Neuromuscular Disorders 34 (2024) 114–122

Contents lists available at ScienceDirect

Neuromuscular Disorders

journal homepage: www.elsevier.com/locate/nmd

270th ENMC International Workshop: Consensus for *SMN2* genetic analysis in SMA patients 10–12 March, 2023, Hoofddorp, the Netherlands

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A B S T R A C T

The 270th ENMC workshop aimed to develop a common procedure to optimize the reliability of *SMN2* gene copy number determination and to reinforce collaborative networks between molecular scientists and clinicians. The workshop involved neuromuscular and clinical experts and representatives of patient advocacy groups and industry. *SMN2* copy number is currently one of the main determinants for therapeutic decision in SMA patients: participants discussed the issues that laboratories may encounter in this molecular test and the cruciality of the accurate determination, due the implications as prognostic factor in symptomatic patients and in individuals identified through newborn screening programmes.

At the end of the workshop, the attendees defined a set of recommendations divided into four topics: SMA molecular prognosis assessment, newborn screening for SMA, *SMN2* copies and treatments, and modifiers and biomarkers. Moreover, the group draw up a series of recommendations for the companies manufacturing laboratory kits, that will help to minimize the risk of errors, regardless of the laboratories' expertise.

1. Introduction and overview

The 270th ENMC workshop on the consensus for *SMN2* genetic analysis in spinal muscular atrophy (SMA) was held from the 10th to the 12th of March 2023, and brought together 20 representatives of patient advocacy groups and industry as well as neuromuscular and clinical experts from eight European countries and the United States.

Following a welcome from Alexandra Breukel, the ENMC Managing Director, and from the organisers of the workshop (Eduardo Tizzano, Enrico Bertini, and Danilo Tiziano), an overview of the topic was presented by **Giovanni Baranello, Eduardo Tizzano,** and **Janbernd Kirschner**.

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease characterised by progressive muscle weakness and atrophy due to alpha motor neuron degeneration in the brainstem and spinal cord. It is caused by biallelic loss of the *SMN1* gene (Survival Motor Neuron 1) [1]. An almost equal paralog gene, called *SMN2*, in the same chromosomic region has been described as the main modifier of SMA disease. The only functional difference between these *SMN* genes is a silent transition in *SMN2* exon 7 that causes exon skipping and the production of a truncated, non-functional protein in the majority of transcripts of the gene [2]. The number of *SMN2* copies may vary in the general population, usually between 1 and 4, but individuals with more than four copies have been reported; the higher the number of *SMN2* copies, the milder the SMA phenotype, even if the correlation is not absolute and some discrepancies may exist [3–5].

Functional and sequence differences of the *SMN2* genes may at least partially explain why patients with the same copy number may have different phenotypes; besides copy number, other factors have been reported, such as the presence of rare modifier variants, single nucleotide polymorphisms (SNPs), and hybrid genes [6–8].

From a clinical point of view, SMA was traditionally classified into types 1–4, based on age of onset and maximum motor acquisition; a further subtype classification was introduced to better define the inter-individual variability of the condition [9,10]. A new functional classification has been subsequently proposed that divides patients into non-sitters, sitters, and walkers [11].

Since 2016, three SMN-dependent treatments have been approved, changing the perspectives of the disease as well as the clinical care of patients. The approval of some of these treatments in several countries was limited to some types of SMA, a specific age group, or a determined number of *SMN2*

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https://doi.org/10.1016/j.nmd.2023.12.008
0960-8966

copies. The introduction of therapies has led to the appearance of new phenotypes, which no longer fall within the criteria of the standard classification. Therefore, the need for a new multi-functional classification system is increasingly evident, to monitor disease response and long-term outcomes [12]. Moreover, it has been demonstrated that the earlier the diagnosis and intervention, the better and more effective the treatment response and the outcome for the patient [13,14]. In this context, newborn screening programs (NBS) have become of crucial importance and have been gradually implemented in many countries around the world [15,16].

In previous years, *SMN2* copy assessment was mainly informational and mostly used to retrospectively elaborate genotype-phenotype correlations; currently, in the treatment era, this genomic biomarker is the main determinant of therapeutic decision, especially in patients who do not display clear signs of SMA. Whereas genetic confirmation of SMA is relatively straightforward (around 97–98 % of patients can be diagnosed with a simple qualitative test), *SMN2* copy number assessment requires quantitative methodologies that are not easily implemented in most laboratories. Along these lines, surprising discordance between laboratories (around 40 %) has been previously reported [17].

The main aim of this workshop was the development of shared guidelines to optimise and standardize the reliability of *SMN2* gene copy number determination among referral laboratories, and the strengthening of *SMN2* gene collaborative networks between laboratory and clinical settings. The results of this workshop will be useful for the definition of standard operating procedures for *SMN2* copy number determination to be spread to all laboratories performing SMA molecular diagnosis and prognosis.

2. SMA NBS in Europe and worldwide

Enrico Bertini introduced the session on SMA NBS, by discussing the results of five years of follow up in the Nurture study, an ongoing trial to evaluate the safety and efficacy of nusinersen in infants with two or three *SMN2* copies, who initiated treatment within the first five weeks of life, before the onset of overt clinical signs or symptoms of SMA [13]. The 10 infants with three *SMN2* copies, who had comparably the best baseline values in WHO, CHOP INTEND, and HINE-2 scores and in CMAP (Compound Motor Action Potentials), showed physiologic motor developmental milestones. Overall, in the group of the 15 infants with two *SMN2* copies, a certain number had comparable lower functional motor performances: 7/15 (46 %) showed some motor delay and 4/15 (26 %) needed respiratory intervention. Similar results were demonstrated in a real-time experience in a cohort of infants with two *SMN2* copies [18]. In conclusion, *SMN2* copy number alone is useful to predict functional motor outcomes in infants diagnosed within few weeks of life. The predictive utility of the baseline levels of serum neurofilaments is still under evaluation, while the CMAP derived by stimulation of the ulnar nerve showed that the mean potential amplitude was lower in newborns with two *SMN2* copies compared to those with three copies, with no overlapping of standard deviation intervals between the two groups [13].

Tamara Dangouloff gave an overview of the worldwide SMA NBS situation since her group conducted two surveys on this topic. In 2021, information was collected from 87 experts from different countries/regions, and nine SMA NBS programs were ongoing at that time: these programs detected 288 SMA newborns out of 3,674,277 newborns screened [15]. The second study is still ongoing [19], but it is already depicting a quickly evolving scenario: the number of NBS programs has expanded rapidly, with at least 30 countries (20 in Europe) having started SMA NBS as official or pilot programs; also the treatment options in the different countries have increased. The proportion of children screened for SMA has grown rapidly, but is still very uneven across the world, largely due to inequalities in access to treatments.

Wolfgang Müller-Felber presented the current situation of SMA NBS in Germany. After a pilot project between 2018 and 2021, nationwide NBS was implemented in 2021. As several laboratories with different methods and numerous paediatric clinics were involved, harmonisation of the entire process was required [20]. Stakeholders defined criteria for clinical centres participating in the program to enable the treatment of affected children within the limited timeframe needed for optimal outcomes. At the beginning of the nationwide screening phase, there were 4/50 false positive results: feedback was provided to the participating laboratories and the method was optimised. The time to confirmation of diagnosis, including estimation of *SMN2* copy number, was the same as the pilot project (median 13 days of life). However, as in this phase more parents opted for gene replacement therapy, which initially required additional time for payer approval, the time to initiation of treatment was slightly longer (19th versus 26th day of life).

As reported by **Monika Gos**, SMA NBS was successfully and quickly implemented in Poland in April 2022 thanks to the good cooperation between clinicians and SMA patient organisations. The countrywide screening is coordinated by a single Department and molecular tests are performed in two labs for the entire Country. By March 9th 2023, 63 newborns had had a positive screening test, with an estimated SMA incidence of 1/7286. The results of the first-tier test from crude DNA extracts [21] were available on the 8th day of life (mean: 9.0 $\pm$ 3.6); those for the confirmation test from venous blood were available on the 14th day of life (mean 15.6 $\pm$ 5.9).

The majority of patients were diagnosed with two or three *SMN2* copies (about 30 % and 40 %, respectively) and were treated with nusinersen or onasemnogene abeparvovec. Two patients were diagnosed with SMA type 0: one of them received nusinersen, the other palliative cares, due to the severe phenotype and the presence of a congenital heart defect. Patients with four or five copies (20 % and 4 %, respectively) did not receive therapy immediately but underwent regular assessment and began treatment when SMA symptoms appeared.

Emanuela Abiusi described the Italian situation by March 2023. Differently from Germany and Poland, the situation was heterogeneous among the different regions of the country, although the national implementation of screening had been promulgated. Following the two-year pilot study limited to the Lazio and Tuscany regions [22,23], five more regions undertook SMA NBS programs, covering about 27 % of Italian newborns. As planned, by the end of 2023 other nine regions will start SMA NBS, bringing the coverage to about 73 %. With the spreading along the national Italian territory, there are several differences among the various programs planned by the different Italian regions, both in terms of techniques and costs/sample; moreover, while some SMA NBS are public healthcare activities, others are still research-based pilot projects.

She also gave an update on SMA NBS in Lazio and Tuscany by the end of the pilot project: 40 % of the overall patient cohort showed signs of SMA at birth (all with *SMN2* $\leq$ 2) but they also detected about 13 % of patients (3/23) with more than four *SMN2* copies. Finally, as they tested the *SMN2* modifier variants c.859G>C (p.Gly287Arg) and c.835-44A>G [24,25], they identified a patient carrying two *SMN2* copies, the c.859G>C variant, and no signs of the condition at birth; the patient was treated as a child with two *SMN2* copies, due to the lack of prospective data on patients bearing the variant.

In Belgium, according to **François Boemer,** a three-year pilot SMA-NBS program was launched in the region of Liège in 2018 and was rapidly expanding to all of southern Belgium [26,27]. During this pilot program, 136,339 neonates were tested and nine SMA cases were identified, with an estimated incidence of 1/13,634 newborns. All patients identified began treatment before the age of two months. A false negative case, compound heterozygous for the *SMN1* deletion and a missense variant (c.815A>G; p.Tyr272Cys), was identified after symptoms appeared at the age of four months. The pilot program is now successfully transformed into an official neonatal screening program in Southern Belgium [28].

Marie-Christine Ouillade described the activities of the European Alliance for Newborn Screening in Spinal Muscular Atrophy, established by SMA Europe, which plays an important role in tracking the implementation of newborn screening for SMA across Europe and supporting relevant efforts. In particular, the Alliance disseminates quality information about SMA NBS (such as the White Paper on Newborn Screening in SMA available from its website), supports national initiatives focused on SMA NBS, and highlights clinical, health-economic, and human considerations. The Alliance also looks into optimizing information being provided to families of children identified through SMA NBS – Marie-Christine stressed that, unlike in the past, the accuracy of *SMN2* determination is crucial for patients when discussing therapy choice. In addition, she underlined the need to collect natural history data of patients treated at neonatal age.

3. *SMN2* copy number and prognosis

Wolfgang Müller-Felber presented the situation of *SMN2* copy number and treatment decisions in SMA NBS patients. There is clear support to treat all children with two and three *SMN2* copies as soon as possible, especially those with two, in whom the treatment is urgently needed because rapid disease onset is expected. Ethical aspects must be considered in patients with one *SMN2* copy, as the decision to treat depends mainly on the expected quality of life; so far, of the few children identified, most remained untreated.

The approach to children carrying $\geq$ 4 *SMN2* copies varies worldwide and ranges from not reporting the result to early treatment recommendation. Considering that the incidence rate of SMA did not increase after the initiation of NBS, it can be assumed that all patients will develop symptoms throughout their lifetime [29]. The reasons why parents decided against treatment were fear of long-term side effects and lack of conviction of the need for treatment. In Germany, 55 % of parents of children with four *SMN2* copies opted for treatment in the pilot studies, whereas 45 % initially took a wait-and-see approach; three of the latter children were lost at follow up. Six of these patients (27 %) developed symptoms between 1.5 and 4 years of age [20].

The choice of a particular drug for treatment depends mainly on regulatory approval criteria. In Germany, most parents of children with two or three *SMN2* copies opted for gene replacement therapy. The psychological burden on families was higher in treated patients compared to patients who underwent a wait-and-see strategy [30].

SMN2 copy number and treatment decision in symptomatic SMA patients was discussed by **Janbernd Kirschner**. The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) hold different positions regarding the regulation of SMA treatments: FDA labels do not take into account the number of copies, whereas EMA does. Within the SMArtCARE registry, the response to nusinersen was evaluated in SMA patients from 64 participating centres. SMA type 1 patients (non-sitters) showed a probability of gaining independent sitting and the improvement was greater in those patients who started the treatment before two years of age [31]. In patients with SMA type 2/3 (sitters), improvement in motor function was more prominent in upper limbs; a correlation between the number of *SMN2* copies and response to nusinersen treatment was shown. Lastly, a ceiling effect in the Hammersmith score was observed in SMA type 3 (walkers) patients treated with nusinersen [32].

In symptomatic patients, age of onset, disease duration, and severity are very important factors in putting forth a prognosis and predicting response to treatment. Although *SMN2* copy number is the most important prognostic marker for disease severity, it is unclear whether it has a predictive value for the clinical course and treatment response in patients who display comparable phenotypes. Therefore, the possible effect of *SMN2* genotype on the response to treatment requires additional research and a deeper analysis, including the identification/analysis of modifier variants and of hybrid structures. Finally, the epigenetic regulation of *SMN2* expression/alternative splicing and other modifier genes, outside the SMN locus, need to be evaluated.

As a representative of SMA Europe, **Kacper Rucinski** described the management of prognosis information from a patient's point of view. Families or patients usually do contact patient groups or organisations at diagnosis and/or when discussing a disease-modifying therapy. In symptomatic patients, the prognostic factors often discussed include duration and evolution of the disease, response to treatment, likelihood of acquiring new or losing existing motor skills, choice of type of treatment according to the age, SMA type, number of *SMN2* copies, access to standard of care. This approach has radically changed with the introduction of the SMA NBS, as the only prognostic factors available to establish the timing of treatment of early-identified patients are clinical evaluation of patient and *SMN2* copies. The possible compliance of the family to the long-term follow-up programs and repeated administration of treatments may orientate the choice of treatment. Lastly, another point addressed was the perception of genetic and clinical information on SMA by families, which is crucially influenced by time and quality of communication of healthcare providers, education and psychological factors of parents, and information from public sources (patients advocacy groups, web information, public noise).

4. *SMN2* genetics and interactions

Pascale Saugier-Veber gave an overview of *SMN1* and *SMN2* copy number in the general population. Given the highly repetitive and homologous sequence found in the human SMN locus, the number of copies of *SMN1* and *SMN2* is variable, both in healthy people and in SMA patients. The *SMN2* copy number varies from zero to up to eight in the general population [33]. Interestingly, this number is significantly higher in patients than in the general population; conversely, a higher number of *SMN1* is associated with decreased *SMN2* copy number in the general population, suggesting *SMN2* to *SMN1* gene conversion [34].

Taking into account the complex genomic architecture of the SMN locus, partial gene deletions and hybrid genes have also been observed. Partial deletions of *SMN1/2* mediated by Alu/Alu rearrangements and lacking exons 7/8 are detected in 20 % of individuals in Caucasian populations [35]. Hybrid genes also support the hypothesis of *SMN1*⇄*SMN2* gene conversion events. As some investigations have found that SMA patients harbouring hybrid genes have milder clinical phenotypes, it is hypothesised that some of these hybrid structures may produce greater amounts of SMN protein than expected [36].

Marta Codina-Solà and **Mar Costa-Roger** provided data regarding *SMN2* copies, point and structural variants, in SMA patients. A meta-analysis of 3393 SMA cases from the literature showed that *SMN2* copy number is a good predictor of SMA type

however, better-than-expected or worse-than-expected patients (i.e., patients who perform better or worse with respect to the expected phenotype, based on *SMN2* copies) were described [5]. The reasons for discrepancies in *SMN2* copies and phenotype-genotype correlations can be technical or biological. On one hand, technical reasons may often cause unexpected results, due to an incorrect estimation of *SMN2* copies; these reasons could include, among others, erroneous information or patient classification, poor quality and/or low DNA concentration, quality, number and type of control samples used for calibration of quantitative assays, and sequence mismatches in the primer hybridisation regions. On the other hand, also biological reasons need to be considered, such as sequence variants with positive or negative effect on alternative splicing/expression, epigenetic regulation of *SMN2* copies, and hybrid genes, among others [17,37,38]. Thus, levels of full-length SMN protein in SMA patients are influenced not only by copy number of *SMN2* (quantity) but also quality (SNVs, structure, etc.).

In order to identify *SMN1* and *SMN2* copy numbers, positive modifiers, and hybrid structures, a specific Next-Generation Sequencing (NGS) method for *SMN1/2* characterisation was developed in their group [7] and more than 400 SMA patients have already been sequenced. They characterised in depth a total of 11 SMA patients with a milder phenotype carrying the c.859G>C modifier [24], finding two different haplotypes associated with the variant, regardless of the ethnic lineage of the patients [39]. Regarding the positive modifier c.835–44A>*G* [25], they found four SMA patients with a milder phenotype and are currently collecting more individuals. Lastly, they identified a total of 45 SMA patients carrying hybrid structures (12 %, 45/372); the NGS assay allowed to identify hybrid structures undetectable by Multiplex Ligation-dependent Probe Amplification (MLPA) (P021 assay, MRC-Holland) as well as to fully characterise the hybrid gene structures.

Ewout Groen presented their ongoing project focused on the application of long-read NGS to *SMN2* gene study. Bearing in mind the aforementioned complexity of the SMN locus, standard short-read sequencing encounters many technical limitations in the characterisation of this region. Using novel, long-read sequencing methods, it is now becoming possible to address these challenges. Recent studies using these technologies, including Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio) sequencing methods, have started to provide further insights into the structure and sequence variation of the SMN locus of healthy, non-SMA individuals. For instance, the Telomere-to-Telomere Consortium (T2T) was able to resolve 6 out of 24 predicted SMN haplotypes in their recently published T2T draft of the human genome [40,41]. In this context, they are starting to combine novel long-read sequencing methods with extensive phenotyping in a cohort of 400 SMA patients. Using both bioinformatic and CRISPR/Cas9-based approaches, it is possible to obtain highly targeted sequencing reads of the *SMN* locus using ONT technology, always dependent on DNA quality. These reads are on average >60 kb, allowing us to cover the entire *SMN* gene length. Using paralogous sequence variants (PSVs), SNPs, and other variants, it is subsequently possible to perform copy-specific analyses to identify variants that differ between *SMN2* copies in individual patients. This approach will be relevant to elucidate further undiscovered genetic variation at the SMN locus, and of particular interest in discrepant SMA patients.

Arthur Burghes described pitfalls and discrepancies in the assessment of *SMN2* copy number. His group has developed a droplet-digital-PCR (ddPCR) assay, used as primary assay. There are some important considerations to improve accuracy of this method such as the use of some locked nucleotides (LNA) in the probes and the inclusion of controls with known *SMN2* copy number in all runs. Interestingly, when comparing a cohort of worse- or better-than-expected patients, tested in different laboratories by other methods, they found a discordance rate of 9 %, although the population analysed was biased.

He also presented a previous study in which they performed an association analysis and identified two variants in *SMN2* (c.835–549A>G and c.835–44A>G) associated with a milder phenotype [42]. In this context, they have performed whole genome sequencing in twelve SMA sib-pairs with the same *SMN2* genotype (five phenotypically concordant and seven discordant) to identify further modifiers outside the *SMN* locus. The association with phenotype severity of variants with a presumed dominant mode of inheritance has been progressively tested and excluded. Other potentially interesting candidates with a recessive pattern of inheritance were identified and are currently under evaluation by targeted sequencing in a wider population of patients.

Henny Lemmink represented the European Molecular Quality Network (EMQN), which aims to help raise and maintain the standards of diagnostic clinical genomic testing, a leading authority in quality assurance and providing quality reports (https://www.emqn.org). Every year, they offer a scheme of three different cases with a request for diagnostic, carrier, or prenatal analysis of DNA samples including the DNA samples and mock clinical referral information. EMQN assess the reports of participating laboratories according to the following criteria: 1) correct *SMN* genotypes in suspected SMA cases; 2) interpretation of results in a clear and concise format; 3) correctly use standard nomenclature; and 4) accuracy regarding patient and sample information and identifiers.

A total of 92 and 99 laboratories participated in 2021 and 2022, respectively. Labs used PCR-based methods to determine *SMN1* and *SMN2* dosage (gene copy number) in exons 7 and 8, being MLPA the most commonly used technique. Real time and fluorescent PCR were also frequently used; other techniques have been introduced, such as targeted NGS, whole exome or genome sequencing. In the context of SMA carrier testing, an important issue was the report of *SMN2* copies. Inexplicably, 20 % of the labs do report *SMN2* gene copy number in SMA carrier analysis, which is not clinically relevant and distracts from the identified number of *SMN1* gene copies. This might lead to confusion to non-expert readers and is a point that all laboratories should be aware of.

Lastly, genotyping errors were reported by different laboratories both in 2021 and 2022. In fact, in the 2022 scheme, five different laboratories reported a total of five *SMN2* genotyping errors and seven labs reported eight critical *SMN1* genotyping errors. These results highlight the importance of reassessing *SMN1/SMN2* testing protocols, including the use of validated positive and negative controls isolated via the same method as patient DNA samples.

5. SMA modifiers and biomarkers beyond *SMN2*

Maria Jędrzejowska introduced the topic of discordant siblings; almost every SMA sibship exhibits mildly different clinical courses, however, the coexistence of various forms of SMA within one pair of siblings is not uncommon.

The exact frequency of this phenomenon is unknown. Among the 303 sibships collected in the Cure SMA database, 15.2 % had discordant phenotypes [43]; the most common was the combination of SMA type 2 and 3 (52.2 %) but extremely discordant phenotypes i.e., SMA type 1 and type 3 or 4 were also rarely reported. The Polish SMA database collected 115 sibships, mainly with chronic forms; discordant phenotypes were observed in 35 sib pairs (30.4 %), mainly suffering from chronic forms.

In very rare cases (>20 reported), asymptomatic subjects with biallelic deletion of *SMN1* have been reported as siblings of patients (or parents as well) with chronic forms of SMA [44–47]. In these families, the predominance of females among asymptomatic subjects was observed. The number of asymptomatic individuals with *SMN1* deletion can be estimated at around 0.5–0.7 %

among first-degree relatives of patients with SMA [46,48,49]. The frequency of this phenomenon in the general population is unknown, however, presumably rare. In the largest population analyses, no asymptomatic subjects with *SMN1* deletions were identified in 68,471 and 107,611 individuals, respectively [50,51].

Most discordant siblings reported in the literature were haploidentical at the SMA locus, suggesting the influence of other phenotype modifiers [44,45,47,52]. An exception was a case described by Prior, having the proband and the sib two and five *SMN2* copies, respectively [48]. Lately, a discordant sibship (a boy affected from SMA type 1 and a girl with type 3a) with different *SMN2* copies (two and three, respectively) was identified in Poland. Genetic differences at the SMN locus may explain the occurrence of extremely different forms in a sibling pair.

Besides *SMN2* copies and other factors that may influence *SMN2* transcription, alternative splicing, translation, and stability (both mRNA and protein), there are other reported genetic modifiers, independent of the SMN locus, that can influence SMA severity. Among these, **Brunhilde Wirth** and her group identified Plastin 3 (*PLS3*) and Neurocalcin Delta (*NCALD*) in SMA discordant families with *SMN1*-deleted siblings being fully asymptomatic [45,53].

PLS3 is an X-linked gene overexpressed in asymptomatic females; its overexpression, either via a transgene or by gene therapy, rescues the SMA phenotype across different species [54]. Recently, the genetic mechanism causing *PLS3* overexpression in females was identified. The gene is located close to the *DXZ4* macrosatellite and Strathmann *et al*., found a strong linear correlation between the length of the repeat units and *PLS3* levels, related to an escape from X-inactivation [55]. Additionally, they identified another epigenetic transcriptional regulator of *PLS3*, chromodomain helicase DNA binding protein 4 (*CHD4*) [55].

NCALD is downregulated in less severely affected patients; for this reason, Wirth's group developed mouse and human *NCALD*/*Ncald* ASOs (antisense oligonucleotides), downregulating gene expression. SMA mice injected with a low dose of SMN ASOs (increasing exon 7 inclusion in *SMN2* mRNA) and *Ncald* ASOs showed improved SMA electrophysiological hallmarks and neuromuscular junction pathology. Moreover, they identified a human *NCALD* ASO that was highly efficient, non-toxic, and well tolerated in motor neurons differentiated from human induced pluripotent SMA stem cells (iPSCs); SMA lines treated with *NCALD* ASOs showed improved growth cone maturation hallmarks and multielectrode array analysis [56].

As reported by **Danilo Tiziano**, currently, there are no effective biomarkers of SMA and further collaborative studies are required. Reliable biomarkers are important, not only to follow the disease course in symptomatic patients but also to monitor the progress of therapies and to make a prediction of prognosis in patients detected by SMA NBS.

SMN products (both transcript levels and proteins) have been extensively studied as pharmacological markers [57,58], however, they display some limitations. Firstly, they are applicable only for systemic treatments, and secondly, the effective correlation of SMN products baseline levels or variations with clinical efficacy has never been effectively demonstrated. Finally, while there is a generalised increase in response to treatments, they are not predictive of response in individual patients.

Neurofilaments (pNf-H and Nf-L) are another promising class of biomarkers in both serum and cerebrospinal fluid [59,60]. It has been shown that patients with two *SMN2* copies show significantly higher serum Nf-L levels than controls [13]. However, patients with a higher number of copies are not significantly different from controls. Furthermore, due to the demonstrated variability of neurofilament levels in healthy subjects, longitudinal data on SMA patients are lacking and necessary. Again, their ability to predict performance in individual patients is limited perhaps only to baseline values in SMA newborns detected by screening and, for this reason, they are not yet suitable for decision making in longitudinal monitoring.

Several miRNAs have been identified as deregulated in serum samples of SMA patients and some showed a response after treatment with nusinersen [61–64]. However, the lack of longitudinal data as well as replication studies, call into question their applicability as biomarkers.

Muscle MRI, although it identifies significant abnormalities in patients, does not show substantial changes after treatment [65]. Moreover, its feasibility for younger patients is still debated.

Finally, CMAP and serum creatinine levels are other candidate biomarkers that have been endorsed following clinical trials and investigations addressed to newborn or infants with SMA [13,60,66,67], however there is still need for collecting data in a real life setting to evaluate the predictive power in individual patients.

6. General questions about *SMN2* copies and phenotypes

Eduardo Tizzano and **Danilo Tiziano** discussed *SMN2* copies in the neonatal setting, taking into account the current NBS scenario. In this context, when a positive case is found, the number of *SMN2* copies will be used as a prognostic tool:

1) SMA cases with one *SMN2* copy will be mainly symptomatic at birth or already *in utero*. In this case, given the high possibility of having a residual disability, the choice is whether to treat or rather opt for palliative cares;
2) SMA cases with two or three *SMN2* copies need to be treated upon identification, regardless of the presence of known positive modifier variants (c.859G>C or c.835-44A>G);
3) For SMA cases with $\geq$ four *SMN2* copies, the debate is still open. Due to the likelihood of late onset, most centres initially opted for a wait-and-see approach [68]. However, in 2020, the Cure SMA Working Group updated their recommendations, arguing that sufficient new clinical data and real-world experience were available to recommend immediate treatment for infants with four *SMN2* copies diagnosed via newborn screening. As an example, in the German NBS study, 5/7 patients with confirmed four *SMN2* who underwent a strict follow-up strategy, showed clinical or electrophysiological disease onset between 1.5 and four years of age; in two of them, there was no complete recovery, despite immediate initiation of treatment after the onset of the first symptoms [20].

Giovanni Baranello and **Susana Quijano-Roy** discussed symptomatic patients with four *SMN2* copies and reported the experiences of the UK and French registries. It turned out that, in several patients, there might be an overestimation of *SMN2* copy number because, despite having four copies, they are non-sitters and with an onset at under one year of age.

Moreover, they introduced the four *SMN2* copies working group, which will be coordinated by Eduardo Tizzano and Susana Quijano-Roy, with the aim of studying the natural history of SMA patients with four *SMN2* copies. This initiative will start with the identification of cases and the collection of preliminary data through a 15-item questionnaire; subsequently, *SMN2* copy number determination will be performed, collecting DNA whenever possible. If discordant results are found, it will be necessary to retest these cases, preferably in a third laboratory.

The group of participants to this workshop proposed retesting all doubtful cases in independent laboratories. In this regard, the following scheme was developed to identify patients suitable for retesting (Table 1).

Table 1
Proposal of patients to be retested based on the disagreement between phenotype and *SMN2* copy number. Cases where a new test is recommended (preferably in another laboratory) are those marked with an x.

	SMA Type				
#SMN2	**SMA 0**	**SMA 1**	**SMA 2**	**SMA 3**	**SMA 4**
1			x	x	X
2	x		(x)	x	X
3	x				X
4	x	x	x		
5	x	x	x	x	X

7. Methodologies/companies: how can we adapt to fill the gaps?

Danilo Tiziano moderated the session dedicated to methodologies for *SMN2* copy assessment. Representatives from companies producing molecular assays for *SMN2* copy number assessment (Asuragen, Bio-Rad, MRC Holland, Thermo Fisher) were invited, independent of the technique sold. Non-commercial assays were also taken into account [69–73].

As already mentioned, *SMN2* copy number is one of the main stop-or-go tools for initiating the treatment of patients, especially if identified in the context of NBS programs. Therefore, an inaccurate determination may be very harmful to patient health. A comparative study of *SMN2* copy number carried out by four reference laboratories on blind samples showed an inter-rater reliability of 0.52; these data indicate the need to make a collective effort to optimise the sensitivity and specificity of the test. Moreover, with the increasing number of NBS programs, the number of labs performing this molecular test will increase, and most having almost no experience in SMA.

The rationale of this session was not to establish which assay is the best, since this information could only come from comparative studies, but rather to obtain a commitment from companies to support scientists in standardising *SMN2* copy number assessment, whatever the assay is. For this reason, at the end of the presentations, the assembly agreed to make its expertise available to develop a series of recommendations for companies (Table 2). The 270th ENMC international workshop marked the beginning of a collaboration between academia and industry for the optimisation of the determination of *SMN2* copy number.

8. Workshop key deliverables

At the end of the workshop, the attendees defined a set of recommendations divided into four topics, to optimise the reliability of *SMN2* gene copy number determination.

SMA molecular prognosis assessment:

- It has been verified that errors in *SMN2* quantification are too frequent, particularly in less-experienced laboratories. Certification and quality controls of the labs are, therefore, strongly recommended;
- The use of validated technology that allows determination of the exact *SMN2* number is recommended;
- It is therefore strongly recommended that the test is carried out using the same DNA extraction procedures and tissue-of-origin for testing samples and controls;
- *SMN2* modifier variants (c.859G>C and c.835-44A>G) should be routinely tested and reported (also in NBS). Sanger sequencing is recommended for the analysis of these variants;
- It is not clear whether hybrid genes have clinical implications in SMA patients. Thus, exploratory collaborative studies are strongly recommended to provide evidence regarding this topic;
- Reports should state the technology used, the exact number of *SMN2* copies (avoiding formulas like *SMN2*≥4) and *SMN2* modifier variants;
- For the management of worse- or better-than-expected cases, retesting the sample by a different method and/or in another laboratory is recommended; if the result is still uncertain, resampling is suggested (see Table 1);
- The group has agreed on the creation of a European network for patients with more than four *SMN2* copies in order to share protocols, exchange biological samples if necessary, and support Centres with less experience in the most complex diagnoses;
- The attendees drew up a series of recommendations for companies producing tests for the determination of *SMN2* copy number that will help minimise the risk of errors, regardless of the expertise of individual laboratories (see Table 2).

SMA NBS:

- All participants agreed on the need to establish a clear workflow, with an optimised communication process, for diagnosis and management of patients identified by SMA NBS. It is strongly recommended that neuromuscular and genetic experts take part in the first meetings with the family, in order to provide more comprehensive information and perform an accurate clinical assessment of the child that is crucial at this stage;
- The confirmatory test and the evaluation of prognostic markers should be performed by expert reference centres, officially identified;
- In case of positive screening results, a consultation with the family should be carried out ideally within three working days and the results of the confirmatory test should be delivered within seven working days. The confirmatory test should be performed on a new sample, collected when the suspicion of SMA is communicated to the family. It is necessary to optimise the administrative path for treatment authorisation that can be adapted globally depending on the health reimbursement system of each country, to prevent possible delays;
- *SMN2* copy assessment should be performed on Dried Blood Spot (DBS) DNA only on purified samples and if quantity

Table 2
Recommendations for companies producing kits for *SMN2* copy number assessment.

1. In the kit protocol, the quantity and quality of DNA required per sample should be specified. The risk of saturation of the assay or of under-amplification is not negligible.
2. It is requested to openly discourage the use of crude DNA extracts from DBS for the determination of *SMN2* copy number.
3. It is necessary to report the exact number of *SMN2* copies, avoiding formulas like *SMN2* ≥ 4.
4. It is necessary to include patients with *SMN2* > 2 in spreadsheets to improve the test yield in patients with a high number of copies.
5. It is also advisable to provide indication on how to obtain verified control samples with high *SMN2* copy number (≥3) to determine the number of copies.

and quality are adequate for quantitative approaches. Control samples should be extracted through the same approach as DBS-DNA;
- Interaction between the screening centre and clinicians is highly recommended, particularly in the case of symptomatic patients;
- SMA NBS should be carried out as soon as possible, also in preterm infants;
- Regarding blood transfusion, risk of DNA contamination is possible and in the ideal situation, DBS for SMA should be collected before transfusion. If collected after transfusion of red blood cell concentrates, test may be perfomed but should be repeated after 2 months since transfusion.
- *SMN2 copies and treatments*:
- In symptomatic patients, access to therapy should be independent of *SMN2* copies. Given the principle of autonomy, treatment initiation is the final decision and responsibility of the family;
- From the biological/physiopathological point of view, early treatment of asymptomatic newborns with four *SMN2* copies patients is advisable to avoid the risk of early disease manifestation that may worsen the therapeutic outcome. Individual risks and benefits should still be discussed with the family;
- Retrospective studies on patients with four copies with re-evaluation of the copy number are highly encouraged. A European patient registry with cases harbouring four *SMN2* gene copies has been proposed to better define the natural history of these patients to obtain the approval of treatments by the Regulatory Agencies;
- The implications of *SMN2* modifier variants and hybrid genes for treatment are not currently known and, therefore there is no indication that the therapeutic approach should be altered. Collaborative studies are recommended to obtain longitudinal data in carriers of these variants;
- Given the lack of high-quality comparative studies, no alternative to single treatment is recommended at present.

Modifiers and biomarkers:

- At present, no reliable biomarkers are known that would allow to assess treatment response and the prognosis (especially in case of patients with four *SMN2* copies). International collaborative studies are highly recommended.
- The deeper study of *SMN2* (by sequencing, structure, and topography) may allow a better correlation with the phenotype. Other genomic modifiers need to be discovered and further investigated.
- Other biomarkers such as muscle MRI, serum neurofilament count and serum creatinine count, are recommended for further investigations and validation.

9. Conclusions

The assessment of *SMN2* gene copy number is currently one of the main determinants for therapeutic decisions for SMA patients, especially those identified via NBS, and requires quantitative methodologies that are not easily implemented in most laboratories. This workshop, involving the stakeholders of the field, provided an overview of the current state of the art of this topic and, more importantly, offered an opportunity to discuss any issues that may lead to erroneous results in *SMN2* copy number determination. The attendees agreed to establish a standard operating procedure for *SMN2* gene copy number assessment and a specific workflow for diagnosis and management of SMA patients identified in neonatal screening as well as collaborative efforts for the creation of a European network that would address discrepancies between expert centres.

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Funding

This workshop was supported by the SMA NBS Alliance.

Declaration of competing interest

E.A. has no conflict of interest to declare M.C.R. has no conflict of interest to declare E.S.B is advisor/consultant for AveXis, Biogen, Edison, Novartis, and Roche; grants from Fondazione Telethon and the Italian Ministry of Health. F.D.T. has received independent research grant on SMA newborn screening by Biogen and serves as consultant to Biogen, Novartis and Roche E.F.T. discloses grant support to conduct CTs and Research on SMA from Ionis/Biogen and Roche and serves as a consultant to AveXis, Novartis, Biogen, Biologix, Cytokinetics, and Roche.

Acknowledgements

The workshop and early-career programme are made possible thanks to the financial support of the European Neuromuscular Centre (ENMC) and its Full Partners: Association Française contre les Myopathies (France), Deutsche Gesellschaft für Muskelkranke (Germany), Muscular Dystrophy Campaign (UK), Muskelsvindfonden (Denmark), Prinses Beatrix Spierfonds (the Netherlands), Schweizerische Stiftung für die Erforschung der Muskelkrankheiten (Switzerland), Spierziekten Nederland (the Netherlands), Telethon Foundation (Italy). In addition, we would like to thank the Associated Partners: Finnish Neuromuscular Association (Finland), Österreichische Muskelforschung (Austria), SMA Europe, and World Duchenne Organisation, and the members of the ENMC Company Forum: Amicus Therapeutics, Astellas, Biogen, Ionis Pharmaceuticals, Lupin Neuroscience, Novartis, PerkinElmer, Roche, Sanofi, and Sarepta.

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