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.

*These authors are equal contributors with a major role in drafting and revising the manuscript and analyzing the data.

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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. 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. 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. 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 hypomorphoic allele that produces mainly transcripts lacking exon 7 (SMN-del7). The SMN-del7 protein is functionally compromised and unstable and therefore rapidly degraded by the ubiquitin-proteasome system. 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. Along these lines, around 40% of samples recently studied by the same methodology in different laboratories yielded discordant results. 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. 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). 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. 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. 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). 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, Ib, and c, IIa and b, IIIa and b, and the milder type IV SMA and even with minimal manifestations, as previously defined. 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.
are endorsed.

facing a presymptomatic patient, 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 Ibb, 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).

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

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

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

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 Ibb, 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.

The main characteristics of methods currently used to quantify SMN2 copies (TaqMan, LightCycler, MLPA, PCR-CE, and digital PCR) are given in table 4. 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 = 1,870) followed by LightCycler in 21.4% (n = 741) and TaqMan in 6.5% (n = 228) and fewer patients with the remaining methodologies. 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 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.

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
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,\textsuperscript{30} and diffuse and progressive brain abnormalities.\textsuperscript{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\textsuperscript{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\textunderscore{017411.3}:c.859G>C\textsuperscript{16} and NM\textunderscore{017411.3}:c.835-44A>G\textsuperscript{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.\textsuperscript{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).\textsuperscript{9} On the other hand, exceptional cases with 2 SMN2 copies may manifest overt disease at birth as usually occurs in type 0 cases.\textsuperscript{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.\textsuperscript{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.\textsuperscript{4,14}

<table>
<thead>
<tr>
<th>SMN2 copy number</th>
<th>Manifestations at birth (clinical category)</th>
<th>Expected correlation</th>
<th>Rationale for recommended actions</th>
<th>Recommended actions and expected phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Not observed (PS)</td>
<td>No\textsuperscript{*}</td>
<td>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.</td>
<td>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&gt;C\textsuperscript{b} and c.835-44A&gt;G). If negative, the patient has &gt;90% probability of developing severe, type I SMA.\textsuperscript{3} If positive, the patient will be virtually a sitter or walker later in their life.\textsuperscript{1,4}</td>
</tr>
<tr>
<td>2</td>
<td>Not observed (PS)</td>
<td>Yes</td>
<td>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.</td>
<td>Test for rare positive variants in SMN2 associated with better-than-expected phenotypes (e.g., c.859G&gt;C\textsuperscript{b} and c.835-44A&gt;G). If negative, the patient has &gt;90% probability of developing type II disease, 35% type III, and 5% type lc. Similar Bayesian estimations can also be calculated.\textsuperscript{4,25} If positive, the patient will be virtually a walker later in their life.\textsuperscript{1,4}</td>
</tr>
<tr>
<td>3</td>
<td>Not observed (PS)</td>
<td>Yes</td>
<td>Neonates with 3 SMN2 copies have a normal appearance and usually without manifestations at least for the first 3 mo of life.</td>
<td>Test for rare positive variants in SMN2 associated with better-than-expected phenotypes (e.g., c.859G&gt;C\textsuperscript{b} and c.835-44A&gt;G). If negative, the patient has about 60% probability of developing type II disease, 35% type III, and 5% type lc. Similar Bayesian estimations can also be calculated.\textsuperscript{4,25} If positive, the patient will be virtually a sitter or walker later in their life.\textsuperscript{1,4}</td>
</tr>
<tr>
<td>≥4</td>
<td>Not observed (PS)</td>
<td>Yes</td>
<td>Neonates with 4 SMN2 copies have a normal appearance. About 14% of SMA cases worldwide have 4 SMN2 copies.\textsuperscript{4}</td>
<td>Retest for SMN2 copy number with a new sample and/or consider another method/laboratory. If copy number is confirmed, the patient has &gt;90% probability of being a walker later in their life (SMA types III or IV).\textsuperscript{3} Test for the rare positive variants is an option.</td>
</tr>
</tbody>
</table>

Abbreviations: PS = presymptomatic; SMA = spinal muscular atrophy.
\textsuperscript{*} In this case, the expected category would be congenital (see text for further details and discussion). Percentages are calculated according to reference 4.
\textsuperscript{b} A commercial test is available for the c.859G>C variant (table 4).
The treatment recommendations for presymptomatic cases with 4 SMN2 copies are still an evolving issue. 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. In our meta-analysis of 3,393 cases, patients with 4 copies accounted for less than 14% of all reported SMA cases. 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. 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. 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 case.4
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. 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. 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>
<thead>
<tr>
<th>Method</th>
<th>Main characteristics</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real-time PCR (TaqMan platform)²²,²³</td>
<td>Multiplex TaqMan real-time quantitative PCR assay.</td>
<td>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.</td>
<td>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.</td>
</tr>
<tr>
<td>Real-time PCR (LightCycler platform)²⁵,²⁶</td>
<td>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).</td>
<td>Low cost. Easy interpretation of results by automated variant reporting software.</td>
<td>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.</td>
</tr>
<tr>
<td>Multiplex ligation-dependent probe amplification, version B¹⁰,¹¹,²⁷</td>
<td>Variation of the multiplex PCR assay that permits amplification of multiple target genes with a single primer pair. Quantitates gene doses.</td>
<td>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 thermocycler 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.</td>
<td>As a relative quantitative assay, normalization of results is necessary, for which control samples are required. Long assay time (24 h minimum).</td>
</tr>
<tr>
<td>AmpliDeX PCR-CE SMN1/SMN2 (asuragen.com)</td>
<td>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.</td>
<td>Robust and sensitive technique. Requires only small amounts of DNA. Simple and rapid workflow (&lt;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).</td>
<td>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.</td>
</tr>
<tr>
<td>Droplet digital PCR (ddPCR)¹⁸,²⁸,²⁹</td>
<td>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.</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>Next-generation sequencing (NGS)¹⁹–²¹</td>
<td>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.</td>
<td>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.</td>
<td></td>
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</table>
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. 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), 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), 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.

### Acknowledgment
The authors thank all colleagues for their confidence, information, and reference of patients for study.

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

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

(Asuragen.com). 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. Other modifier genes and whole genomic data may complete possible influences. SMA = spinal muscular atrophy; SNV = single nucleotide variant.

**Figure** The iceberg representation of the genetic factors that influence SMA phenotype
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Disclosure
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Publication history

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Practical guidelines to manage discordant situations of SMN2 copy number in patients with spinal muscular atrophy
Ivon Cuscó, Sara Bernal, Laura Blasco-Pérez, et al.
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