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Intragenic variants in the *SMN1* gene determine the clinical phenotype in 5q spinal muscular atrophy

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Abstract

Objective

The aim of the study was to report the proportion of homozygous and compound heterozygous variants in the survival motor neuron 1 (*SMN1*) gene in a large population of patients with spinal muscular atrophy (SMA) and to correlate the severity of the disease with the presence of specific intragenic variants in *SMN1* and with the *SMN2* copy number.

Methods

Four hundred fifty Brazilian patients with SMA were included in a retrospective study, and clinical data were analyzed compared with genetic data; the *SMN2* copy number was obtained by multiplex ligation-dependent probe amplification and pathogenic variants in *SMN1* by next-generation sequencing.

Results

Four hundred two patients (89.3%) presented homozygous exon 7-*SMN1* deletion, and 48 (10.7%) were compound heterozygous for the common deletion in one allele and a point mutation in the other allele. Recurrent variants in exons 3 and 6 (c.460C>T, c.770_780dup and c.734_735insC) accounted for almost 80% of compound heterozygous patients. Another recurrent pathogenic variant was c.5C>G at exon 1. Patients with c.770_780dup and c.734_735insC had a clinical phenotype correlated with *SMN2* copy number, whereas the variants c.460C>T and c.5C>G determined a milder phenotype independently of the *SMN2* copies.

Conclusions

Patients with specific pathogenic variants (c.460C>T and c.5C>G) presented a milder phenotype, and the *SMN2* copy number did not correlate with disease severity in this group.

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Glossary

MLPA = multiplex ligation-dependent probe amplification; **NGS** = next-generation sequencing; **SMA** = spinal muscular atrophy; **SMN1** = survival motor neuron 1.

Spinal muscular atrophy (SMA) is a neurodegenerative disease of lower motor neurons, leading to progressive weakness associated with ventilatory insufficiency. The most common form of SMA is caused by mutations in the survival motor neuron 1 (*SMN1*) gene located at 5q13.[1] Exon 7 of *SMN1* is not detectable in approximately 96% of patients with SMA, and approximately 4% of patients have a combination of the deletion and an intragenic mutation in the second allele.[1,2] The centromeric homologous *SMN2* gene cannot compensate for the *SMN1* defect because a single nucleotide polymorphism located in exon 7 causes exon skipping in about 90% of *SMN2* transcripts leading to a nonfunctional SMN protein.[3]

The *SMN2* copy number varies, and several studies have demonstrated a strong correlation between the number of *SMN2* copies and SMA severity.[4–6] However, other genetic and environmental factors also influence the clinical severity of the disease. One example is the variant c.859G>C in exon 7 of *SMN2*, which acts as a positive modifier that results in an approximate 20% increase in full-length *SMN* RNA.[7,8] In addition, intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some patients with SMA.[9]

The aim of this study was to determine the proportion of homozygous and compound heterozygous deletions in the *SMN1* gene in a Brazilian population of patients with SMA and to correlate the severity of the disease with the presence of specific intragenic variants in *SMN1* and with the *SMN2* copy number.

Methods

A national collaborative historical cohort study was conducted to describe the clinical and molecular features of consecutive patients with SMA referred to 12 neuromuscular centers in Brazil, from January 2016 to July 2019. The eligibility criterion was the presence of *SMN1* mutations in patients with the SMA phenotype. General and clinical data of the patients were obtained through the records including age at birth, age at onset, SMA subtype, ventilatory status, use of gastrostomy, and sitting, standing, and walking capacity.

All patients fulfilled the diagnostic criteria of proximal SMA defined by the International SMA Consortium and were classified into the 5 main clinical phenotypes of SMA, from 0 to 4.[10,11] All patients had a genetic confirmation of *SMN1* gene mutation in both alleles. DNA used in the analysis was extracted from leukocytes from peripheral blood or from oral swab. Copy number variation in the *SMN1* and *SMN2* genes was detected by the multiplex ligation-dependent probe amplification (SALSA MLPA kit P060-SMA, MRC Holland) method for both *SMN1* and *SMN2*. Patients who failed to show homozygous absence of *SMN1* exon 7 by MLPA were submitted to sequencing of *SMN1/SMN2* genes by next-generation sequencing (NGS). The methodology used was exon capture with Agilent Mendelics Custom Panel V3 followed by sequencing with Illumina HiSeq.

Sequence variations were compared with data available in the Human Gene Mutation Database and ClinVar with reference to the GRCh37 version of the human genome. Variants were classified according to the 2015 American College of Medical Genetics and Genomics criteria. PolyPhen-2, Sorting Intolerant From Tolerant, Combined Annotation Dependent Depletion, Mendelian Clinically Applicable Pathogenicity score, MutationTaster, Human Splicing Finder v3.0, and ESEfinder v.3.0 were used for in silico analysis. Phylogenetic conservation was estimated with Genomic Evolutionary Rate Profiling, and allele frequencies were searched on the Genome Aggregation Database and the 1000 genomes browser.

Standard protocol approvals, registrations, and patient consents

The study was approved by the institutional ethics committees of the local centers. Informed consent was obtained for genetic studies from the patients or their legal representatives.

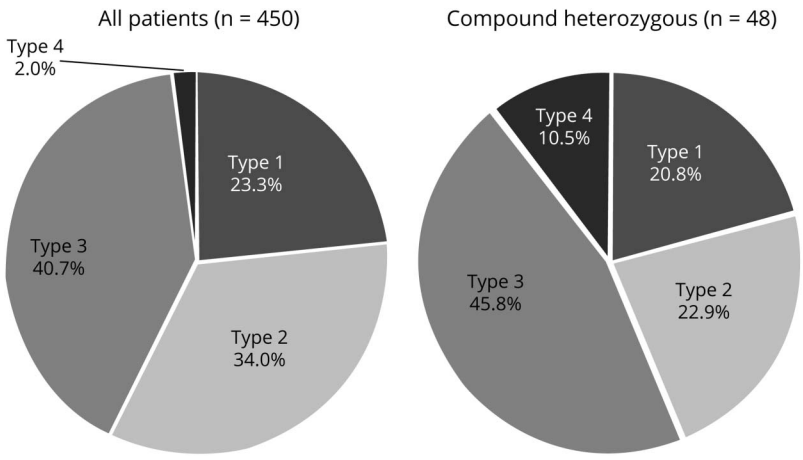
Data availability

Data corresponding to the total number of patients, distribution in SMA types, distribution of the variants in *SMN1*, and *SMN2* copy number are available in figures 1–4. Individual details of heterozygous patients are fully shown in table 1. Any data not published within the article are available in a public repository. Methods and the statistical plan are also fully reported in the Methods section. Details regarding the statistical analysis by logistic regression are shown in table 2.

Statistical analysis

For descriptive purposes, categorical variables were expressed as absolute and relative frequencies and then compared with Pearson χ^2 in a univariate analysis. Continuous variables were expressed as the mean and SD and then compared with the Student *t* test. The mild phenotype was defined as SMA type 3 or 4. To identify factors associated with SMA phenotypes (sex, *SMN2* copy number, and specific intragenic variants), Firth logistic regression analysis was performed for the heterozygous patients. The significant variables at the 0.10 level in the univariate analysis were included on the multivariate model. The results were expressed as odds ratios, coefficients, and 95% confidence intervals.

Figure 1 Prevalence of types of SMA



Prevalence of types of SMA in the studied population (left chart) compared with the prevalence of SMA types in heterozygous patients (right chart). Note the significant increase in the proportion of patients with mild phenotypes (types 3 and 4) in the heterozygote group. SMA = spinal muscular atrophy.

All tests were 2 tailed, and final p values less than 0.05 were considered to be statistically significant. All analyses were performed with Statistical Package for Social Sciences software, version 20 (SPSS, IBM Statistics, Chicago, IL).

Results

A total of 450 patients with SMA with genetic confirmation were identified. Among them, 105 (23.3%) were classified as type 1 (3 of them were SMA type 0/1a), 153 (34%) as type 2, 183 (40.7%) as type 3, and 9 (2%) as type 4 (figure 1). Of the patients, homozygous deletion of *SMN1* exon 7 was detected in 402 (89.3%), and 48 patients (10.7%) had 1 nondeleted allele of *SMN1* exon 7 with an intragenic variant (figure 2). No patient was homozygous for intragenic variants. The quantification of the *SMN2* copy number was possible in all 450 patients tested by MLPA.

Within the group of 48 compound heterozygous patients, 10 (20.8%) were type 1, 11 (22.9%) type 2, 22 (45.8%) type 3, and 5 (10.5%) type 4 (figure 1).

As expected, the *SMN2* copy number presented an inverse correlation with the severity of phenotype in the homozygous group. A greater proportion of patients with SMA type 1 carried 2 copies of *SMN2* (72.5%), most patients with SMA type 2 had 3 copies (65%), and patients with type 3 presented 3 or 4 copies of *SMN2* (69.6% and 30.5%, respectively) (figure 3).

A total of 7 different pathogenic variants were identified in *SMN1/SMN2* (figure 2 and table 1), 4 of which were recurrent variants. The most frequent variant was the c.460C>T (p.Gln154*). This variant was identified in 16 patients and has not been previously reported in the literature; its allelic frequency in large databases (Genome Aggregation Database and Exome Aggregation Consortium) is zero, indicating that it is a pathogenic variant. The variant c.770_780dup (p.Gly261-Leufs*) was identified in 13 patients, the variant c.5C>G (p.Ala2Gly) in 9 patients and the variant c.734_735insC (p.Pro246Thrfs*) in 7 patients. General clinical data of the compound heterozygous patients are presented in table 1.

In the group of compound heterozygous patients, we did not find the variants c.859G>C and A-44G that are considered positive modifiers.[7,8,12] In these patients, it was possible to state that the variant c.859G>C was not detected because in our database, there are controls with this variant identified using the same NGS methodology. Because of methodological limitation, intragenic variants A-549G and C-1897T were not analyzed. All variables that were classically associated with disease severity (sex and *SMN2* copy number), along with pathogenic variants that showed statistical significance ($p < 0.05$) in correlation with the phenotype in the univariate analysis, were included in the multivariate logistic regression analysis model (table 2). Although all patients carrying the variant c.5C>G presented as SMA type 2 or 3, we did not find a statistical significance association with the mild phenotype in the univariate analysis ($p = 0.71$), so these patients were not included in the multivariate analysis. In heterozygous patients, no clear correlation existed between the clinical phenotype and the *SMN2* copy number ($p = 0.498$). However, as occurs in homozygous patients, the male sex showed an association with a more severe phenotype ($p = 0.041$). Several heterozygous patients with type 2 or 3 SMA carried only 1 *SMN2* copy, and most patients with types 3 and 4 SMA carried 2 copies (figure 4). In these patients, we could demonstrate by logistic regression that the most important factor in determining the phenotype was the pathogenic variant that the patients presented, with variant c. 460C>T related to the mild phenotype ($p = 0.014$), whereas variants c.770_780dup and c.734_735insC were related to more severe phenotypes depending on *SMN2* copy number (tables 1 and 2). Although the variant c.5C>G was not entered in the multivariate

Table 1 Genetic and clinical data of patients with SMA with compound heterozygous variants in *SMN1*

Pt	c.DNA variant/effect on protein/localization	SMA type	*SMN2* copy number	Sex/age[a] (y)	Age at disease onset	Use of IV or N BIPAP	Functional status[a]/age at loss of walking
1	c.460C>T/p.Gln154*/exon 3	3	1	M/39	2 y	No	Wheelchair/36 y
2		3	4	M/38	6 y	No	Walking
3		3	3	M/32	13 y	No	Wheelchair/21 y
4		3	1	M/30	2 y	No	Wheelchair/11 y
5		3	2	F/29	6 y	No	Wheelchair/12 y
6		3	2	M/26	5 y	No	Wheelchair/18 y
7		3	3	M/26	8 y	N BIPAP	Wheelchair/17 y
8		3	2	M/19	6 y	No	Walking with assistance
9		3	2	M/18	13 y	No	Walking
10		3	2	M/16	4 y	No	Walking with assistance
11		3	2	F/14	2 y	No	Walking with assistance
12		3	3	F/14	4 y	No	Walking with assistance
13		4	2	M/40	18 y	No	Wheelchair/32 y
14		4	2	F/39	18 y	No	Walking
15		4	2	F/30	25 y	No	Walking
16		4	2	M/19	16 y	No	Walking
17	c.770_780dup/p.Gly261Leufs*8/ exon 6	1	2	F/11	1 mo	IV	Nonsitter
18		1	2	F/died 1 y 5 mo	1 mo	IV	Nonsitter
19		1	2	M/died 1 y 6 mo	5 mo	IV	Nonsitter
20		1	2	F/1 y 6 mo	3 mo	IV	Nonsitter
21		1	2	F/died 9 mo	3 mo	IV	Nonsitter
22		2	2	F/34	1 y	No	Wheelchair
23		2	2	F/19	8 mo	N BIPAP	Wheelchair
24		2	3	M/12	11 mo	No	Wheelchair
25		2	3	M/8	1 y 5 mo	No	Wheelchair
26		2	3	F/4	7 mo	No	Wheelchair
27		3	3	F/18	1 y 6 mo	No	Wheelchair/7 y
28		3	3	F/13	2 y	No	Wheelchair/11 y
29		4	4	F/39	26 y	No	Walking
30	c.5C>G/p.Ala2Gly/exon 1	2	1	M/12	7 mo	N BIPAP	Nonsitter
31		2	1	M/died 9 y	1 y 2 mo	IV	Nonsitter
32		2	1	M/9	9 mo	IV	Nonsitter
33		3	1	F/23	11 y	No	Wheelchair/21 y
34		3	1	M/20	5 y	No	Walking with assistance
35		3	1	F/19	3 y	N BIPAP	Wheelchair/11 y
36		3	1	F/12	1 y 5 mo	IV	Wheelchair/4 y

Continued

Table 1 Genetic and clinical data of patients with SMA with compound heterozygous variants in *SMN1* *(continued)*

Pt	c.DNA variant/effect on protein/localization	SMA type	*SMN2* copy number	Sex/age[a] (y)	Age at disease onset	Use of IV or N BIPAP	Functional status[a]/age at loss of walking
37		3	1	F/7	1 y 6 mo	No	Walking
38		3	1	F/6	3 y	No	Wheelchair/6 y
39	c.734_735insC/ p.Pro246Thrfs*10/exon 6	1	2	F/13	4 mo	IV	Nonsitter
40		1	2	F/8	4 mo	IV	Nonsitter
41		1	2	F/1	4 mo	N BIPAP	Nonsitter
42		1	2	M/1	2 mo	N BIPAP	Nonsitter
43		2	3	M/17	7 mo	N BIPAP	Wheelchair
44		2	3	F/4	6 mo	No	Wheelchair
45		3	3	F/27	2 y	No	Wheelchair/21 y
46	c.469C>T/p.Gln157*/exon 3	2	3	F/25	1 y 6 mo	No	Wheelchair
47	c.806T>C/p.Met269Thr/exon 6	3	1	M/14	5 y	No	Wheelchair/13 y
48	c.346A>T/P.Ile116Phe/exon 3	1	2	M/died 11 mo	3 mo	IV	Nonsitter

Abbreviations: IV = invasive ventilation and tracheostomized; N BIPAP = night BIPAP use; Pt = patient; SMA = spinal muscular atrophy.
[a] At last evaluation.

analysis, based on the model criteria, all patients with this variant had 1 *SMN2* copy, but none of them had type 1 SMA, which one may expect.

Discussion

We report on the largest cohort of patients with SMA in Latin America and bring new insights regarding the molecular diagnosis in SMA. The presence of compound heterozygous mutations occurred in approximately 10.7% of the patients with SMA, a frequency higher than that reported in the literature. This fact draws attention to our population, especially with the future advent of neonatal screening for the diagnosis and treatment of presymptomatic patients. Patients with milder phenotypes tend to have a compound heterozygous genotype more often than patients with type 1 SMA, and in our study, the frequency of type 1 SMA was lower than commonly reported in the literature[10], most probably related to the higher mortality in this group of patients. More interesting is the finding that most of the patients with SMA type 4 (55.5%) were compound heterozygous, most of them carrying the same variant (p.Gln154*). These data reinforce the idea that diagnosis in SMA type 4 is more difficult even from the molecular point of view, and the average time from symptom onset to diagnosis in this group was 10 years, ranging from 2 to 18 years (data not shown in table).

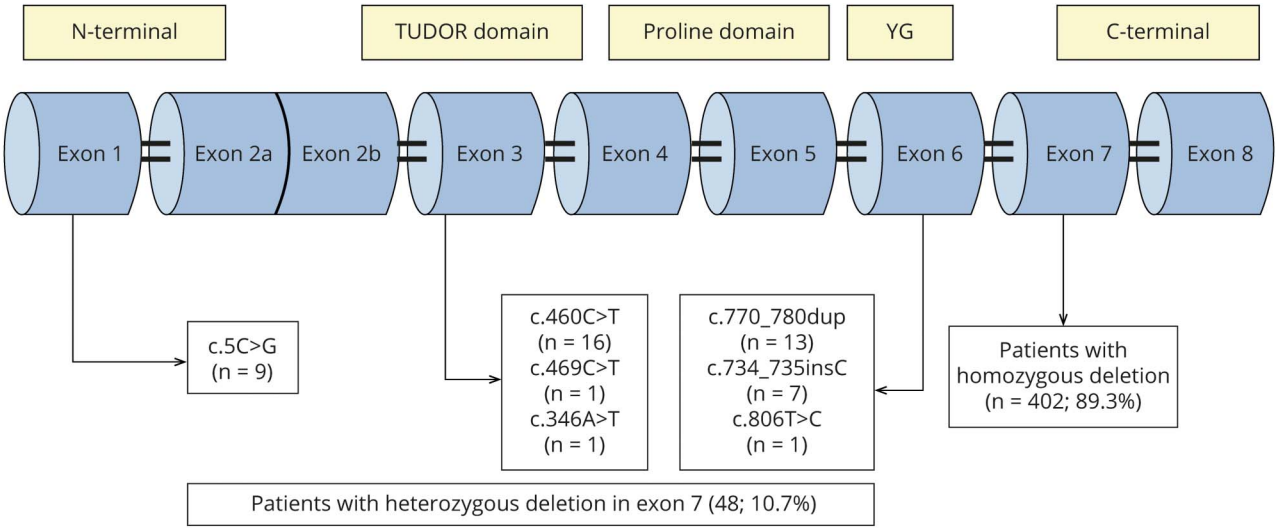
Knowledge of the molecular portrait of the population is important to understand the clinical profile and natural history of patients and to assist in the therapeutic planning,

Table 2 Multivariate analysis identifying factors associated with mild phenotype (SMA types 3 and 4) in heterozygous patients (n = 48)

Variables	Coefficient	SE	Chi square	Odds ratio	95% CI	*p* Value
Sex (male)	−1.960	1.091	4.174	0.14	−4.55 to −0.07	0.041
***SMN2* copy number**	0.466	0.689	0.460	1.59	−0.84 to 1.98	0.498
c.460C>T	3.288	1.682	6.022	26.78	0.58 to 8.33	0.014
c.770_780dup	−2.731	1.456	4.642	0.06	−6.10 to −0.22	0.031
c.734_735insC	−3.010	1.586	4.895	0.04	−6.61 to −0.31	0.027

Abbreviations: CI = confidence interval; SE = standard error; SMA = spinal muscular atrophy.

Figure 2 Characterization of *SMN1* mutations in the Brazilian population

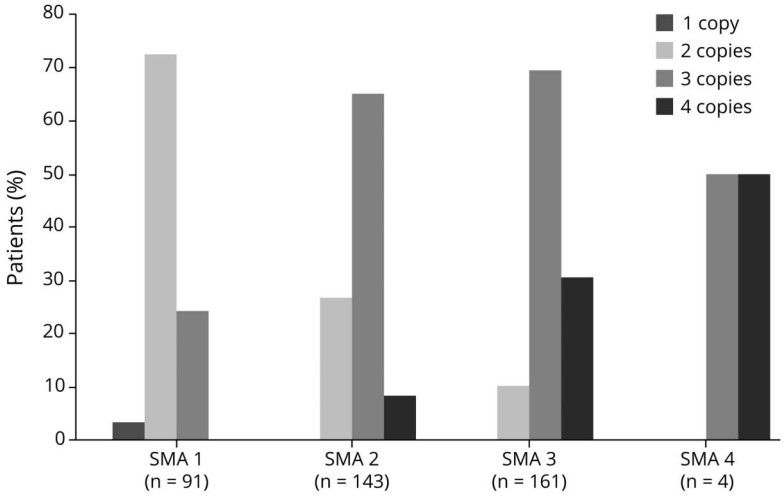


Among 450 patients with SMA, 402 (89.3%) had a homozygous deletion in exon 7, whereas 48 (10.7%) retained 1 copy of the *SMN1* gene with point mutations, most of them localized at exons 1, 3, and 6. SMA = spinal muscular atrophy; *SMN1* = survival motor neuron 1; YG = YG domain.

considering that part of the currently available specific therapies modulate the *SMN2* gene expression.[13] The correlation between the copy number of the *SMN2* gene and the phenotype followed a similar pattern to that reported in the literature,[4–6] as patients with SMA type 1 usually carried 2 copies, whereas type 2 patients usually carried 3 copies and types 3 and 4 presented with 3 or 4 copies. As reported in the literature, such a correlation is not strict because to a lesser extent, patients with SMA type 1 had 3 copies and type 3 and 4 phenotypes had 2 copies.[4,5] The lack of exact correlation must be related to other phenotypic modifiers, such as the presence of the variants c.859G>C in exon 7 and A-44G, A-549G, and C-1897T in intron 6 of *SMN2* that act as positive modifier.[7,8,12] Analysis of these variants in the *SMN2* gene was not performed in our homozygous patients, and in the group of compound heterozygous patients, we did not find the variants c.859G>C and A-44G.

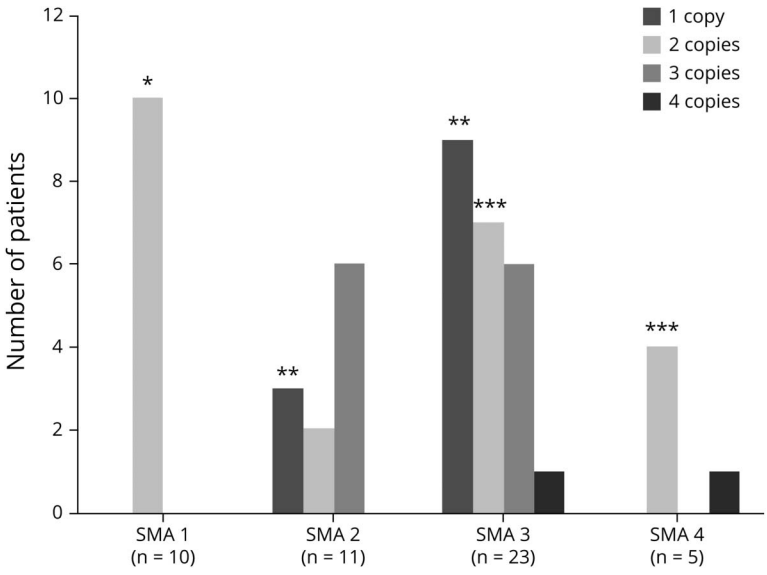
We identified 4 recurrent variants in our population: 3 nonsense and 1 missense variants. The most common variant identified was c.460C>T, located at exon 3, that creates a premature stop codon at residue 154 (p.Gln154*). Exon 3 is responsible for encoding the tudor domain of protein, which interacts with a complex of Sm proteins, kaolin, fibrillarin, and ribonucleoproteins.[14] This information confirms that exon 3 is a hot spot for small mutations; in addition, it supports the

Figure 3 Correlation between clinical phenotype of homozygous SMA patients X *SMN2* copy number



Correlation between clinical phenotype of SMA X *SMN2* copy number in patients (%) with homozygous deletion of *SMN1* detected by MLPA (n = 402). Data from SMA type 0 are not shown, as all 3 patients carried only 1 *SMN2* copy. SMA = spinal muscular atrophy.

Figure 4 Correlation between clinical phenotype of compound heterozygous SMA patients X *SMN2* copy number



Correlation between clinical phenotype X *SMN2* copy number in patients with specific intragenic variants (n = 48). *All patients with SMA type 1 presented with variants c. 770_780dup or c.734_745insC and carried 2 copies of *SMN2*. **Most patients with SMA types 2 and 3 carrying only 1 *SMN2* copy presented the c.5>G variant. ***The variant c.460C>T was present in most patients with SMA types 3 or 4 carrying only 2 copies of *SMN2*. SMA = spinal muscular atrophy.

importance of the tudor domain for SMN functioning.[15,16] This variant was the most common variant in our population, suggesting a founder effect by a common ancestor. More detailed ancestral haplotype studies would be needed to confirm this hypothesis.

Among the 16 patients with this variant, 9 were still walking (table 1), and of those patients who lost the ability to walk, the mean age of gait loss was 19 years (11–36 years), compatible with an SMA type 3b phenotype.[11] Of interest, all patients presented a milder phenotype (SMA types 3 and 4) even in the presence of 1 or 2 copies of *SMN2*. There is no clear explanation for the milder phenotype in this situation. Sossi et al.[17] reported patients with mutations in exon 3 of *SMN1* leading to premature termination codons and skipping of the entire exon generating in-frame transcripts and a better phenotype. However, many studies have shown severe missense mutations occurring in exon 3 that cause severe phenotypes and thus confirming that amino acids and the tudor domain located at exon 3 are essential to SMN function.[15,16,18–20] A possibility to explain the milder phenotype associated with the c.460C>T is the presence of positive modifier variants in *SMN2*. The presence of the variants c.859G> C and A-44G was negative in these cases; however, we cannot exclude the possibility that other untested modifier variants are involved in the modulation of the clinical phenotype.

The second most common variant was c.770_780dup (p.Gly261Leufs*), located at exon 6, and has been previously reported to be associated with SMA.[19,21,22] Parsons et al.[21] showed through real-time PCR that the 11-bp duplication in exon 6 was associated with the *SMN1* locus and not *SMN2* and was sufficient to produce a severe type 1 SMA phenotype. In a Portuguese SMA population, this variant was the most frequent intragenic mutation in a total of 14 heterozygous unrelated patients, which leads to the hypothesis that this mutation migrated from the Portuguese population to Brazil[22]. In our study, as in an SMA Portuguese population study, all patients carrying this variant exhibited a strict correlation between the phenotype and the *SMN2* copy number, with patients with 2 *SMN2* copies presenting as SMA type 1, patients with 3 copies presenting as SMA type 2 or 3, and a patient with a very mild phenotype (SMA type 4) with the presence of 4 *SMN2* copies (table 1).

The third most common variant was the missense c.5C>G (p.Ala2Gly), located at exon 1. This variant was first reported in 3 patients with SMA, all of whom had only 1 *SMN2* copy, and 2 of whom had a mild phenotype (type 3 SMA).[18] More recently, 3 additional patients with this variant, a mild phenotype and only 1 *SMN2* copy were also reported.[23] Another mutation in the same location, the p.Ala2Val variant, has already been described, and all patients had only 1 *SMN2* copy with a mild phenotype (type 3 SMA).[9] Thus, in the presence of this variant, the *SMN2* copy number did not correlate with the phenotype. The mutation effect of p.Ala2Val, as well as p.Ala2Gly, appears to be much less deleterious.

A deleterious effect of the c.5C>G variant has been demonstrated in an SMA mouse model, although the mutation did not produce a total loss of protein function.[24] The authors reported that in the absence of the *SMN2* gene, the transgenic *SMN* c.5C>G mutant is unable to rescue the embryonic lethality, but in its presence, the c.5C>G transgene delays the onset of motor neuron loss, resulting in mice with mild SMA. They concluded that only in the presence of low levels of full-length SMN is the c.5C>G transgene able to form partially functional higher-order SMN complexes essential for its

functions, demonstrating the importance of SMN levels in SMA, even if the protein is expressed from a mutant allele.[24] Similarly, in Brazilian patients carrying this variant, none presented a severe phenotype of the disease, such as type 0 or 1, even in the presence of only 1 *SMN2* copy. Patients presented with type 2 or 3 SMA with a typical age at disease onset (table 1). Of interest, 3 patients with this variant were in need of continuous invasive ventilation before age 10 years, which would not be expected due to the disease duration presented by patients. This may represent a phenotypically characteristic effect of the c.5C>G variant on this population, although deficient respiratory care in our population cannot be ruled out.

The last recurrent variant identified in our population was c.734_735insC (p.Pro246Thrfs*). This variant was not previously reported in the literature as a recurrent variant in patients with SMA. As occurs with the c.770_780dup variant, this mutation is located at exon 6, and all patients carrying this variant also exhibited a strict correlation between the phenotype and the *SMN2* copy number. These data lead us to suppose that this mutation severely compromises protein function at the YG domain of interaction with protein Gemin3.[25] Accordingly, the clinical phenotype of those patients appears to be dependent exclusively on the *SMN2* copy number.

Our study has some limitations as it was a retrospective study, and there was a bias of survival because the most severe cases (SMA type 1) had a low prevalence in our cohort. Another limitation is that NGS cannot distinguish whether pathogenic variants are located in *SMN1* or *SMN2*, although it is a reliable test that can be used in real life. The large number of patients with intragenic small variants identified in our population could be explained by the current access to the large-scale sequencing of *SMN* genes, especially for patients with clinical features of SMA but negative in the MLPA assays.

We report a large cohort with important findings, such as a higher frequency of compound heterozygous patients with SMA in the Brazilian population than reported in the literature. Most of these patients have a milder phenotype, and the *SMN2* copy number did not correlate well with the severity of disease, as observed in patients with homozygous deletion in *SMN1* exon 7. The result of our study indicates a specific point mutation spectrum in the Brazilian population and may help in the search for an early diagnosis in the era of therapies, especially in newborn screening. Variants in exons 3 and 6 (c.460C>T, c.770_780dup, c.734_735insC, c806T>C, and c.346A>T) accounted for almost 80% of our patients. In patients with exon 7 deletion in 1 *SMN1* allele, analysis of exons 3 and 6 should be prompt, as it seems to be a specific hot spot for small mutations and should be sequenced first. If the patient presents with mild phenotype and only 1 copy of *SMN2*, a search for the c.5C>G variant at exon 1 should be performed.

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Appendix *(continued)*

Name	Location	Contribution
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Continued

Appendix *(continued)*

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Intragenic variants in the *SMN1* gene determine the clinical phenotype in 5q spinal muscular atrophy

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