SAMtools v0.1.19, GATKv3.2 and the 1000 Genomes project. All variants thus identified were confirmed using Sanger sequencing. The impact of the mutation on the structure and function of the protein was predicted by in silico analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/)

### Table 1 SMA classification

| SMA type and subtypes | Age at onset | Highest achieved motor milestones |
|-----------------------|--------------|----------------------------------|
| 1                     | 0–6 months   | Never acquires ability to sit unsupported |
| 1a                    | Prenatal/neonatal | Symptoms in prenatal and/or neonatal (first month) period, no head control |
| 1b                    | 1–3 months   | No head control and no ability to roll over |
| 1c                    | 3–6 months   | Will usually acquire additional motor skills, such as head control or rolling from supine to prone, or at least to one side at any stage in life. Patients with SMA Type 1c are reported to survive into adulthood with or without respiratory support |
| 2                     | 6–18 months  | Able to sit unsupported, not able to walk unsupported |
| 2a                    |              | Unsupervised sitting but not able to stand or walk with help |
| 2b                    |              | In addition to unsupported sitting also able to stand or walk with help, but not unassisted |
| 3                     | >18 months   | Able to walk unsupported |
| 3a                    | 18–36 months |                                     |
| 3b                    | >36 months   |                                     |
| 4                     | During adulthood, i.e. ≥18 years | Able to walk unsupported |

### Figure 1 Representation SMN alleles including hybrid SMN1–SMN2 genes.

- **A** Non-deleted alleles
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8
  - $\text{SMN2}$: exon 1-6, exon 7, exon 8

- **B** SMN1-deleted alleles
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8

- **C** Single hybrid allele
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8

- **D** Double hybrid allele
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8

- **E** Extra SMN2 exon 8
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8

- **F** Deletion $\text{SMN2}$ exon 8
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8

- **G** Deletion $\text{SMN2}$ exon 1-6
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8

- **H** Deletion $\text{SMN2}$ exon 7-8
  - $\text{SMN1}$: exon 1-6, exon 7, exon 8

**Presumed good alleles**

| SMN1     | SMN2     |
|----------|----------|
| exon 1-6 | exon 1-6 |
| exon 7   | exon 7   |
| exon 8   | exon 8   |
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variation correlated to age. Levels of each TBP showed interpolate variability and experiments (Vandesompele SMN (2016)). The assays were carried out using QX200™ Droplet Digital PCR System (Bio-Rad). In brief, 22-μl reactions contained 1 μl of cDNA, 1 μl of 20× assay mix (TBP/HRPT1, HEX labelled), 227 nM SMN probe (FAM), 818 nM of forward and reverse SMN primer, 11 μl of 2× droplet digital PCR Supermix for probes (no dUTP) and 6.95 μl of RNase/DNase free water. We mixed the reaction mix with droplet generation oil (#186-4110; Bio-Rad, Hercules, CA, USA) and partitioned its droplets in an automated droplet generator (Bio-Rad). Polymerase chain reaction amplification for SMN1, SMN2 and SMN2Δ7 in combination with TBP and HRPT1 reference genes was performed using a Bio-Rad T100 thermal cycler. After amplification, we analysed the droplets in a QX200 droplet reader as per the manufacturer’s protocol. mRNA concentrations were calculated as copies per nanogram of cDNA. Reference probes were used to check the stability of probe measures in each plate. We decided to use both measures to calculate the mean levels of SMN, although TBP showed interpolate variability and HRPT1 showed variation correlated to age. Levels of each SMN product (SMN1, SMN2, SMN2 delta7) were analysed using the geometric mean of the SMN levels of two separate experiments (Vandesompele et al., 2002).

**statistical analysis**

Normality was tested with the Kolmorogov–Smirnov and Shapiro–Wilk tests. Multivariate analyses were checked and corrected for co-linearity. Univariate and multivariate tests, including dichotomous data, were performed using (multivariate) logistic regression. Comparison of data between SMA types, (discordant) pairs and/or SMN2 copy number and variants was performed using Kruskal–Wallis (KW), Jonckheere Terpstra (JT) or Mann–Whitney (MW) U-test (continuous data) or Chi-square/Fisher’s exact analysis (dichotomous data). We used IBM SPSS v23 for all statistical analyses.

**data availability statement**

Anonymized data that support the findings of this study are available from the corresponding author upon reasonable request.

**results**

We enrolled 286 patients with a genetically confirmed diagnosis of SMA and 53 parents (24 trios: both parents and child and 5 pairs: single parent and child). A total of 56 patients in our cohort were analysed as part of 25 families, which either included one or more siblings or one or more second-degree relatives. SMN1 and SMN2 copy numbers were determined in all patients and in all parents whose DNA was available (Table 2).

**SMN1 copy number status and gene variations**

Two hundred eighty-four patients (99%) had a homozygous deletion of SMN1 Exon 7. Two patients had a heterozygous deletion of SMN1 with a small mutation of SMN1 on the other allele (Fig. 2). One of these patients, with SMA Type 1c, had a heterozygous deletion of SMN1 on one allele and an 11-nucleotide duplication in Exon 6 (c.770-780dup p. Gly261Leufs*8) leading to a frame shift mutation on the other allele (Parsons et al., 1996; Parsons et al., 1998; Martin et al., 2002; Clermont et al., 2004; Alias et al., 2009). The other patient, with SMA Type 3a, had a heterozygous deletion of SMN1 and a point mutation in Exon 4 (c.542A>G; p.Asp181Gly) in the other allele. Using in silico mRNA analysis, the c.542A>G mutation was predicted to create a new splice-donor site within Exon 4 of SMN1 leading to an truncated transcript, introducing a premature stop codon (Wadman et al., 2017). Three of 53 (6%) parents were carriers of two SMN1 copies. After confirmation of parental status, this suggests the presence of two SMN1 copies on one allele and a deletion of SMN1 on the other allele or a de novo SMN1 deletion (Wirth, 2000). One parent of a patient with severe SMA Type 1a had zero copies of SMN2.

**SMN2 copy number status**

SMN2 copy numbers varied from one to five gene copies. Copy number prevalence in the patient cohort was 1%,
11%, 58%, 29% and 1% for 1–5 copies, respectively (Table 2). In 201 patients (70%), the SMN2 copy number corresponded with the expected clinical phenotype, i.e. 1–4 copies with SMA Types 1a, 1b, 2 and 3 or 4, respectively. SMN2 copy number correlated with SMA type ($X^2 P < 0.01$), age at onset (Spearman's rho 0.7, $P < 0.01$) and NAIP1 copy number ($X^2 P < 0.01$).

### Sequence variation in SMN2

We used Sanger sequencing to determine variation in SMN2 in 252 patients. Sequencing revealed six exonic and four intronic SMN2 variants (Fig. 2). *In silico* analysis of these variants suggested effects ranging from benign to likely damaging (Supplementary Table 3). Variants in Intron 1 (c.1–14C>T; c.81+45C>T) resulted in an altered SMN2 copy composition with a lower copy number of exons (1–6 compared to Exons 7–8), correlating with a more severe phenotype. The two variants in Exon 7 were associated with a more severe (c.838_840del) or a benign (c.859G>C) clinical phenotype, in comparison to what was expected based upon SMN2 copy number. There was no clear association between the other SMN2 variants and SMA phenotype.

### NAIPI1 gene copy number and mutation analysis

NAIPI1 copy number varied between zero and four copies (Table 2). NAIPI1 copy number correlated with SMA type and SMN2 copy number ($X^2 P < 0.05$). In addition, compared to SMN2 copy number, the NAIPI copy number had no additional value in predicting the SMA phenotype. NAIPI sequencing revealed two mutations [c.134A>G(H45Y); c.3503C>T[R1168K/R1330K]] in two unrelated patients presenting with different degrees of severity.

### Family analysis of SMA type and SMN2 copy number

Next, we investigated the relationship between type of SMA and SMN2 copy number in related patients. We included 25 different families, including 56 siblings and first-degree relatives (Fig. 3). Fifty-three patients (95%) shared the same number of SMN2 gene copies, but clinical phenotypes were discordant in 34 patients (60%) from 14 families (e.g. siblings with SMA Types 2a and 2b or siblings with SMA Types 2b or 3a).

| Gender (F:M) | Total SMA (n = 286) | SMA Type 1 (n = 59) | SMA Type 2 (n = 120) | SMA Type 3 (n = 98) | SMA Type 4 (n = 9) | Parents (n = 53) |
|-------------|---------------------|---------------------|---------------------|---------------------|---------------------|------------------|
| Total       | 151:135             | 28:32               | 73:47               | 46:51               | 4:5                 | 29:24            |
| Median age in years at inclusion (range) | 14.9 (0.2–78) | 1.3 (0.2–62) | 13.3 (0.4–67) | 32.7 (27–77.5) | 47.4 (36–70) | NA               |
| Median age in years at onset (range) | 1 (0–43) | 0.3 (0–1.5) | 0.8 (0.3–8.8) | 2.2 (1–17.5) | 31 (21–43) | NA               |

**SMN1 copy number, n (%)**
- 0: 284 (99.5)
- 1: 2 (0.5)
- 2: 0 (0)

**SMN2 copy number, n (%)**
- 0: 284 (99.5)
- 1: 2 (0.5)
- 2: 0 (0)
- 3: 165 (57)
- 4: 84 (30)
- 5: 4 (1)

**Hybrid SMN1–SMN2, n (%)**
- None: 258 (90)
- Single: 25 (9)
- Double: 3 (1)

**Partial duplication or deletion SMN2, n (%)**
- None: 276 (96)
- Deletion Exons 1–6: 1 (0.5)
- Deletion Exons 7–8: 5 (2)
- Extra SMN2 Exon 8: 1 (0.5)
- Deletion SMN2 Exon 8: 3 (1)

**NAIP1 copy number, n (%)**
- 0: 25 (9)
- 1: 161 (59)
- 2: 75 (27.5)
- 3: 10 (4)
- 4: 1 (0.5)

F = female; M = male; NA = not applicable; NAIP = NLR family apoptosis inhibitor protein.

1Including one patient with the deletion of SMN1 on one allele and frame shift mutation in Exon 6 (c.770-780dup; G261Lfs*8) in the other allele.

2Including one patient with the deletion of SMN1 on one allele and a point mutation in Exon 4 (c.542A>G; D181G).
Partial deletions and conversions of SMN2

We used MLPA data and Sanger sequencing ($n = 3$) to investigate the presence of partial gene deletions and conversions (see Materials and Methods section and Fig. 1). We found a single hybrid gene copy of SMN1–SMN2 in 25 patients and a double hybrid gene in 3 patients, as confirmed by Sanger sequencing (Fig. 3 and Table 2). We could confirm paternal or maternal inheritance of hybrid gene copies in 10 cases, but this could not be determined in the other patients because insufficient DNA was available from the parents. Two patients carried double hybrid gene copies with one hybrid gene copy inherited from each parent. Moreover, we identified structural abnormalities other than hybrid genes. One patient with SMA Type 1c had a deletion of Exons 1–6 in two SMN2 gene copies and an additional two SMN2 copies with Exons 1–8, probably because of a mutation in the promoter region (c.1 = 14C>T) and Intron 1 (c.81 + 45C>T) in two copies. Five patients (carrying 2–4 SMN2 copies) had a deletion of Exons 7–8 in one of their SMN2 copies. We also detected this partial deletion of SMN (i.e. Exons 7–8) in nine parents (17%) and two controls (5%). Both parents of a patient with SMA Type 1a (harbouring one copy of SMN2) carried only one SMN1 copy with one functional SMN2 copy (Exons 1–8). Their other SMN2 copies contained only SMN Exons 1–6.

**Figure 2 Genetic variation in SMN1 and SMN2.** (A) SMN locus and base pair differences between SMN1 and SMN2. The exact location of the SMN and NAIP genes in relation to each other is still unclear. (B) Representation of SMN1 and SMN2. Mutations are shown for SMN1 (upper notations) and SMN2 (lower notations). Mutations shown in red are novel variants reported in this article. Numbering refers to standard. Exon and intron sizes are not to scale (for a full list of references to previously published variants, see Supplementary Table 4).

**Figure 3 Family trees of dis- and concordant families.** Pedigree chart of 25 families with 2 or more children affected with SMA who were included in this study. Colours of the pedigrees reflect SMA Types 1c–4. The numbers inside the pedigree reflect SMN2 copy numbers. ‘U’ indicates that the SMN2 copy number status is unknown. SMN2 copy number was the same in 53 patients (95%), but clinical phenotypes were discordant in 34 patients (60%).

**SMN expression analysis**

We next investigated the effect of genetic variation (including the presence of partial deletions and hybrid
genes) on SMN mRNA expression in a cohort of 109 patients (Fig. 4). SMN1 expression was completely absent in all patients with a homozygous deletion of SMN1 copy number status. Mean levels of SMN1 expression differed between carriers and controls (MW \( P < 0.01 \)) (Fig. 4A). Full-length SMN2 and delta7 SMN2 expression levels were higher in patients compared to controls or carriers (KW \( P < 0.01 \)). Age correlated with SMN2 full-length levels (Spearman’s rho −0.26, \( P < 0.01 \)), but not delta7 SMN2 (Spearman’s rho −0.18, \( P = 0.06 \)). There was no correlation between SMN expression levels and SMA type (KW SMN2 FL KW \( P = 0.9 \); delta7 SMN2 KW \( P = 0.7 \)) but levels of full-length SMN2 and delta7 SMN2 differed between patients with varying SMN2 copy numbers (JT SMN2 FL \( P < 0.05 \); delta7 SMN2 \( P < 0.01 \)) (Fig. 4B). Full-length SMN2 expression was higher in a double hybrid gene background than in a single hybrid gene background in 3 versus 33 patients with SMA, respectively (MW \( P = 0.03 \)) (Fig. 4C). SMN2 expression levels in two patients with the c.859G>C mutation were not significantly different from those in patients without this variant (MW \( P = 0.2 \)) (Fig. 4D).

**SMN2 variation in relation to clinical phenotype and disease course**

Two hundred one patients (70%) had an SMN2 copy number that corresponded with the expected clinical phenotype. One copy of SMN2 was associated with neonatal onset SMA Type 1a (\( n = 3 \)) and two SMN2 copies with SMA Type 1b if c.859G>C was absent (95%). On a two or three SMN2 copy background, neither the presence of a hybrid SMN1–SMN2 gene nor the NAIP copy number was predictive or correlated with a milder (1c) or more severe (1b) than expected phenotype. At the milder end of the SMA clinical spectrum, four or five copies of SMN2 were almost always associated with SMA Types 3 or 4 (87%). Deleted, converted or duplicated NAIP copies (e.g. 0, 1/2 or 3/4 copies) were identified across all SMA types and were not associated with a specific phenotype.

Eighty-two patients (29%) had a more severe (51%) or milder (49%) phenotype than expected based on SMN2 copy number (see Materials and Methods section). All patients with SMA Type 4 in our cohort (\( n = 9 \)) carried four SMN2 copies, which are usually associated with SMA Type 3 (Piepers et al., 2008). Three out of four patients with two SMN2 copies who did not have SMA Type 1 but SMA Types 2a, 2b or 3b all had a c.859G>C mutation. The fourth patient had SMA Type 3a and an extra copy of SMN2 with only Exons 1–6. Twenty-eight patients with SMA Type 1c carried three copies of SMN2, and one had even four copies with a double mutation in the promoter region.

Patients with a hybrid SMN1–SMN2 gene (\( n = 28 \)) showed a milder disease course compared to patients with the same SMN2 copy number, but no statistical analysis was possible using these individual clinical parameters. None of the patients with SMA Types 2 or 3 on a three or four copy SMN2 background with a hybrid SMN1–SMN2 gene needed respiratory support (mean age 29 years; median 9 years; range 2–69), in contrast to 20% (\( n = 24 \)) of patients without a hybrid gene with SMA Types 2 or 3 and 3 or 4 SMN2 copies (start of ventilation: mean age of 21 years, median 14 years; range 2–62 years).

**Discussion**

The approval of therapies and the emergence of neonatal screening programmes urgently require a better understanding of genetic variants that underlie clinical heterogeneity in SMA. Our study aimed to explore the variability in the SMN locus in more detail than before, including an analysis of SMN2 and NAIP1 sequences, copy number variation, (partial) deletions or duplications and their relation to SMA severity. We show that SMN2 copies are structurally different between patients and identified SMN2 variants that explain clinical variability in individual cases. More importantly, we identified SMN1–SMN2 hybrid genes as a relatively frequent and important structural variation in SMN2 copies, between and even within patients.

Our study confirms that SMN2 copy number is the most important severity modifier in SMA. We observed the expected association of SMN2 copy numbers with specific SMA types (i.e. SMA Type 1: two copies; SMA Type 2: three copies; SMA Types 3 and 4: four copies) in 70% of cases (Lefebvre et al., 1995; Feldkotter et al., 2002; Wirth et al., 2006; Rudnik-Schoneborn et al., 2009; Calucho et al., 2018). The strongest correlation of SMN2 copy number and SMA type is present at both ends of the severity spectrum (Calucho et al., 2018). For example, neonatal onset (SMA Type 0/1a) is virtually always associated with one SMN2 copy and the majority of children with SMA Type 1b carry two SMN2 copies (Mercuri et al., 2012; Calucho et al., 2018). Patients with late-onset and mild SMA (Types 3b and 4) mostly have four or more SMN2 copies. In patients with three SMN2 copies, the most prevalent copy number in this cohort, clinical variation is much more pronounced, ranging from patients with no ability to sit independently (SMA Type 1c) to ambulant patients with early onset (SMA Type 3a). The fact that SMN2 copy number variation is insufficient to explain all relevant clinical variation is further illustrated by the 60% of siblings with discordant phenotypes but similar SMN2 copy numbers in 95% of our families. It suggests the presence of other genetic variants that influence SMA severity, either within or outside the SMN locus (Jones et al., 2019).
Figure 4 Expression levels of SMN1 and SMN2. (A) SMN1 expression levels differ between patients, carriers (= parents) and controls (P < 0.01). SMN1 levels were non-detectable in patients with a homozygous deletion of SMN1. (B) SMN2 expression levels (SMN2 full-length upper panel, SMN2 delta7 lower panel) show a correlation with the SMN2 copy numbers (e.g. higher SMN2 copy number correlates with higher SMN2 expression levels) (SMN2 FL KW P = 0.02; SMN2 delta7 KW P = 0.09), also when analysed within the SMA types. (C) Hybrid genes resulted in higher levels of SMN2 full length if analysed within the same SMN2 copy number (KW P = 0.06). SMN2 full-length levels (upper panel) were higher in a double hybrid gene background compared to levels on a single hybrid background in patients with SMA (MW P = 0.03). (D) No difference was found in expression levels of patients with (n = 110) or without (n = 2) a c.859G>C mutation (MW P = 0.2). SMN expression levels were presented as number of copies per 75 ng RNA. Panels B–D present data of SMA patients only.
Because specific mutations in SMN2 that modify severity have been reported, we first assessed intragenic variation in a relatively large cohort of well-defined patients (Prior et al., 2009; Bernal et al., 2010; Harahap et al., 2013; Wu et al., 2017; Ruhno et al., 2019). We identified 10 single-nucleotide variants in SMN2, including five novel ones that are SMN2 specific (i.e. they have not been reported in the SMN1 sequence) (Hahnen and Wirth, 1996; Wirth et al., 1997; Alias et al., 2009; Jędrzejowska et al., 2014). Four of these variants had severity-modifying effects. We found previously described polymorphisms in SMN2 Exons 2a and 3 (c.84C>T and c.462A>G, respectively) in 30% (n=88) of patients without a clear correlation with the phenotype (Ruhno et al., 2019). We documented a strategic mutation in the promoter region of the SMN2 gene (c.1–14C>T) that explained the clinical phenotype (SMA Type 1) in the presence of four SMN2 copies. With extended MLPA analyses, we confirmed that this mutation abrogated the function of at least two SMN2 copies. Mutations in Exon 7 of SMN2 showed more clear associations with the clinical phenotype. We detected a deletion of three nucleotides (c.838_840del) in a child with SMA Type 1b contrast to a previous report, however, we were unable to confirm a positive effect on SMN2 expression levels in our patients with a c.859G>C mutation (Vezain et al., 2010). The presence of heterozygous mutations at c.859, as shown in our current and previous studies, implies that not all patients’ SMN2 copies contain this SNP and copies of SMN2 are, therefore, different (Prior et al., 2009; Bernal et al., 2010; Calucho et al., 2018). The modifying effect of the c.859G>C mutation occurred in patients with fewer SMN2 copies than expected (e.g. two copies and SMA Type 3b). The lack of correlation between the SMN2 variants and SMN protein expression levels may suggest the presence of other isoforms of SMN, which are currently unable to detect (Singh et al., 2012; Harahap et al., 2018).

Our MLPA results show structural heterogeneity of the SMN locus beyond copy number variation. We identified hybrid SMN genes and partial deletions of SMN2 in 12% of our patients. Trio analysis showed that this variation was often inherited. Hybrid SMN1–SMN2 genes were found in patients with a relatively mild disease course compared to patients with the same SMN2 copy number. The correlation with a better clinical phenotype was supported by the observation that patients with a double hybrid gene showed higher expression levels of full-length SMN2, suggesting a more efficient transcription of the hybrid gene. The mechanism behind the up-regulation of SMN protein expression is currently not well understood. The molecular architecture of hybrid genes may also vary (Wu et al., 2017), e.g. conversion of an exon with or without intronic sequences, which may have additional effects on the transcription and clinical phenotype. Other patients carried partial deletions of SMN2 Exons 1–6 or 7–8, strongly suggesting further structural heterogeneity between SMN2 copies. Since these deletions are not rare, we think that similar SMN2 copy numbers encompass a much larger genetic and functional heterogeneity that provides a likely explanation for clinical variation. Deletion junctions resulting in the partial deletion of Exons 7–8 have recently been described in 10% of patients in a cohort of 217 SMA patients (Ruhno et al., 2019). We detected deletions of Exons 7–8 in a much lower percentage, i.e. 2% of patients in our cohort. SMN locus rearrangements vary considerably between populations, including the loss of Exons 7–8, which provides a likely explanation for this discrepancy (Vijzelaar et al., 2019; Vorster et al., 2020).

Our findings are of particular relevance in relation to present genetic therapies. Although inter-sample variation of the MLPA in our repeated analysis was very low (3%), for eight out of 286 patients (3%), it was not possible to determine whether they had three or four copies. This may raise difficulties in some countries, where there is no reimbursement of antisense-oligonucleotide (ASO) therapy for patients with more than three SMN2 copies, or in prenatal screening programmes where a similar cut-off might be used (Baker et al., 2019; Muller-Felber et al., 2020). Intronic sequence variation is a possible explanation for differences in treatment response. We analysed flanking intronic regions of up to 100 reads but did not detect intronic variation, including the previously described positive modifier in Intron 6 (44A>G) (Wu et al., 2017; Ruhno et al., 2019). Moreover, none of the 10 detected SMN2 mutations in our cohort were located in the flanking regions of Introns 6 or 7, which represent the target sites of SMN2 splicing modulating ASOs or small molecules currently in development (Singh et al., 2006; Calder et al., 2016; Fletcher et al., 2017). Although we cannot exclude the presence of other deep intronic variations (in)directly influencing these targeted therapies, our current data suggest that genetic variation at the target sites of therapies is rare. Structural variability, as illustrated by the presence of hybrid genes, may, however, reflect the presence of DNA sequences in patients who are more or less susceptible to gene-targeted therapies. The exact DNA sequences and mechanisms associated with this variability remain to be determined. Recent technological advances allow for the increasingly detailed analysis of highly complex genetic regions such as the SMN locus, including approaches based on
improved analysis of current short-read sequencing methods, optical mapping and long-read sequencing (van Dijk et al., 2018; Ho et al., 2020). Indeed, these approaches have already been shown to be applicable to the SMN locus and SMA in proof-of-concept studies (Ebbert et al., 2019; Chen et al., 2020). Combining these novel methods with a large, well-phenotyped cohort of patients in future studies will be required to obtain a complete picture of the genetic variability that underlies clinical variation in SMA.

This study shows that gene copies of SMN2 are structurally different between and also within patients. This may have implications for current counselling and treatment practices. With currently available sequencing and genotyping methods, obtaining genotype–phenotype correlations and predictions for individual patients remains a challenge.

### Supplementary material

Supplementary material is available at Brain Communications online.

### Acknowledgements

The authors wish to thank Yana van der Weegen and Elske Mak-Nienhuis for their contributions to the sample preparation and sequencing of SMN2.

### Funding

This work was supported by a grant from the Prinses Beatrix Spierfonds (WAR08-24).

### Competing interests

R.I.W., M.D.J., M.S., C.A.W., C.A.D.C., J.M., P.S., J.S., R.V., H.H.L. and E.J.N.G. report no disclosures or competing interests. L.H.v.d.B. reports grants from ALS Foundation Netherlands, The Netherlands Organization for Health Research and Development [Vici scheme; and funded through the EU Joint Programme—Neurodegenerative Disease Research, JPND (SOPHIA, STRENGTH, ALS-CarE projects)], personal fees from Shire, Biogen, Cytokinetics and Treeway, outside the submitted work. W.L.v.d.P. received grants from non-profit entities Prinses Beatrix Spierfonds, Stichting Spieren voor Spieren, Vriendenloterij. His employer received fees for his membership of the Novartis Data Monitoring Committee (Branaplam) and ad hoc consultancy for Biogen and Avexis.

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