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Practical guidelines to manage discordant situations of *SMN2* copy number in patients with spinal muscular atrophy

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Abstract

Objective

Assessment of *SMN2* copy number in patients with spinal muscular atrophy (SMA) is essential to establish careful genotype-phenotype correlations and predict disease evolution. This issue is becoming crucial in the present scenario of therapeutic advances with the perspective of SMA neonatal screening and early diagnosis to initiate treatment, as this value is critical to stratify patients for clinical trials and to define those eligible to receive medication. Several technical pitfalls and interindividual variations may account for reported discrepancies in the estimation of *SMN2* copy number and establishment of phenotype-genotype correlations.

Methods

We propose a management guide based on a sequence of specified actions once *SMN2* copy number is determined for a given patient. Regardless of the method used to estimate the number of *SMN2* copies, our approach focuses on the manifestations of the patient to recommend how to proceed in each case.

Results

We defined situations according to *SMN2* copy number in a presymptomatic scenario of screening, in which we predict the possible evolution, and when a symptomatic patient is genetically confirmed. Unexpected discordant cases include patients having a single *SMN2* copy but noncongenital disease forms, 2 *SMN2* copies compatible with type II or III SMA, and 3 or 4 copies of the gene showing more severe disease than expected.

Conclusions

Our proposed guideline would help to systematically identify discordant SMA cases that warrant further genetic investigation. The *SMN2* gene, as the main modifier of SMA phenotype, deserves a more in-depth study to provide more accurate genotype-phenotype correlations.

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Glossary

FL-SMN = full-length SMN; **MLPA** = multiplex ligation-dependent probe amplification; **NGS** = next-generation sequencing; **SMA** = spinal muscular atrophy; **SMN** = survival motor neuron; **SMN-del7** = *SMN2* transcripts lacking exon 7; **SNV** = single nucleotide variant.

Spinal muscular atrophy (SMA) is a neuromuscular disorder with a global incidence of approximately 1:11,000 live births and a worldwide carrier frequency of 1:51.[1] According to age at onset and achieved motor abilities, patients with SMA are usually classified into type I (never sit), II (never walk unaided), or III (achieve independent walking abilities). Independent of the clinical severity, all forms of SMA are caused by loss or homozygous loss-of-function pathogenic variants of the *SMN1* gene, located at 5q13.[2,3] The number of copies of *SMN2*, the highly homologous paralog of *SMN1*, is currently the most important modifier of disease phenotype; in most patients with SMA, this number varies between 1 and 5.[4] In fact, both *SMN1* and *SMN2* encode, in principle, the same survival motor neuron (SMN) protein. However, a single C→T transition in exon 7 disrupts an exon splicing enhancer and/or creates a splicing silencer, and as a consequence, *SMN2* works as a hypomorphic allele that produces mainly transcripts lacking exon 7 (SMN-del7).[5] The SMN-del7 protein is functionally compromised and unstable and therefore rapidly degraded by the ubiquitin-proteasome system.[6] Thus, the SMA phenotype is ultimately due to insufficient levels of full-length SMN (FL-SMN) protein.

On confirmation of biallelic deletion or pathogenic variants of the *SMN1* gene in a given patient, the number of *SMN2* copies is usually determined and reported. In previous years, this figure was mainly informative and mostly used to elaborate genotype-phenotype correlations rather than to predict a particular phenotype. However, recent advances in SMA therapeutics have strengthened the importance of estimating as accurately as possible the number of *SMN2* copies for all patients with SMA. Indeed, whereas genetic confirmation of SMA is relatively straightforward (95% of the patients can be diagnosed with a simple qualitative test), the assessment of *SMN2* copy number requires a quantitative methodology that is not easily implemented in most laboratories. Issues of DNA sample quality, calibration controls, and expertise to resolve ambiguous cases have been previously discussed.[4] Along these lines, around 40% of samples recently studied by the same methodology in different laboratories yielded discordant results.[7] Furthermore, intrinsic biological factors are also a source of discrepancies and add complexity to understanding how a specific *SMN2* genotype influences the final phenotype in a given patient.[4]

Numerous studies have shown that the higher the number of copies of *SMN2*, the larger the amount of FL-SMN protein produced, and thus the milder the associated SMA phenotype. However, this correlation is not absolute, and some patients with 2 copies of *SMN2* have mild SMA phenotypes, whereas some with 4 or more copies of the gene have been described as type I or II (reviewed in Calucho et al., 2018).[4] Thus, accurate estimation of *SMN2* copy number is essential in the present scenario of therapeutic advances with 3 specific SMA therapies already approved—nusinersen, *onasemnogene abeparvovec,* and risdiplam—and with the perspective of SMA neonatal screening and early diagnosis to initiate treatment.[8,9] We propose a practical guide for the management of discordant SMA cases based on systematic specified actions once *SMN2* copy number has been determined for a given patient. Our approach is independent of the method used to estimate *SMN2* copy number and focuses on the manifestations of the patient to decide how to proceed in each case.

Methods

This guideline can be applied to the vast majority of genetically confirmed SMA cases with biallelic deletion of *SMN1* and to patients who may need further analysis (e.g., those with hybrid *SMN2-SMN1* genes or pathogenic *SMN1* variants). We base the current guideline on our previously published meta-analysis of SMA genotype-phenotype correlations and in our continued multidisciplinary experience with patients referred to our consultation, both national (Spain) and international.[4] Briefly, our approach considers the initial report of *SMN2* copy number for a given patient, which is in turn based on a quantitative analysis by multiplex ligation-dependent probe amplification (MLPA) using a mixture of specific probes for the SMA locus (P021-B SMA MLPA kit, a new version of the MLPA kit that includes probes for all exons of the *SMN* genes, in addition to introns 6 and 7).[10,11] However, our proposed guide can be applied to any report regardless of the method used for *SMN2* analysis. Starting with the estimated *SMN2* copy number reported, we then focus on the manifestations of the patient and how to proceed in case of an unexpected discordance. An unambiguous assignment of the SMA type by motor milestones criteria (0 "congenital," I "never sit," II "never walk," or III "walker") was initially widely established for simplicity. However, when necessary, these categories were further refined into subtypes Ia, b, and c, IIa and b, IIIa and b, and the milder type IV SMA and even with minimal manifestations, as previously defined.[9,12] Altogether, we distinguish up to 10 different clinical diagnostic categories to which genetically confirmed cases may be ascribed (table 1) to establish genotype-phenotype correlations and define possible discrepancies.

Data availability

All data and scripts used to generate the analyses of this article are available on request unless the type of request compromises ethical standards or legal requirements.

Table 1 Spinal muscular atrophy (SMA) major clinical diagnostic categories in genetically confirmed cases

Clinical categories/ SMA type	Main clinical description
PS	Presymptomatic cases (identified at birth by newborn screening or previous affected sibling)
0/Ia	Congenital cases/patients with early manifestations within the first weeks of life
Ib	Patients with manifestations within first 3 mo of life
Ic	Children capable of head control, nonsitters
IIa	Sitters who are not able to stand up
IIb	Sitters who are able to stand up, but not to walk independently
IIIa	Onset before age 3 y, short-term walkers
IIIb	Onset after age 3 y, long-term walkers
IV	Walkers with weakness initiated in adult life
MM	Patients with only MMs (include also asymptomatics)

Abbreviations: MM = minimal manifestation; PS = presymptomatic.
Based on references 4, 9, and 12.

Results

We defined several discordant situations according to *SMN2* copy number in patients with a specific phenotype in 2 different scenarios: (1) presymptomatic diagnosis of a case detected in a newborn screening program or because of a previous SMA family history and (2) when a symptomatic patient is genetically confirmed. The spectrum of possible situations includes from 1 to 4 or more *SMN2* copies. A genetically confirmed neonate is considered presymptomatic based mainly on the absence of hypotonia, weakness, hypo- or areflexia, or fasciculations. Other manifestations may be more subtle and therefore not clearly noticeable.[9,13] In the second scenario, according to the patient's phenotype, different discrepancies are discussed. We defined recommendations according to the reported literature and our own experience, as follows.

Guideline in a neonatal screening: asymptomatic context

The different situations that could be encountered when facing a presymptomatic patient, the number of *SMN2* copies, the predicted phenotypes and suggested actions in each situation, and their rationale are given in table 2. Patients with 1 *SMN2* copy usually present a congenital SMA form, and the discordance refers to their presenting without symptoms in the neonatal period. On the other hand, an apparently normal neonate should be expected to have at least 2 *SMN2* copies, and different predictions and actions are endorsed.

Guidelines in a symptomatic context

The different situations of symptomatic patients, the number of *SMN2* copies, the observed phenotypes and the rationale, and actions suggested in each case are summarized in table 3. Unexpected discordant cases include patients having (1) a single *SMN2* copy but noncongenital disease forms (types Ib, II, or even III), (2) 2 *SMN2* copies with type II or III SMA, (3) 3 copies of the gene with severe disease forms (type Ia and b), and (4) at least 4 *SMN2* copies but more severe SMA (types I or II).

Discussion

We have developed a practical guide for management and advice to help in the interpretation and resolution of discordant SMA cases according to the number of *SMN2* copies and phenotype. Our approach applies to virtually all genetically confirmed cases and is independent of the method used to determine *SMN2* copy number (table 4), but focuses instead on the manifestations of the patient. We suggest several recommendations to rapidly define the course of actions for a given SMA patient. *SMN2* copy number estimation is essential to establish accurate genotype-phenotype correlations, to predict disease evolution, to stratify patients for clinical trials, and to define those eligible for a given treatment. However, in some patients, this information may be insufficient to correlate with the observed phenotype. So far, the number of copies of the *SMN2* gene and the presence of rare *SMN2* variants (e.g., NM_017411.3:c.859G>C and NM_017411.3:c.835-44A>G) remain the major modifiers of SMA disease phenotype.[14–17]

The main characteristics of methods currently used to quantitate *SMN2* copies (TaqMan, LightCycler, MLPA, PCR-CE, and digital PCR) are given in table 4.[10,11,18–29] In a meta-analysis of 33 studies published from 1999 to 2017, in which *SMN2* copy number was reported for a total of 3,393 patients with SMA, MLPA was used in 54% of patients (n = 1870) followed by LightCycler in 21.4% (n = 741) and TaqMan in 6.5% (n = 228) and fewer patients with the remaining methodologies.[4,22,27] All these different methodologies have advantages and disadvantages, and there are technical aspects beyond the method itself that have to be considered such as DNA sample quality and interpretation and control issues. Digital PCR approaches[28] and novel protocols using next-generation sequencing (NGS) may help with the resolution of particularly difficult cases. Noteworthy, NGS methodologies allow a thorough analysis of *SMN2* copies at the genomic level including also introns and allowing a better investigation of the equivalency and quality of the *SMN2* copies. In addition, NGS provides valuable information that may be validated to establish more comprehensive genotype-phenotype correlations.[19–21]

A virtually asymptomatic neonate with a single *SMN2* copy is an obviously unexpected situation. As indicated in table 2, congenital type 0 cases have only 1 *SMN2* copy, which is insufficient to rescue the phenotype of the disease at the prenatal stage. In these patients, SMA manifests usually at

Table 2 Suggested course of actions in SMA cases identified during newborn screening

SMN2 copy number	Manifestations at birth (clinical category)	Expected correlation	Rationale for recommended actions	Recommended actions and expected phenotype
1	Not observed (PS)	No[a]	Presence of only 1 *SMN2* copy is usually associated with congenital SMA. If a child is asymptomatic at birth and remains so for the first weeks of life, this would suggest an error in the previous *SMN2* quantitation or the presence of a positive modifier single nucleotide variant in the single gene copy.	Retest for *SMN2* copy number with a new sample and/or consider another method/laboratory. If the presence of a single *SMN2* copy is confirmed, test for rare positive variants associated with better-than-expected phenotypes (e.g., c.859G>C[b] and c.835-44A>G), e.g., by Sanger sequencing, or perform next-generation sequencing (NGS) analysis.[19–21]
2	Not observed (PS)	Yes	Neonates with 2 *SMN2* copies usually have a normal appearance. There is a latency period in which SMA symptoms may not be detectable. However, subtle manifestations of the disease might appear shortly after birth.	Test for rare positive variants in *SMN2* associated with better-than-expected phenotypes (e.g., c.859G>C[b] and c.835-44A>G). If negative, the patient has >90% probability of developing severe, type I SMA.[4] If positive, the patient will be virtually a sitter or walker later in their life[4,14]
3	Not observed (PS)	Yes	Neonates with 3 *SMN2* copies have a normal appearance and usually without manifestations at least for the first 3 mo of life.	Test for rare positive variants in *SMN2* associated with better-than-expected phenotypes (e.g., c.859G>C[b] and c.835-44A>G). If negative, the patient has about 60% probability of developing type II disease, 35% type III, and 5% type Ic. Similar Bayesian estimations can also be calculated.[4,25] If positive, the patient will be virtually a walker later in their life[4,14]
≥4	Not observed (PS)	Yes	Neonates with 4 *SMN2* copies have a normal appearance. About 14% of SMA cases worldwide have 4 *SMN2* copies.[4]	Retest for *SMN2* copy number with a new sample and/or consider another method/laboratory. If copy number is confirmed, the patient has >90% probability of being a walker later in their life (SMA types III or IV).[4] Test for the rare positive variants is an option.

Abbreviations: PS = presymptomatic; SMA = spinal muscular atrophy.
[a] In this case, the expected category would be congenital (see text for further details and discussion). Percentages are calculated according to reference 4.
[b] A commercial test is available for the c.859G>C variant (table 4).

birth with at least marked hypotonia and weakness, but more commonly with a complex clinical picture that includes in addition respiratory problems, contractures, cardiac malformation, vascular necrosis,[30] and diffuse and progressive brain abnormalities.[31] If the patient does not manifest any of these symptoms, the most likely explanation is an erroneous determination of *SMN2* copy number, which should be excluded. Retesting with a new DNA sample, eventually using a different method or performing the analysis in a different laboratory, might solve the issue. However, if the presence of only 1 *SMN2* copy is confirmed, it is possible that single nucleotide variants (SNVs) of this single gene copy or a potential *SMN2-SMN1* hybrid structure[32] make it functionally superactive, i.e., capable of generating more full-length mRNA transcripts and FL-SMN protein than wild-type *SMN2* and thus to at least partly rescue the phenotype. Thus, testing for known positive variants such as NM_017411.3:c.859G>C[16] and NM_017411.3:c.835-44A>G[15] is recommended. If negative, it would be interesting to conduct an *SMN2* NGS study of the patient to unravel changes that may act as positive modifiers of disease severity. Along these lines, at least 10 SMA cases with 1 *SMN2* copy and type II or even III disease have been reported or personally communicated to date.[4,33] Unfortunately, these apparently discrepant cases have not been further studied, and it remains to be seen whether these phenotype-genotype discrepancies are due to technical or biological reasons.

Genetically confirmed SMA cases of newborns with 2 *SMN2* copies have a high probability (>90%) of developing type I disease, but they usually have a normal appearance at birth. There is a latency period—from 1 to several weeks—in which clear symptoms of weakness and hypotonia may not be detectable. However, subtle or less evident manifestations may appear early after birth such as hypo- or areflexia, weak cry, diaphragmatic breathing, feeding problems, and dysautonomic manifestations (i.e., increase of sweating and irregular skin responses to temperature changes).[9] On the other hand, exceptional cases with 2 *SMN2* copies may manifest overt disease at birth as usually occurs in type 0 cases.[34] Thus, and considering the continuous spectrum of phenotypes in SMA, it would be difficult to differentiate between congenital type 0 and type Ia disease, and both categories could be merged into type 0/Ia disease.[12]

To better predict the evolution of patients with 2 *SMN2* copies, it would be advisable to test for the presence of rare positive variants mentioned above. Indeed, in our experience, around 40% of cases with 2 *SMN2* copies and a milder phenotype (types II or III) may harbor one of these SNVs.[4,14]

Table 3 Suggested course of actions in symptomatic SMA cases, for whom phenotypes and genotypes are not correlated (see text for further details and discussion)

SMN2 copy number	Observed manifestations/ milestones clinical category	Expected clinical category	Rationale for recommended actions	Recommended actions
1	Patients with type I, II, or III SMA	0	Patients with 1 *SMN2* copy usually present congenital SMA. Patients with typical type I, II, or even III disease forms might point to an error in the initial *SMN2* copy number determination or to the presence of a positive modifier in their single *SMN2* copy.	Retest for *SMN2* copy number with a new sample and/or consider another method/laboratory. If the presence of a single *SMN2* copy is confirmed, test for SNVs in the gene that have been previously associated with better-than-expected phenotypes (e.g., c.859G>C[a] and c.835-44A>G), e.g., by Sanger sequencing, or perform next-generation sequencing (NGS) analysis.[19–21]
2	Typical type II or type III patients	Ia, Ib, Ic	The vast majority of patients with 2 *SMN2* copies have typical type I disease. Exceptions are usually due to the presence of positive *SMN2* modifiers.	Retest for *SMN2* copy number with a new sample and/or consider another method/laboratory. If confirmed, test for rare variants in *SMN2* that have been previously associated with better-than-expected phenotypes (e.g., c.859G>C[a] and c.835-44A>G). If negative, perform NGS analysis to detect novel SNVs or other changes that could be positive modifiers of disease severity.[19–21]
3	Type I cases with disease onset before the age of 3 mo (Ia; Ib)	Ic, IIa, IIb, IIIa, IIIb	Type Ic cases usually manifest disease between 3 and 6 mo of life, and have 3 *SMN2* copies. A typical type Ib patient has 2 copies of the gene. Three copies are also detected in type II and III patients.	Retest for *SMN2* copy number with a new sample and/or consider another method/laboratory. If *SMN2* copy number is confirmed, perform further studies to identify SNVs or partial intragenic deletions that could act as negative phenotype modifiers (e.g., complete MLPA,[11] NGS[19–21]).
≥4	Non-walkers, either type I or II	IIIa, IIIb, IV, MM	Most reported cases of type I or II SMA patients with 4 *SMN2* copies are due to pitfalls in the quantitation of *SMN2* copy number.	Retest for *SMN2* copy number with a new sample and/or consider another method/laboratory. If *SMN2* copy number is confirmed, perform further studies to identify SNVs or partial intragenic deletions that could act as negative phenotype modifiers (e.g., complete MLPA,[11] NGS[19–21]).

Abbreviations: MLPA = multiplex ligation-dependent probe amplification; MM = minimal manifestation; NGS = next-generation sequencing; SMA = spinal muscular atrophy; SNV = single nucleotide variant.
[a] A commercial test is available for the c.859G>C variant (table 4).

Negative variants in *SMN2* have not been discovered, but warrant further investigation.

In patients with 3 *SMN2* copies, our previous meta-analysis revealed that about 60% of cases develop type II disease, 35% type III, but 5% still had the more severe type Ic SMA.[4] Therefore, all neonates with 3 gene copies would be expected to have a normal appearance and to remain essentially asymptomatic at least for the first 3 months of life. The NURTURE study of presymptomatic patients with 2 or 3 *SMN2* copies treated with nusinersen has shown that patients with 3 gene copies treated in the neonatal period have in general a better evolution.[13] Again, here it is advisable to check for rare positive variants to better predict the expected outcomes.

The treatment recommendations for presymptomatic cases with 4 *SMN2* copies are still an evolving issue.[8,35,36] Based on available evidence, and in the absence of a reliable biomarker of disease evolution, in the United States, it has been recently recommended to initiate treatment of all infants with 4 copies of *SMN2*.[35] In our meta-analysis of 3,393 cases, patients with 4 copies accounted for less than 14% of all reported SMA cases.[4] In the light of this finding, it is rather surprising that in a recent pilot newborn screening study, 15 of 37 detected cases (40%) had 4 *SMN2* copies.[36] Excluding technical issues with *SMN2* quantitation, if these results are reproduced in other newborn screening studies, it would be tempting to speculate that a certain number of individuals in the general population with 0,4 genotype (i.e., no *SMN1* gene but 4 *SMN2* copies) remain with minimal symptoms or asymptomatic throughout their lives and thus undetected. Preliminary results of the SMA newborn screening program in Australia reported 9 positive cases, but none had 4 *SMN2* copies.[37] It is important to highlight that copy number studies in positive patients detected by newborn screening should be performed in expertise centers and with a validated methodology. In the shared decision to immediately start treatment of neonates with 4 *SMN2* copies or delay the initiation of treatment, several alternatives—each with advantages and disadvantages—have to be considered (outlined in table 5). Whatever decision is taken, it is important to recall that disease onset in these patients before the first year of life is rather unlikely, giving the health care team and the parents more time to weigh advantages and disadvantages of each

Table 4 Major features of the more commonly used methods to determine *SMN2* copy number

Method	Main characteristics	Advantages	Disadvantages
Real-time PCR (TaqMan platform)[22,23]	Multiplex TaqMan real-time quantitative PCR assay.	Fast, robust, and sensitive technique. Low cost. Requires only small amounts of DNA. Easy interpretation of results by automated variant reporting software. DNA quality important, but not as limiting as in other techniques. Neither a standard curve nor control samples are necessary.	Assays are performed in triplicate and an internal control is necessary to normalize results, as the method is based on relative quantitation. Only the number of copies of *SMN2* exon 7 is determined. Real-time PCR technology is not always available in a routine laboratory.
Real-time PCR (LightCycler platform)[25,26]	Quantitative assay on the basis of real-time PCR, performed with a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) by using the fluorescence resonance energy transfer technique (PCR products are based on the use of SYBR Green).	Low cost. Easy interpretation of results by automated variant reporting software.	As a relative quantitative assay, it requires standard curves to normalize results. DNA quality is limiting, large amounts of DNA are necessary, and the process is laborious. Only the number of copies of *SMN2* exon 7 is determined.
Multiple ligation-dependent probe amplification, version B1[10,11,27]	Variation of the multiplex PCR assay that permits amplification of multiple target genes with a single primer pair. Quantitates gene doses.	Low cost. Robust and sensitive. Requires only small amounts of DNA and its quality is not as limiting as in other techniques. Does not require sophisticated logistic (a therrmocycler and a vertical electrophoresis sequencer). Easy interpretation of results by automated variant reporting, free software (Coffalyser). *SMN1* and *SMN2* exons analyzed in the same experiment, detection of exonic hybrid genes, and partial intragenic deletions. False positive deletions may result from mutations located in regions of hybridization of the probes	As a relative quantitative assay, normalization of results is necessary, for which control samples are required. Long assay time (24 h minimum).
AmplideX PCR-CE *SMN1/SMN2* (asuragen.com)	Quantitative method based on a multiplex PCR and separation by capillary electrophoresis to calculate copy number of exon 7 in *SMN1* and *SMN2* genes, using specific fluorescently labeled primers. Quantitation is based on the peak area ratio of the target gene to an endogenous control, normalized to a calibration sample.	Robust and sensitive technique. Requires only small amounts of DNA. Simple and rapid workflow (<4 h). Does not require sophisticated technology or structural logistics (a thermocycler and a vertical electrophoresis sequencer). Easy interpretation of results by automated variant reporting software. Scalable design that allows the study of additional variants, such as hybrid genes, silent carriers, and 1 *SMN2* modifier variant (PLUS KIT).	As a relative quantitative assay, normalization is necessary, which requires calibration samples. Analysis limited to exon 7 of *SMN1* and *SMN2* genes, therefore unable to detect partial deletions.
Droplet digital PCR (ddPCR)[18,28,29]	DNA is partitioned into thousands of droplets that are subsequently amplified, and fluorescently labeled probe signals within each droplet are recorded as either positive or negative, depending on the presence or absence of a nucleotide target.	Eliminates the need for standard curves by using references or endogenous controls. As an absolute quantitation method of exons 7 and 8 in *SMN1* or *SMN2*, there is no need for normalization. Requires extremely low DNA concentrations (e.g., from dried blood spots).	The special technology necessary to perform ddPCR is not available in most laboratories. High costs limit determination of the copy number of all *SMN2* exons. Therefore, partial intragenic *SMN2* deletions are not detected.
Next-generation sequencing (NGS)[19–21]	Non–Sanger-based high-throughput DNA sequencing technologies (several platforms are currently available).	Allows analysis of complete genes (exons and introns) and to detect rearrangements and point mutations.	Not available in several diagnostic laboratories. Laborious process and longer assay times. Interpretation of results requires specialized bioinformatics tools and usually a bioinformatician. Quantitative studies using NGS are not very robust when *SMN1* and *SMN2* genes coexist due to their extremely high homology.

therapeutic alternative. Some parents may want to move forward without further testing, but it is crucial that an expert team adequately communicates about the disease and manages their expectations.[8] The implementation of neonatal screening in different regions will help to better define protocols of follow-up and validate biomarkers of disease progression such as levels of plasma phosphorylated neurofilament heavy chain in these patients.[38]

Different approaches should be considered when dealing with symptomatic cases. Here, most of the discrepancies should be initially faced with a retesting of the patient with a new sample, a different method or even in a second laboratory. According to the results of this second test, it might be advisable to continue testing for known variants in *SMN2*. A recent SMA test that includes testing for the NM_017411.3:c.859G>C variant has been made commercially available (table 4)

Table 5 Factors to be considered when deciding to treat neonates with genetically confirmed spinal muscular atrophy with 4 *SMN2* copies

Factors to consider	Treat presymptomatically	Treat when symptoms appear
Disease appearance and complications	Avoid possible long-term disease complications	Risk of long-term disease complications
Opportunity of treatment	Some patients might be unnecessarily treated for a long time	Depends on rescue of disease manifestations
Time to initiate therapy	Predicted large therapeutic window	Therapeutic window might be too short or lost
Adverse events when continuous therapy	Risk of treatment complications	Reduced risk of treatment complications
Economic aspects	Higher cost of therapy	Higher cost of managing morbidity
Quality of life (QoL) issues	Effect of years of treatment on QoL	Effect of disease on QoL
Parent and family expectations	Unease of treating a healthy baby	Increased stress during follow-up waiting for the imminent onset of manifestations

(asuragen.com). Furthermore, a new version of the SMA MLPA kit including all exons of the *SMN1* and *SMN2* genes has been reported. This new version of the kit would allow detection of some intragenic or 5′ terminal deletions that were previously extremely difficult to detect.[11] However, not all cases might be resolved with an accurate *SMN2* copy number assessment or checking for known variants by Sanger sequencing. If the results of all these studies are not categorical, *SMN2* NGS studies should be considered to determine whether the *SMN2* copies are functionally identical (table 4).[19–21]

Certainly, the *SMN2* gene, as the main modifier of SMA phenotype, deserves a more in-depth study beyond the current standard copy number determination. We believe that in terms of its impact on SMA phenotype, *SMN2* copy number might be considered as the tip of an iceberg of which other genetic and epigenetic features, most notably SNVs, represent the submerged part with relevant effects to phenotype of the patients with SMA (figure). A number of other genes have been proposed as candidate modifiers of the SMA phenotype including methylation status of *SMN2* (reviewed in Maretina et al., 2018),[39] although none of them are yet validated in clinical practice. Given that *SMN2* variants modify the disease phenotype and that transcripts derived from *SMN2* are targets for splicing modifiers in the therapeutic scenario, it is essential to gain a thorough insight into the complete *SMN2* sequences of discordant patients. Furthermore, we need to unveil possible linkages between specific *SMN2* variants and factors involved in *SMN2* splicing, on the one hand, and responses to treatment, on the other hand. In patients receiving expensive treatments, their efficacy should be periodically assessed to decide whether to continue treatment or to look for alternatives. Responses to treatment may vary in patients with SMA (from responders to slow responders to nonresponders),[40] but it is currently unknown whether specific features of their *SMN2* genes are directly correlated with these responses. Discovery and validation of positive and negative genetic markers remain thus an urgent matter in SMA research. New SMA classifications may need to be adopted in line with the current scenario of early genetic diagnosis, therapeutic intervention, and evolving phenotypes.[41] In this context, time to development of different manifestations and age at treatment initiation are becoming crucial as predictors of the trajectory of the disease.[9,42] In this envisaged perspective, a better and clearer definition of the *SMN2* genotype (copies and sequence) in each patient would be extremely relevant. Along these lines, our proposed guideline would help to systematically and rigorously identify discordant SMA cases that warrant further genetic investigation.

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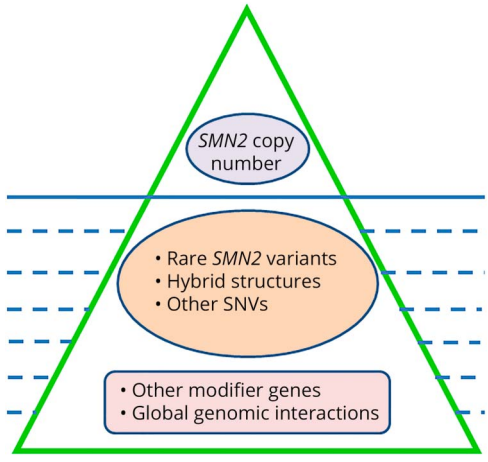
Figure The iceberg representation of the genetic factors that influence SMA phenotype



SMN2 copy number might be considered as the tip of an iceberg of which other *SMN2* genetic and epigenetic features, most notably *SMN2* SNVs, represent the submerged part. Other modifier genes and whole genomic data may complete possible influences. SMA = spinal muscular atrophy; SNV = single nucleotide variant.

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Disclosure

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