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SPINAL MUSCULAR ATROPHY AND THE IMPACT OF NEWBORN SCREENING IN PATIENT MANAGEMENT: A NARRATIVE REVIEW

L. RINALDI[1], G. RODELLA[2], L. RUBERT[2,3], A. BORDUGO[4], E. RIGOTTI[2], A. PIETROBELLI[2,5], G. CANTALUPO[1,5]

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[1]Child Neuropsychiatry Unit, University Hospital of Verona, Verona, Italy
[2]Pediatric Unit, University Hospital of Verona, Verona, Italy
[3]Inherited Metabolic Disease Unit, Pediatric Clinic C, University Hospital of Verona, Verona, Italy
[4]Regional Centre for Rare Diseases, University Hospital of Udine, Udine, Italy
[5]Department of Engineering for Innovation Medicine, Innovation Biomedicine Section, University of Verona, Verona, Italy

Corresponding Author
Livia Rinaldi, MD; e-mail: livia.rinaldi@studenti.univr.it

***ABSTRACT* – Objective:** The clinical spectrum of SMA has changed due to advances in the treatment with disease-modifying therapies, which should be started as soon as possible to improve therapy efficacy and the disease's prognosis. This work aims to present the state of the art of the SMA clinical pathway, outlining the importance of newborn screening (NBS) in patient management.

Materials and Methods: The literature search was conducted on PubMed, using the following keywords: *spinal muscular atrophy, genetics, diagnosis, SMA disease-modifying therapies, SMA newborn screening, SMN1, SMN2, SMN2 copy number.* Relevant articles published in English from 2010 to 2024 were selected.

Results: NBS can enable early diagnosis and early initiation of therapy, thus changing the prognosis and clinical pathway of the disease. As a result, significant improvement in the quality of life of patients suffering from SMA and their families can be observed.

Conclusions: A well-established NBS system shows strong potential and social impact along with adequate support in the follow-up phases of SMA diagnosis and clinical management.

Keywords: Spinal muscular atrophy, Newborn screening, SMN1, SMN2, Disease-modifying therapy, Neuromuscular disease.

List of abbreviations: CMAP - compound muscle action potential; DBS - dried blood spots; NBS - newborn screening; NF - Neurofilament; pNF-H - Phosphorylated Neurofilament heavy chain; qPCR – quantitative polymerase chain reaction; SMA - spinal muscular atrophy.

INTRODUCTION

Newborn screening (NBS) programs are an ensemble of tests designed to minimize or prevent serious health conditions that can cause disabilities or death via presymptomatic diagnosis. In the last decades, the spectrum of diseases screened has significantly expanded, including inborn error of metabolism, endocrine, hematologic, immunologic, cardiovascular, and hearing loss diseases[1-3].

SMA (OMIM# 253300, 253550, 253400 and 271150) is the second most common recessive disorder in the pediatric population after cystic fibrosis, with an overall incidence of 1/10,000 live births[4]; its prevalence is approximately 1–2 per 100,000[5], and carriers' prevalence is about 1 in 54[6]. The frequency of carrier status is highest in White and Asian populations (around 1 in 50) and lowest in Black (1 in 100) and Hispanic (1 in 76) populations, with a *de novo* variation rate of 2%[4,7]. Before the availability of disease-modifying therapies, SMA was the most common genetic cause of child mortality[8]. For this reason, secondary prevention plans have begun to include SMA in the NBS program over the last decade[3].

This narrative review aims to describe the state of the art of SMA genetics, diagnosis, and clinical pathway, outlining the role of NBS on patient management and outcomes, as well as its socioeconomic impact.

MATERIALS AND METHODS

The literature search was conducted on the PubMed database, selecting papers published in English from 2010 to 2024. The following search strategies were applied:

1. (*Spinal muscular atrophy) AND (genetics);* number of records = 2,227
2. (*Spinal muscular atrophy) AND (diagnosis);* number of records = 2,994
3. *(Spinal muscular atrophy) AND (disease-modifying therapies);* number of records = 244
4. *(Spinal muscular atrophy) AND (newborn screening);* number of records = 301
5. *(SMN1) OR (SMN2) OR ("SMN2 copy number")*; number of records = 1,658

As judged by the authors, relevant articles published before this time range were also included in the analysis. Articles were screened (full-text or abstract only) based on their relevance to the topic (SMA). Duplicates and unrelated topic publications were excluded. From a total of 7,424 articles, 7,356 were excluded due to duplicates, unrelated topics, or because they were redundant. Four papers published before 2010 were also appraised as deemed relevant by the authors. As a result, 72 articles were selected (**Figure 1**).

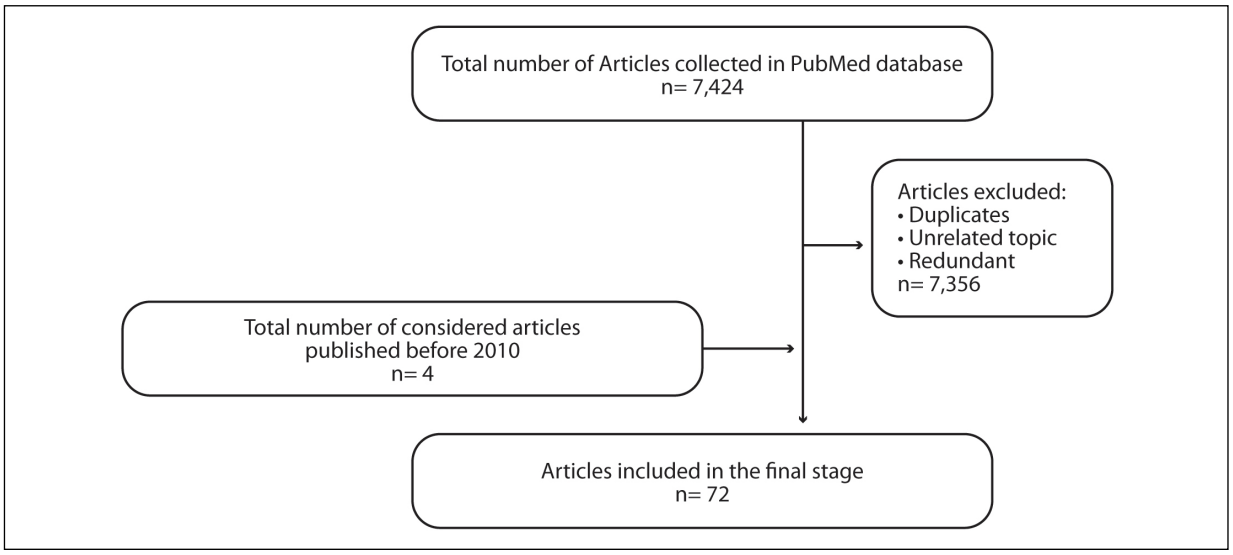

Figure 1. Literature research flowchart.

RESULTS

As a result of the literature research, 72 papers focusing on SMA and NBS features were selected (**Supplementary Table 1**). In particular, the genetics, the clinical pathway from diagnosis to treatment, the impact of NBS on SMA patient management and treatment, and the socioeconomic benefits are reported.

Genetics and Diagnosis of SMA

SMA is caused by deletions (95% of cases) or mutations of the *SMN1* gene (NC_000005.10) located on 5q13. Chromosome 5 contains two highly homologous copies of SMN, a telomeric copy (*SMN1*) and a centromeric copy (*SMN2*)[9]. *SMN1* produces a full-length mRNA that encodes the functional SMN protein. In contrast, *SMN2* encodes only around 10% to 15% of full-length transcript[10], resulting in a non-functional transcript that is quickly degraded, thus leading to motor neuronal death[11]. The number of *SMN2* copies varies between 0 and 8[12], which strongly correlates with the phenotype diversity[11,13]; for instance, a larger copy number is associated with milder phenotypes[14].

Most patients inherit *SMN1* deletion from their parents; in 2% of cases, *de novo* deletions have been found in one of the two alleles[9,15], and approximately 3–4% of patients carry small pathogenic variants (missense, small deletion or duplication, splicing mutation) that abolish SMN protein production and occur in a compound heterozygous state with deletion[9,15-17]. The clinical classification of SMA is determined by the highest motor milestones reached and the age of onset (**Table 1**)[7].

Table 1. Clinical classification of SMA. Source: Keinath et al[22], 2021. Adapted with permission from Dove Press under a Creative Commons CC BY-NC 4.0 license.

SMA type	Copies *SMN2*	Percent of cases	Onset	Motor Milestones prior to disease-modifying therapy	Clinical Features	Natural history prior to disease-modifying therapy
0	1	Rare, < 1%	Prenatal, at birth	Non-sitter, no head control	Generalized weakness, hypotonia, respiratory failure, poor feeding, contractures	Death within weeks of birth
1	1-2	45%	0-6 mo	Non-sitter	Proximal predominant weakness, respiratory insufficiency, poor feeding, tongue fasciculations	Death by age 2
2	3	20%	6-18 mo	Sits independently, never stands or ambulates	Proximal predominant weakness, tongue fasciculations, minipolymyoclonus, scoliosis	Most alive at 25 years
3	3-4	30%	A: 18 mo – 3 yr B: 3-30 yr	Ambulates independently	Proximal, lower extremity predominant weakness, abnormal gait	Normal lifespan
4	4or more	<5%	> 30 yr	Ambulates independently	Maintain ability to ambulate	Normal lifespan

SMA type 0 and type 1A are the rarest and most severe phenotypes; they are characterized by areflexia and hypotonia at birth or in the first week of life and by a history of decreased movements in utero. SMA type 1B and 1C are the most common phenotypes, representing approximately 45% of cases[18,19], with normal cognitive functions[20,21]. SMA type 2 represents about 20% of cases, whereas SMA type 3 has an onset ranging from 18 months to adulthood and represents 15% of cases. Children achieve the ability to stand or walk without support for 10 meters or more (walkers)[22]. SMA type 4 refers to individuals with muscle weakness in the second or third decade of life. Most patients maintain ambulation,

and scoliosis is less prominent. New trajectories and phenotypes of the disease have been observed since the introduction of disease-modifying therapies.

SMA diagnosis is based on genetic testing, which detects the homozygous absence of *SMN1* and the number of *SMN2* copies with high sensitivity and absolute specificity[16]. The absence of both full *SMN1* copies will provide a diagnosis of SMA. If only one full copy is present and the clinical phenotype is compatible with SMA, the remaining *SMN1* gene should be sequenced for other point mutations. If both full *SMN1* copies are present, a diagnosis of SMA is highly unlikely, but the *SMN1* gene should be sequenced if there is a striking typical phenotype or consanguinity[23]. The genetic bases of SMA are very homogeneous since 95–98% of the disease is caused by a biallelic deletion of *SMN1*. This peculiar condition has enabled the development of very sensitive, specific, and relatively inexpensive genetic tests[24].

Multiplex ligation-dependent probe amplification (MLPA) of *SMN1* and *SMN2* is the gold standard of genetic testing for SMA, which allows the identification of SMA patients with a homozygous *SMN1* deletion, SMA patients with one *SMN1* copy who might be compound heterozygous for small pathogenic variants of *SMN1*, the exact number of *SMN2* copies, and healthy heterozygous carriers. However, 2–4% of SMA patients who carry point mutations will not be diagnosed with this type of test[16]. Other genetic analysis concerns the quantitative polymerase chain reaction (qPCR) or next-generation sequencing[25-27]. Homozygous *SMN1* deletions can also be identified through qPCR followed by restriction digest. This method is faster and less expensive than MLPA. However, it does not allow quantification of *SMN1* or *SMN2* copy numbers, which are relevant for identifying heterozygous deletions of *SMN1* and for the prognosis and therapeutic approaches[23].

Features of newborn screening for SMA

NBS for SMA consists of searching for *SMN1* deletion and determining the number of *SMN2* copies, with 97–98% sensitivity, 100% specificity[28], and predictive prognostic value higher than 80%.

NBS for SMA is performed using real-time qPCR on DNA isolated from dried blood spots (DBS). The blood sample is collected in the first 3 days of life. The screening test result requires confirmation and evaluation of the *SMN2* gene copy number, usually performed using the MPLA technique. Genetic tests applied to NBS must ensure sufficient amounts of DNA extracted from the DBS and be appropriate to detect *SMN1* deletion. In SMA, *SMN1* and *SMN2* are highly homologous, and homologous recombination may occur between the two genes, resulting in false positives if positions beyond exon 7 are analyzed[29]. Therefore, it is necessary to establish and validate a DNA cleanup procedure that considers intra- and inter-assay variabilities. A protocol for genetic tests should be designed to be suitable for rapidly extracting DNA from standard DBS to test it in multiplex qPCR assays; the molecular genetic screening procedure should be operated by one person who analyzes up to 2000 samples using qPCR cyclers. It is essential to identify and eliminate possible sources and effects of contamination and to avoid false-positive results by optimizing assay design[30].

The purpose of the screening program is to detect people with serious or life-threatening disorders that are treatable and have a recognizable latent or early symptomatic stage in order to start an early disease-modifying treatment. The identification of infants with SMA before the onset of clinical symptoms has been accomplished by NBS, allowing infants to be treated before the loss of motor neurons and resulting in improved clinical outcomes[31]. One of the first pilot studies on NBS was conducted in Taiwan; out of 120,267 newborns, seven cases of SMA were identified[32]. Another early study was conducted in three hospitals in New York between January 2016 and January 2017; it allowed the identification of one case of SMA in 3,826 newborns tested[33]. Several other pilots and national programs were subsequently developed in Australia, Japan, and the USA. In Europe, the first countries were Belgium and Germany. The Belgian study identified SMA in five of 35,000 newborns, indicating a higher incidence of the disorder in Europe than previously assumed[34]. A similar incidence was confirmed by the German studies that detected SMA in 1:7,350 births (38/278,970 newborns) and identified four copies of *SMN2* in 40% of newborns with confirmed SMA at the biomolecular level[35]. In 2023, the first pilot study conducted in Italy showed 15 patients/90,885 newborns (incidence 1:6059) having the following SMN2 genotypes: 1 (one patient), 2 (eight patients), 2+c.859G>C variant (one patient), 3 (three patients), 4 (one patient) or 6 copies (one patient). Six patients (40%) showed signs suggestive of SMA at birth[36]. However, in SMA type 1 patients, the window for beneficial therapeutic intervention is very small, as data show that 95% of motor units are lost in the first 6 months of life[37]. Given the SMA recurrence risk of 25%, genetic counseling and prenatal diagnosis should be offered to couples who have previously had a child with SMA[38].

The USA was the first country to gradually implement NBS for SMA for the entire population[39] and many pilot programs have been activated wordwide[40-45]. In December 2020, NBS programs for SMA were available in Taiwan, USA, Germany, Belgium, Australia, Italy, Russia, Canada, and Japan[45]. The Advisory Committee on Heritable Disorders in Newborns and Children added NBS for SMA to the Recommended Uniform Screening Panel (RUSP) in July 2018[1].

The results of the first Italian pilot study provided promising inputs to update the current molecular diagnostic scenario for SMA and paved the way for national NBS implementation[36,43]. NBS has currently been activated in 12 Italian regions (Abruzzo, Campania, Friuli-Venezia Giulia, Lazio, Liguria, Lombardia, Puglia, Piemonte, Trentino-Alto Adige, Toscana, Val D'Aosta, Veneto). Other four regions plan to start with pilot screening programs to add SMA to the current regional NBS panel.

Diagnostic confirmation and management

In case of a positive result from DBS, the test is repeated on a new DNA sample from the same DBS card; if the diagnosis is confirmed, the screening laboratory informs the referral center for treatment of SMA on the same day. The family is involved in a care pathway dedicated to presymptomatic patients at the SMA referral center. There, parents will meet with a dedicated multidisciplinary team and genetic counseling and must sign a novel informed consent to allow the center to perform a confirmatory, prognostic MLPA test on fresh blood samples[36]. A first neurological and electrophysiological evaluation is performed, and the therapy is discussed. According to the approved national guidelines, the therapy starts within the first 3 weeks of life (**Figure 2**).

In 2018, Glascock et al[46] published a report titled "Treatment Algorithm for Infants with SMA Detect-

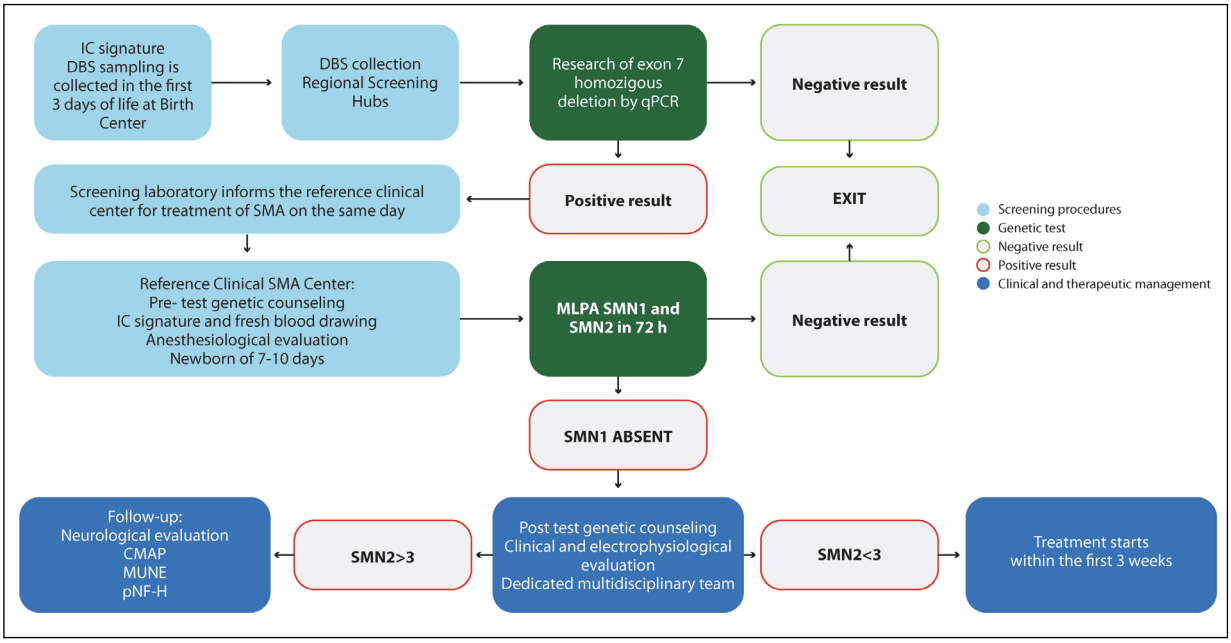

Figure 2. Clinical pathway from the screening to the start of the treatment.

ed by Newborn Screening", mainly based on *SMN2* copy number. Patients with two or three *SMN2* copies were treated immediately; patients with ≥4 *SMN2* copies were included in a strict clinical follow-up to detect the first signs of the disease. However, in 2020, the same group published a revised version of the above algorithm that also suggests treating patients with four or more *SMN2* copy numbers as soon as possible[47]. The patient is enrolled in standardized follow-up every 2–4 months, during which motor scale evaluation and electrophysiological exams are repeated and scored, ensuring supportive therapies and pneumological/nutritional/orthopedic counseling if necessary. The assessment of motor milestones is necessary for the diagnosis, the design of the rehabilitative intervention[23], and the evaluation of the

therapy effects. The most common functional motor outcome measurements used in the assessment of SMA are Hammersmith Infant Neurological Examination Section 2, Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders, Hammersmith Functional Motor Scale Expanded, Revised Upper Limb Module, and Motor Function Measurement[48].

During the first evaluation, a complete clinical assessment is necessary to establish the specialists to be involved in future follow-ups: respiratory, gastrointestinal, nutritional, and deglutition assessment and care must be provided to patients; in most severe cases, palliative care specialists are involved since earliest stages of diagnosis[49].

Electrophysiological and molecular biomarkers

Although neurophysiology testing is no longer required for diagnosis, compound muscle action potential (CMAP) can be used to indirectly measure disease progression across all patients with SMA[7,50]. CMAP is reduced in patients with SMA, correlating with reproducibly decreased functional motor scores in several studies[51,52]. Thus, CMAP could be used as a disease severity indicator in addition to the number of *SMN2* copies. Patients with severe forms of SMA (≤2 copies of *SMN2*) show smaller and sometimes undetectable CMAP amplitudes than those with milder genotypes (>2 copies of *SMN2*). This measurement shows a different pattern of decline depending on the phenotype. The most crucial predictive value of CMAP amplitude is to identify those children who are asymptomatic clinically but symptomatic electrophysiologically, especially in the case of two copies of the *SMN2* gene. A recent study showed increased and maintained CMAP in treated children with infantile-onset disease (known as CMAP respondents) compared with untreated patients[53,54]. The motor unit number estimation has a prognostic and predictive value and can be used to monitor disease progression[37]. The motor unit number estimation is a quantification of the number of motor units innervating a muscle and is also decreased in SMA, correlating with the number of *SMN2* copies[55]. Electromyography is abnormal in all forms of SMA[56]; the disease severity and rate of progression directly influence the degree of abnormalities detected. Another putative biomarker that could be used during the disease follow-up due to its prognostic value is the phosphorylated neurofilament heavy chain (pNF-H)[57]. Neurofilament (NF) is a cytoskeletal protein that regulates axonal caliber and maintains the structural integrity of the axon. It is released from neurons following injury, and elevated NF levels can be detected in both the blood and cerebrospinal fluid barrier. High levels of pNFs are found at birth in babies with two *SMN2* copies, even before the onset of symptoms. These biomarkers later decline to attest to the destruction of the pool of motor neurons[58]. Data show that symptomatic SMA type 1 patients have higher levels of plasma pNF-H than healthy controls and that higher pNF-H levels correlated positively with earlier onset of symptoms and inversely with motor function at the start of nusinersen treatment. Furthermore, pNF-H levels decrease during nusinersen therapy, and the reduction is more pronounced the earlier the treatment is started[59]. Using validated biomarkers could be useful in predicting the clinical course of the disease and the response to any drug treatment; this would help clinical decision-making and reduce time and resources for clinical drug development[60].

THE POTENTIAL OF NEWBORN SCREENING FOR SMA

Early initiation of disease-modifying therapy

Decoding the genetic background and identifying the disease-causing mutations of *SMN1*[9] paved the way for targeted medical approaches to treat SMA[15,60]. Disease-modifying therapies cross the traditional subtypes of SMA[60], either by modifying the splicing of *SMN2* or by replacing the *SMN1* gene[7,15], thus increasing the full-length SMN protein level.

Several innovative drugs have recently been developed to improve or alleviate symptoms in many patients[8,61]. Three treatments have been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for treating SMA.

Nusinersen was the first drug approved by the FDA in December 2016 and by the EMA in May 2017 to treat all types of SMA in both pediatric and adult patients[62]. Nusinersen is an antisense oligonucleotide that binds intron 7 of pre-RNA *SMN2* to modulate the splicing of *SMN2* and enhance the production of a functional SMN protein[63,64]. This therapy is injected intrathecally (12 mg) through four loading doses over 2 months,

followed by maintenance every 2 months. Phase II studies showed subjects safely tolerating multiple intrathecal injections and presenting improvement in motor function and achievement of motor milestones[65]. The efficacy and safety of nusinersen have been confirmed by recent clinical trials and real-world data[53]. Moreover, the NURTURE clinical trial results showed how early treatment may maximize efficacy. In NURTURE, patients with two and three copies of *SMN2* were treated presymptomatically, before 6 weeks of age for the first dose; they managed to reach higher motor function at each observation after treatment, with the latest data showing that 23/25 (92%) of patients achieved walking with assistance, and 22/25 (88%) of them achieved walking independently[58]. Onasemnogene abeparvovec was approved by the FDA in May 2019 as a one-time treatment for patients under 2 years of age with SMA and by the EMA in June 2020 for all patients with a biallelic mutation in *SMN1* and a clinical diagnosis of SMA type 1 or up to 3 *SMN2* copies[66]. Onasemnogene abeparvovec is a recombinant AAV9 viral vector encoding human SMN protein under the control of the cytomegalovirus enhancer/chicken-β-actin hybrid promoter, injected intravenously in a single dose as the administration of a virus-carried drug stimulates permanent immunity. An immunomodulatory regimen with prednisolone is required before and after intravenous infusion to decrease the response to the AAV9 capsid and mitigate hepatotoxicity[67]. In a phase III clinical trial, 22 patients with symptomatic SMA type 1 received onasemnogene. Of them, 13 infants achieved independent sitting at 18 months of age; 91% of patients did not require permanent ventilation by 14 months of age, compared with only 26% of the untreated group[68]. SPR1NT clinical trial enrolled SMA infants under 6 weeks to evaluate the efficacy and safety of onasemnogene abeparvovec in presymptomatic patients. Interim results showed that patients treated during the disease-free period reached motor milestones earlier than those treated after symptoms onset[69]. Risdiplam is a small-molecule splice modulator that binds directly to *SMN2* pre-mRNA, promoting exon 7 inclusion and full-length SMN protein production[70]. Risdiplam is orally administered with a weight-modulated dose; it is the first SMA disease-modifying therapy administered at home, whose efficacy was observed in two trials, FIREFISH (SMA1)[71] and SUNFISH[72]. This drug was approved by the FDA in 2020 for treating patients older than 2 months and further extended to all ages in 2022. The EMA approved risdiplam for treating people with SMA types 1, 2, or 3 with up to 4 copies of *SMN2* above the age of 2 months in 2021[73] and for people of all ages in 2023. Presymptomatic patients have been enrolled in the ongoing clinical trial RAINBOWFISH, whose interim results show that most children can achieve motor milestones similar to those of not-affected children and within the WHO normal developmental window[74].

Disease-modifying drugs should be administered at the earliest possible stage of the disease progression to improve functional outcomes and quality of life. This enforces the importance and potential of NBS applications for SMA to identify presymptomatic patients and possibly optimize the effect of innovative therapies[8].

Socioeconomic advantages of NBS

Implementing NBS would contribute to reducing SMA morbidity, a relevant cost-reducing factor for the healthcare system. An analysis in the Netherlands reported that implementing NBS for SMA led to cost savings of €12,014,949 per annual cohort of newborns over a lifetime horizon[75]. In a study carried out in the UK, cost savings of £62,191,531 were reported per annual cohort of newborns and an estimated gain in quality-adjusted life years of 529 years over their lifetime[76]. By improving health outcomes for patients with SMA and thus reducing morbidity, NBS represents a cost-saving approach compared with no screening. A multicentric study on the cost and quality of life of patients with SMA (treated and untreated) conducted by Dangouloff et al[57] demonstrated that total financial costs were lower for treated patients identified by early screening than for treated patients identified after symptom onset; moreover, direct financial costs (consultation, examinations, medication, hospitalization) were much lower in treated patients identified by early screening.

Furthermore, decreasing the severity of disease progression also reduces social and economic costs related to the psychosocial burden of patients with symptomatic SMA and their caregivers[77], thus leading to an improved quality of life.

As an alternative to NBS, population screening could also be considered through carrier identification or prenatal testing. However, both methods have disadvantages and are less suitable than NBS. Compared with NBS, carrier screening has lower sensitivity (93%), as the analysis involves each parental couple rather than individuals and has higher costs, which makes carrier screening not easily scalable to the high-throughput platform. Prenatal diagnosis is performed on DNA extracted from either chorionic villus sampling specimens or amniotic fluids, leading to higher miscarriage risk, as well as ethical issues[78,79].

DISCUSSION

The SMA screening is essential in changing the natural history of the disease. New treatments cannot prevent the disease onset. However, early intervention of a multidisciplinary team to treat children detected by NBS allows a more favorable evolution of SMA, with less need for intensive care and hospitalization. Despite the increase in SMA NBS programs, many countries haven't yet activated them, and more data on clinical management and follow up, together with clear guidelines and protocols, are still required. This would improve the clinical management pathway for patients and their families and would also facilitate the study of diseases and available approaches. For instance, the actual DBS is not able to find point mutations[16], thus preventing NBS from detecting 2–4% of symptomatic or presymptomatic patients, and territorial healthcare professionals who usually deal only marginally with these children should be aware of this limitation. Along with the implementation of NBS, another crucial aspect concerns the accessibility of children detected with SMA to specialized centers to start the treatment in time, assuming that neuronal injury is likely to occur asymptomatically in the uterus[80]. Direct contact between the screening laboratory and the SMA centers should be ensured to reduce the stress of the affected families and save time from consulting non-specialized centers unable to provide pharmacotherapy[40,81]. Re-contacting families to make diagnostic confirmations is the first step of a pathway that starts with discussing the suspected diagnosis of a rare genetic disease. Communicating the diagnosis is a medical act that constitutes an important moment in the process of parental acceptance of the child's disease and should be done only when certainty of diagnosis is reached. The diagnosis must be explained by an expert in the disease, the newborn's physiology, and the chronic disabilities. Parents must be involved in each decisional step, and professional skills are necessary to manage parents' feelings, such as anxiety and the need for explanation. Therefore, it is crucial to establish an empathetic and trusting relationship from the first meeting through a multidisciplinary involvement (neuropsychiatrist, pediatrician, pneumologist, palliative care specialist, nutritionist, psychologist).

CONCLUSIONS

Given the genetic characteristics of SMA, the new therapeutic possibilities, and the promising results of pilot screening projects worldwide, NBS for this disease is a great opportunity to change the prognosis and is an obligation that institutions are urged to fulfill. Early diagnosis of the condition must be perceived as urgent, considering the implications of delay on prognosis and therapeutic outcomes. Specific centers must coordinate NBS and subsequent steps with a multidisciplinary team of experts[5,6] and highly qualified staff located throughout the national territory. Homogeneous standards of care and follow-up must be pursued to guarantee the same quality of care for every infant born in the Italian territory; this goal could be achieved through the creation of a data network between centers.

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No artificial intelligence-assisted technologies were used in the production of this article.

Authors' contributions:

Study conception and design: Dr. Giulia Rodella and Dr. Livia Rinaldi; collection and interpretation of data: Dr. Giulia Rodella and Dr Livia Rinaldi; manuscript drafting: Dr. Giulia Rodella, Dr. Livia Rinaldi, Dr. Angelo Pietrobelli, Dr. Gaetano Cantalupo; approval to submit: all the authors.

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ORCID ID:
Gaetano Cantalupo: https://orcid.org/0000-0003-1343-8434
Angelo Pietrobelli: https://orcid.org/0000-0002-9912-3232
Andrea Bordugo: https://orcid.org/0000-0003-1927-8187
Giulia Rodella: https://orcid.org/0000-0001-9254-1231
Erika Rigotti: https://orcid.org/0009-0005-1639-8951
Livia Rinaldi: https://orcid.org/0000-0003-2407-3119

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