Original research

Experience of a 2-year spinal muscular atrophy NBS pilot study in Italy: towards specific guidelines and standard operating procedures for the molecular diagnosis

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► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jmg-2022-108873).

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Received 12 August 2022
Accepted 6 November 2022

To cite: Abiusi E, Vaisfeld A, Fiori S, *et al*. *J Med Genet* Epub ahead of print: [please include Day Month Year]. doi:10.1136/jmg-2022-108873

ABSTRACT

Background Spinal muscular atrophy (SMA) is due to the homozygous absence of *SMN1* in around 97% of patients, independent of the severity (classically ranked into types I–III). The high genetic homogeneity, coupled with the excellent results of presymptomatic treatments of patients with each of the three disease-modifying therapies available, makes SMA one of the golden candidates to genetic newborn screening (NBS) (SMA-NBS). The implementation of SMA in NBS national programmes occurring in some countries is an arising new issue that the scientific community has to address. We report here the results of the first Italian SMA-NBS project and provide some proposals for updating the current molecular diagnostic scenario.

Methods The screening test was performed by an in-house-developed qPCR assay, amplifying *SMN1* and *SMN2*. Molecular prognosis was assessed on fresh blood samples.

Results We found 15 patients/90885 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. We also discuss some unusual cases we found.

Conclusion The molecular diagnosis of SMA needs to adapt to the new era of the disease with specific guidelines and standard operating procedures. In detail, SMA diagnosis should be felt as a true medical urgency due to therapeutic implications; *SMN2* copy assessment needs to be standardised; commercially available tests need to be improved for higher *SMN2* copies determination; and the *SMN2* splicing-modifier variants should be routinely tested in SMA-NBS.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The scenario of spinal muscular atrophy (SMA) has been revolutionised over the past few years by the availability of three different disease modifying-modifying therapies (DMTs) that have radically changed the trajectories of the disease and the quality of life of patients. It has been demonstrated that if DMTs are administered before the onset of symptoms, most patients achieve motor milestones within the expected range; these exciting results are prompting the implementation of newborn screening (NBS) programmes for SMA in many countries.

WHAT THIS STUDY ADDS

⇒ In our study, besides the epidemiological data of SMA in our regions (the highest incidence reported so far), we discuss the critical aspects of SMA-NBS and molecular diagnosis of SMA, also based on some unusual findings we experienced.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Molecular diagnosis of SMA is at an epochal turn: great efforts are required from the community of specialised diagnostic laboratories to adapt to the requests coming from clinicians and society in terms of turnaround time of the results and accuracy of the molecular prognosis. We enlight here aspects that have not been discussed enough in studies published so far; one above all is the standardisation of *SMN2* copy number assessment, the main decision point for treatment start.

INTRODUCTION

The trajectories of spinal muscular atrophy (SMA; OMIM# 253300, 253550, 253400 and 271150) have been revolutionised by the registration of three disease-modifying therapies (DMTs).[1] Classically ranked into types I–III, based on age of onset and maximum motor milestone achieved, SMA is an autosomal recessive neuromuscular disorder characterised by the degeneration of the α-motor neurons of the ventral horns of the spinal cord.

The outcomes of DMTs have brought out new paradigms for SMA: the clinical improvement and the increase in life expectancy of patients have uncovered new phenotypes not fulfilling the criteria classically used for classification.[1] Additionally, the earliest the treatment starts, the better is the outcome so that, if established in the presymptomatic phase, most patients acquire motor developmental milestones within the expected age range.[2]

The new era of SMA is prompting both the development of a new nosology for the condition and the implementation of NBS programmes strongly supported by both patients' advocacy groups and the scientific community,[3] and the fulfilment of the Wilson and Jungner criteria.[4 5] Irrespective of the phenotypical severity, patients with SMA share the homozygous loss of *SMN1*[6] due to deletion/gene conversion events in 97%–98% of cases; 2%–3% of subjects are compound heterozygotes for the deletion in one *SMN1* allele and small pathogenic variants in the other.[7]

In presymptomatic patients, prognostic biomarkers are crucial for therapeutic decisions: the main phenotypical modifier of SMA is *SMN2*, a hypomorphic copy of *SMN1*, mainly transcribed into an isoform lacking exon 7 and translated into an unstable protein. The main functional difference between *SMN1* and *SMN2* is a C>T transition in position +6 of exon 7. The number of *SMN2* genes is variable in patients, generally from two to four, and is inversely related with disease severity.[8] Some rare variants of *SMN2* can modify the efficiency of exon 7 inclusion in mature transcripts[9]; two of them, rs121909192 and rs1454173648, are associated with a milder phenotype.[10 11]

We report here the results of the first Italian SMA-NBS pilot project, a 2-year prospective multicentric pilot study carried out in the regions of Lazio and Tuscany. This was an epidemiological study aimed at the identification of patients with presymptomatic SMA and at the definition of the incidence of the condition in the two regions, in view of its inclusion in the list of diseases covered by the National Health System NBS.

MATERIALS AND METHODS

Study workflow and management of positive tests

The study workflow is schematised in figure 1; we exploited the pre-existing networks in both regions, developed for the metabolic newborn screening (M-NBS). On information of families by the local health personnel and informed consent, blood was sampled on a dedicated customised dried blood spot (DBS; Ahlstrom 226 grade paper card, PerkinElmer). Samples for SMA-NBS were collected simultaneously to those for the M-NBS at 48 or 72 hours of life. Informative sheets and consent forms have been made available, in Italian and seven other languages (Hindi, Chinese, English, French, Spanish, Arabic and Romanian) for the most common non-Italian mother-tongue citizens or residents. The English version of informative sheets and informed consent forms are available in the online supplemental materials.

Daily blood samples and informed consent forms were sent from 54 of the 55 regional birth centres in a dedicated envelope to the regional screening centre hubs and forwarded the day after to the reference centre. One birth centre did not consent to participate in the study due to shortage of personnel.

The NBS test was performed within 0–3 days after the arrival of samples to the reference centre (4–8 days of life); SMA and non- SMA babies exited the study after the NBS test.

Following the already established M-NBS policy, in case of negative screening, no information was provided to families. For positive babies, a confirmation test was performed on a new DNA sample from the same DBS card, and on the same day, the family was invited for a multidisciplinary counselling with medical geneticists and child neurologists to provide information regarding SMA and therapeutic options and to perform the first neurological assessment of the child. At this stage, the patient was included in a care pathway dedicated to presymptomatic patients with SMA at the Gemelli Foundation University Hospital or Meyer Children's University Hospital, for children born in Lazio or Tuscany, respectively. Confirmatory and prognostic molecular tests were performed on a fresh blood sample, collected on signature of a novel informed consent form; this strategy was chosen also to rule out sampling mismatch at the birth centre, since we did not expect false positive results. Within few days, the family was provided with the official report of the genetic tests, in the context of a second multidisciplinary counselling aimed at providing information regarding prognostic elements, therapeutic options and reproductive risk for the couple and their families. The costs of these procedures were covered by the National Health System, in a standard diagnostic workflow.

Patients with two *SMN2* or three copies were treated immediately; patients with *SMN2* of ≥4 were included in a strict clinical follow-up to detect the first signs of the disease (figure 1).[12] According to Italian law, the costs of allowed DMTs are covered by a dedicated national fund for innovative drugs for 3 years following the registration; subsequently, the costs are covered by the regional governments following hospital prescription.

Molecular analyses

gDNA extraction from DBS and qPCR setting were performed by the Sciclone NGS Instrument (PerkinElmer) using customised scripts and protocols. Two 3.5 mm diameter spots per sample (the first blood-moistened, the second blank to clean the puncher and to reduce cross-contamination of samples) were picked by the DBS Puncher (PerkinElmer) in a 96-well plate. DNA was extracted by the DNA Blood Spot LH kit (PerkinElmer) with modifications to the manufacturer's protocol (see online supplemental materials). DNA was amplified in ViiA 7 or 7900HT Real Time PCR instruments (Applied Biosystems). For each plate, we extracted and amplified four control DNA samples: subjects with both *SMN1* and *SMN2*, with the homozygous loss of *SMN2* or *SMN1*, and negative control (two blank spots).

To detect *SMN1* absence, we modified our *in-house*-developed qPCR assay reported elsewhere, routinely used in our unit for *SMN2* copy number assessment, carrier screening and second-level diagnoses.[13] In particular, in a final volume of 9 µL, we mixed 3 µL of Taqman Universal mastermix, no UNG (P/N 4440049, Thermo Fisher Scientific), 400 nmol of each primer (Thermo Fisher Scientific), 100 nmol of *SMN1* and *SMN2* Taqman MGB probes (Thermo Fisher Scientific) and 3 µL of gDNA. The PCR cycle was 95°C 10′, 68°C 10′, followed by 35 amplification cycles (95°C 15″, 68°C 60″). In the present setting, the test was used as purely qualitative (presence/homozygous absence of *SMN1*) rather than semiquantitative to prevent possible identification of heterozygous deletions of *SMN1*. Samples with Ct of ≥30 were considered amplification failures. These latter samples were re-extracted using a half blood spot; if failing again, samples were diluted (1/10) and tested manually or, finally, extracted in Chelex 100 medium (BioRad Laboratories).

For confirmation of positive samples, gDNA was extracted from fresh blood using the Wizard DNA extraction kit (Promega), according to the manufacturer's protocol. The diagnostic workflow included the confirmation of homozygous *SMN1* deletion by RFLP-PCR,[14] *SMN2* copy number assessment

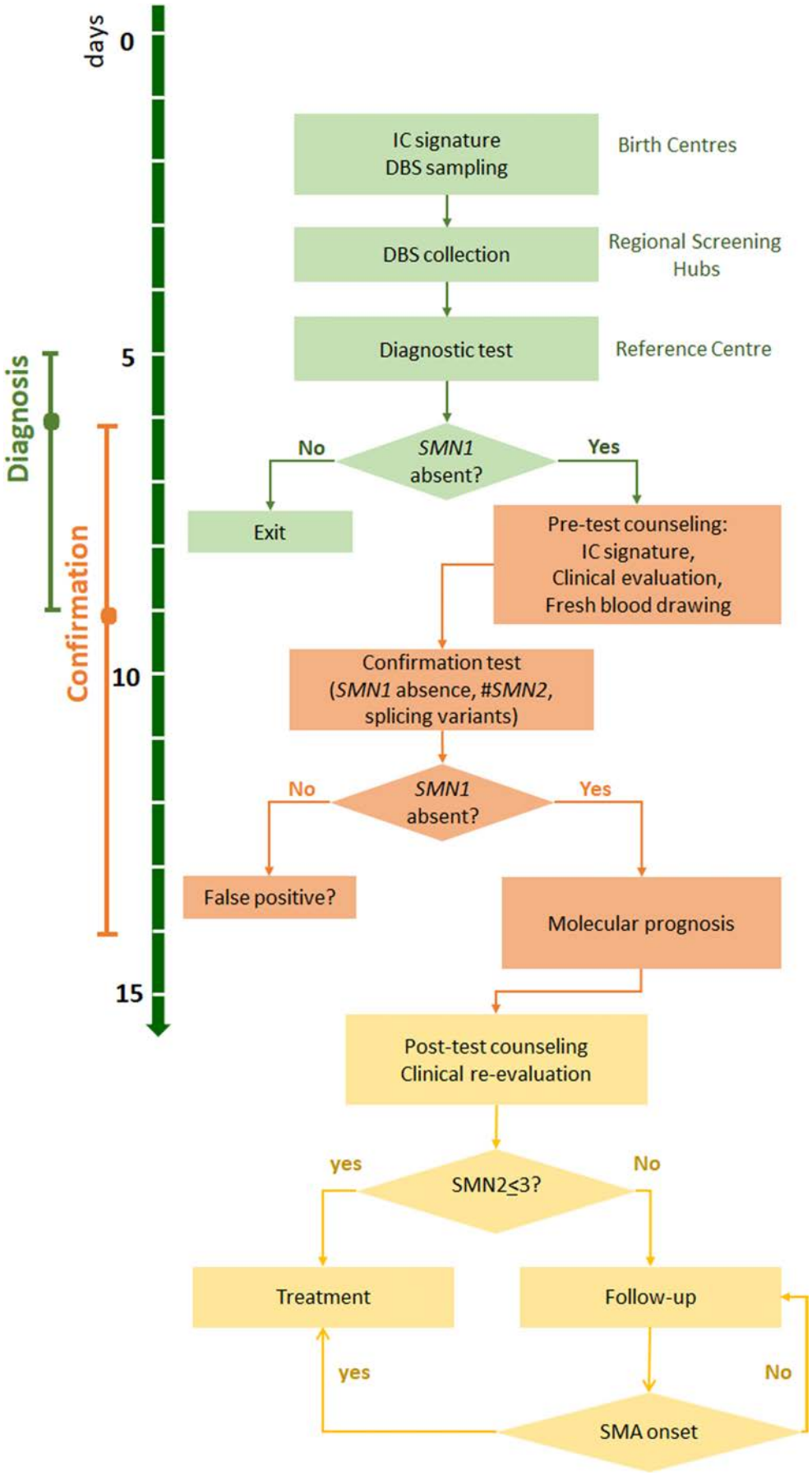

Figure 1 Workflow of the pilot study: the diagnostic (in green), the confirmation (in orange) and the follow-up phases (in yellow), respectively. IC, informed consent; DBS, dried blood spot; SMA, spinal muscular atrophy.

by semiquantitative qPCR[15] and identification of exon 7 splicing-modifier variants (rs121909192 and rs1454173648).[16]

RESULTS

Population data

The official starting of the study was on 5 September 2019. The study ended with children born on 4 September 2021 and therefore was almost entirely performed during the COVID-19 pandemic. The participation of the birth centres started gradually; the enrolment in Tuscany birth centres started on 16 March 2020 due to administrative issues. The birth centre that did not adhere to the study accounts for >10% of births in Lazio.

We enrolled 90 885 newborns; the mean compliance of families was 91% (calculated as indicated in online supplemental materials and methods), beyond the value estimated during study design. In 2020, the number of live births was reduced by −5.06% compared with 2019, slightly higher than the trend of 2014–2019 (median −4.68%/year); based on data of M-NBS centres, natality remained substantially unchanged in 2021. The global failure rate was 2.1%. Failures were concentrated in the first months of the study and were substantially reduced after an alert was sent to birth centres to avoid the use of heparin-coated capillaries for blood sampling.[17] Subsequently, the failure dropped to 0.5% (479 samples) that required manual DNA extraction. All samples were successfully screeened, and no resampling was required.

Molecular epidemiology of patients identified in NBS

We identified 15 patients with the homozygous deletion of *SMN1*, providing an incidence of 1/6059 (15/90 885): the first patient was previously identified during amniocentesis. The others were from families unaware of SMA; one symptomatic child was diagnosed at 40 days on M-NBS DBS since initially parents refused to participate in the study. So far, we are not aware of additional patients, born during the pilot study, who were missed in the SMA-NBS.

The clinical and molecular data of patients are reported in table 1. Of the 15 patients with SMA, one had one *SMN2* copy; eight had two copies, one had two *SMN2* and the c.859G>C variant[10]; three had two *SMN2* copies; one had four copies and one had six copies (figure 2A).

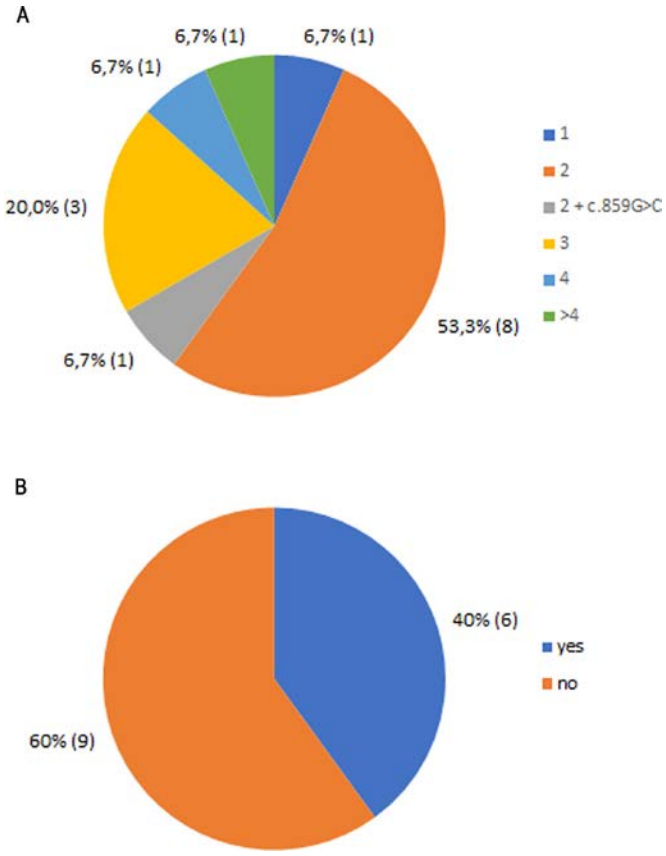

Figure 2 Clinical and molecular data of patients identified through SMA-NBS during the pilot project: (A) molecular prognosis (*SMN2* copy number and splicing variants), (B) presence of signs suggestive of SMA at the first visit. Data are indicated as % (n). NBS, newborn screening; SMA, spinal muscular atrophy.

Six patients (all men, 40%, figure 2B) were symptomatic or showed signs suggestive of disease onset at the first visit; the clinical details of these patients have already been reported.[12] The median age at the diagnosis of SMA was 6 days of life (range: 5–9, excluding the child with late diagnosis); the turnaround

Table 1 Clinical and molecular data of patients identified through SMA-NBS

Patient number	Sex	#*SMN2*	Age at diagnosis (days)	Official report (days)	Symptoms at birth	DTR	CHOP-INTEND Scale score	Age at treatment (days)
1	M	1	6	7	Yes	–	0	13
2	M	2	5	10	No	+	52	17
3	F	2	7	13	No	+	60	17
4	M	2	7	11	Yes	–	28	18
5	M	2	3	5	Yes	–	24	13
6	M	2	5	7	Yes	±	48	12
7	M	2	6	9	Yes	–	26	30
8	M	2	4	11	Yes	–	29	13
9*	M	2	47	48	No	+	6	62
10	F	2+c.859G>C	8	15	No	+	44	21
11	F	3	6	12	No	+	60	16
12	M	3	8	11	No	+	52	11
13	M	3	5	11	No	+	47	51
14	M	4	9	16	No	+	43	–
15	F	6	7	21	No	+	42	–

*Patient identified on M-NBS dried blood spot sample. At birth, the parents refused the participation to pilot project but accepted later, once mild signs were observed at the first visit.
CHOP-INTEND, Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; DTR, deep tendon reflex; F, female; M, male; M-NBS, metabolic newborn screening; NBS, newborn screening; SMA, spinal muscular atrophy.

time of the confirmatory test was 1–3 working days; the family was provided with an official report at a median age of 11 days (range: 7–21 days, figure 1). Thirteen out of 15 patients started DMTs immediately on diagnosis; the median age at treatment was 16.5 days of life (range: 11–62).

Management of floppy infants

During the pilot project, six newborns were indicated by birth centres as floppy infants. Three were not included in the NBS, who were from the birth centre that did not participate the study. For all patients we requested a fresh blood sample for second-level test and qPCR analysis. One child (16.7%) had the homozygous deletion of *SMN1*; one had a diagnosis of Prader-Willi syndrome; three of the four remaining had two *SMN1* copies and were not further tested. The last baby had a clinical picture suggestive of SMA (areflexia, almost absent active movements and respiratory insufficiency): to rule out possible homozygosity/compound heterozygosity for small variants, even though parents were unrelated, we sequenced the coding region of *SMN1*/*SMN2* and found no pathogenic variants. We also excluded the presence of pathogenic variants in *BICD2*[18] or *IGHMBP2*.[19] The baby died at 11 days of age for respiratory insufficiency. For all patients, the referral centre was provided with the official report within 5 days from the arrival of the sample.

SMA-NBS implementation in Lazio and Tuscany

After the end of the pilot project, both regions are prosecuting the SMA-NBS on a regional healthcare base: the screening test is performed by the regional NBS centres; the confirmation test is performed in the Catholic University/Policlinico Gemelli. The care pathway of patients is unchanged compared with the pilot. During the first 8 months, we found 3/49 887 newborns with SMA, with 2, 4 and 5 *SMN2* copies, respectively (1/16 629, non-significant vs pilot-NBS: $\chi^2=2.7719$, $p=0.096$). The three patients did not display clinical signs at the first visits. Only the patient with 2 *SMN2* copies started the DMT. We visited and performed the genetic test of the older siblings of patients with four and five *SMN2*. The sister of the child with four *SMN2* copies displayed mild signs of SMA at the age of 3 years and 10 months (ambulation on tiptoes) and had the homozygous absence of *SMN1*. The family preferred not to start any DMT yet.

DISCUSSION

We have performed a pilot project by using, for first-level test, an in-house qPCR assay. Differently from the commercial tests for SMA-NBS, our assay coamplifies and differentiates *SMN1* and *SMN2* by the C-T transition in exon 7. The advantages of this approach are the lower costs/sample, the availability of an internal amplification control (*SMN1* or *SMN2*) and, more importantly, the lower risk of false negatives since subjects with the homozygous deletion of *SMN2* (about 7% of the general population) cannot be affected from SMA. As in other similar studies, we used our qPCR assay only for qualitative assessment, for at least two reasons: (1) we decided to prevent the detection of heterozygous *SMN1* deletions for both ethical (predictive test on a minor) and technical issues (search of potential small pathogenic variants of the other *SMN1* allele) and (2) as demonstrated by the case of the family with a *SMN1* point variant (online supplemental materials and online supplemental figure 1) in our hands, the quality of DNA samples from DBS is not adequate for semiquantitative approaches. We also obtained highly inaccurate results in the set-up phase of the assay, when testing *SMN2* copies in DNA samples from DBS of patients with known copy number (data not shown). These findings are likely related to quality and quantity of DNA obtained from DBS and to the presence of molecules interfering with DNA amplification and/or fluorescence emission, found in blood and in the Guthrie paper.[20 21] This issue is likely more prominent in the case of protocols based on heat shock of DBS punches only, without DNA extraction and purification. For these reasons, we performed semiquantitative approaches on DNA from fresh blood only.

The number of newborns enrolled in the pilot project was lower than estimated during study design (see online supplemental methods), even though sufficient for epidemiological purposes. While on one side the compliance of families has been above expectations, the lack of participation of the largest birth centre and the denatality rate of about 5%/year that has been occurring in Italy since 2015 had a negative impact on recruitment. The global impact of the SARS-CoV-2 pandemic on natality is unknown, although in our regions the number of newborns in 2021 (approximately 61 700) was comparable to that of 2020 (60 362).

With the regional implementation, compliance of families for SMA-NBS has raised up to 98%–99%, likely due to two reasons: (1) the informed consent form was simplified, and (2) the idea to participate in a pilot study may have negatively influenced the parental choice. Differently from the M-NBS, for which the passive consent strategy is applied, the European General Data Protection Regulation (EU-GDPR) imposes the active consent for any genetic screening test. A specific adaptation of the EU-GDPR to genetic NBS in general would probably be advisable to maximise participation, as we can expect in the next future an increase in the number of conditions undergoing genetic NBS. This will have a relevant impact on the workload of the birth centre personnel, to provide families with adequate information and to get informed consent signed. In the specific case of SMA, the NBS provides the opportunity of a dramatically different treatment outcome. This could be considered as a life-saving intervention, not requiring specific parental consent.

In our population, the incidence of SMA was 1/6059, one of the highest in the NBS studies published so far. The range of the reported incidences is very wide and possibly at least in part explained by duration and size of the different studies (table 2).[22–29] In our opinion, it can be hardly attributed to the ethnicity of the populations analysed, although a higher frequency of SMA carriers has been reported in Caucasians compared with Asians or Hispanics.[30] Indeed, we can expect an admixture of genetic pools in the countries where the studies have been performed, due to migration flows, that could have mitigated the possible effect of ethnicity. Kay *et al*[25] ascribed the low incidence of SMA in New York State to the performance of expanded carrier screening programmes. However, these would have likely led to an increase in preimplantation or prenatal diagnoses that could have been tracked. Kay *et al*'s data could also be explained by false negative results, not yet identified, either due to technical issues or to small *SMN1* variants. Also the actual incidence of SMA in the Australian population cannot be properly estimated, since Kariyawasam *et al*[24] did not include patients with *SMN2* ≥4 that in our population and other populations account for 15%–35% of the patients identified.

Table 2 Population characteristics of SMA-NBS studies published so far; in the last two columns, there is the comparison of the incidence of each study compared with the present one (expressed by χ^2 statistics and p value)

				#*SMN2*					
Country	Incidence	False positives	False negatives	2	3	≥4	Reference	χ^2	P value
Taiwan	1/17 181 (7/120 267)	8	–	3	2	2	Chien *et al*[22]	5.67	0.017
Germany	1/6910 (43/213 279)	–	–	17	10	16	Vill *et al*[23]	0.45	0.50
Australia	1/11 544 (9/103 903)	1	–	6	3	0	Kariyawasam *et al*[24]	2.4	0.12
USA (New York)	1/28 137 (8/225 093)	–	–	3	3	2	Kay *et al*[25]	14.92	0.0001
Belgium	1/13 634 (10/136 339)	–	1 (small mutation)	5	3	2	Boemer *et al*[26]	4.17	0.04
USA (Massachussets)	1/19 941 (9/179 476)	1	–	7	0	2	Hale *et al*[27]	8.97	0.003
USA (Wisconsin)	1/10 164 (6/60 984)	–	–	2	2	2	Baker *et al*[28]	1.17	0.28
Latvia	1/5206 (2/10 411)	–	–	1	1	0	Gailite *et al*[29]	0.0408	0.84
Present study	1/6059 (15/90 885)	–	–	8	3	2			

Of the 15 patients identified through the SMA-NBS, six showed signs compatible with or clearly suggestive of disease onset at the first visit, although some of these patients displayed mild specific signs and were categorised as pauci-symptomatics.[12] All symptomatic patients had two *SMN2* copies and, intriguingly, were men (table 1). Our present cohort is too small to draw any conclusion on sex differences in SMA that so far remain anecdotal: men are usually thought more severely affected.

In the absence of SMA-NBS, most non-specific signs would have been underestimated until the appearance of clear disease manifestations. In these patients, signs of SMA became more evident in the few days between diagnosis and treatment start; the latter led to a quick improvement of the clinical picture. Our findings clearly indicate that, due to the risk of quick evolution of the clinical signs, the molecular diagnosis of SMA (even if precocious as in the case of NBS) should be felt as a true urgency by both clinicians and genetic laboratory scientists. Consequently, the turnaround time should be shortened to the minimum possible.

One further aspect that will require specific attention is the follow-up of subjects with *SMN2* of >4: during SMA-NBS, we found two subjects with six and five *SMN2*, in the pilot project and in the implementation phase, respectively. To our knowledge, only three patients with six copies (out of 3393) have been reported in the meta-analysis of Calucho *et al*.[8] In 1999, we reported a mild type IV patient with eight copies as determined by fluorescence in situ hybridization (FISH) on interphase nuclei.[31] When retested by qPCR, this subject had four *SMN2* copies. On finding the very rare genotype with six *SMN2* copies, we preferred to ask for an independent evaluation of our results and to perform the parental segregation of *SMN2* genes (see online supplemental figure 2). This led to a delay in providing the family with the report, but we were confident that the number of *SMN2* copies was >4.

High copy number genotypes are rare among patients; on the other hand, the frequency of subjects who have SMA by genetic test but display no sign or very mild signs of the disease in their life course is unknown. This infrequent situation raises the issue of counselling, follow-up and treatment strategy of these subjects, when identified in the SMA-NBS setting. Treatment is probably the most delicate topic: while the indication to start DMTs as soon as possible is well established for patients with two or three *SMN2* copies, the best approach for those with 4 *SMN2*, and even more for subjects with *SMN2* of >4, is still a matter of discussion in the scientific community.[32–34] It would be extremely useful to perform population studies among elder subjects (eg, patients belonging to other study cohorts, such as for Alzheimer disease or other late-onset conditions) in order to define the frequency of homozygotes for the *SMN1* deletion among subjects who do not display signs of SMA.

The standardisation of *SMN2* copy number determination is another crucial unmet issue in the molecular diagnosis of SMA, even more important in the NBS era. In the current scenario, *SMN2* copy number coupled with the clinical assessment is the main decision-making point for treatment start. This matter is very relevant for patients with *SMN2* of ≥4 since, regardless of the technique used, semiquantitative approaches lose accuracy for samples with a higher copy number, unless controls with the appropriate *SMN2* copies are included in the experimental settings. In our hands and with our in-house assay, comparable quality and quantity of controls and test DNAs are the other crucial node. This issue is likely independent of the technique used for *SMN2* assessment and would be addressed by comparative blind studies among laboratories. Most commercial tests have been conceived for carrier testing rather than for *SMN2* copy number determination, and thus, some adaptations of the protocols are also required. Inaccurate genotyping has a severe impact on the therapeutic outcome of the individual patients and will also have universal implications: we are now in the real-world phase of the postmarketing evaluation of DMTs. It is likely that presymptomatic patients will be stratified according to the *SMN2* copy number for the long-term evaluation of treatments, as in the case of the Nurture, Str1ve-US/EU or Rainbowfish studies.[2 35–37] Beside the purely scientific and clinical aspects, these data will also be useful to payers in the forthcoming years, coupled with SMA incidence figures, to establish the reimbursement policies of DMTs in single countries.

We can expect that, with the spread of SMA-NBS, the number of molecular centres involved in the diagnosis of SMA will increase markedly, mostly having limited experience in this field. In our opinion, it is crucial at this stage, quite embryonal for SMA-NBS, that the SMA scientific community provides both first-level and second-level diagnostic centres with unequivocal guidelines and standard

operating procedures for *SMN1/2* molecular analyses, arising from consensus documents and comparative studies among different laboratories.

Differently from the other published studies, for the molecular prognosis, we also tested the *SMN2* variants rs121909192 (c.859G>C; p.Gly287Arg) and rs1454173648 (c.A-44G) that increase the inclusion of exon 7 in *SMN2* mRNA and may significantly modify the prognosis.[10 11] The possible functional impact of other *SMN2* variants on SMA phenotype is still in the exploratory phase.[9] We identified one patient with two *SMN2* copies, the rs121909192 variant and no signs of the condition at birth[12]; due to the lack of prospective data on individuals with such genotype, the patient has been treated immediately on the basis of *SMN2* copy number only. In our opinion, testing of these variants should be implemented routinely in NBS programmes, and the therapeutic outcome of patients with one or the other variant needs to be evaluated separately due to the potentially different prognosis (see also Cuscò *et al*).[35] For this purpose, international cohorts are required to collect sufficiently large numbers of patients.

So far, we have no evidence of false negatives. We can exclude the possibility that type I SMA cases were missed, since over 10 months have elapsed since the end of the study, but we cannot rule out chronic forms of SMA. All samples identified at the first-tier screening were confirmed on fresh blood samples, showing absence of false positives in this series. The only exception was the case reported in the supplemental materials of a child that we interpreted as a mosaic *SMN1* homozygous loss (online supplemental figure 3). To our knowledge, mosaic deletion of *SMN1* has been reported twice in the literature in two carriers,[38 39] but no patient has been reported so far; thus, the prevalence is completely unknown, although it is likely a rare event. At any rate, this finding prompts to pay particular attention to potential false-positive results, in order to rule out possible mosaicisms. As in any mosaic condition, the phenotypical outcome for the child was unpredictable; thus, clinical follow-up would have been, in our opinion, advisable; unfortunately, the patient was lost at follow-up, and parental carrier testing could not be performed.

The management of the floppy infant is another issue that, in our opinion, would require some changes related to the effect of the early DMTs in SMA. In our very small cohort, 1/6 newborns were affected. The real prevalence of SMA among floppy infants is unknown, ranging from 2% to 7% according to two retrospective studies performed at the turn of *SMN1* identification,[40 41] likely leading to an underestimation of patients. In these patients, we preferred to determine *SMN1* copy number on fresh blood directly instead of searching for homozygous *SMN1* absence, to overcome the risk of missing carriers of small *SMN1* pathogenic variants. This approach required the set-up of a collaborative network between birth centres and diagnostic laboratories in order to get the 'floppy infant alert'. Similarly, the management of uncommon cases, such as the familiarity for known *SMN1* small variants (see online supplemental figure 1), requires the tight collaboration between neonatologists and geneticists since additional non-routine molecular tests are required.

In conclusion, following the marketing of DMTs, the clinical scenario of SMA has changed rapidly and has requested an effort to the referral clinical centres to adapt the management of patients to the presymptomatic diagnosis. The 'new' patients with SMA will be subjects with a molecular diagnosis rather than with a clinical picture. As a consequence, the nosology, the natural history of the disease and the standards of care need to be rewritten. It is important that the molecular diagnostic centres adapt to this new scenario: (1) the diagnosis of SMA should be felt as a true urgency with unprecedented short turnaround time for genetic tests[42]; (2) due to the lack of phenotypical feedback in presymptomatic patients, the accuracy of *SMN2* copy number determination needs to be improved and validated by comparative blind studies among referral laboratories; (3) the commercially available semiquantitative tests for *SMN1/SMN2* copy number need to be improved for higher *SMN2* copy number by including proper controls; (4) the *SMN2* splicing-modifier variants should be routinely tested in SMA-NBS; (5) the SMA community should provide non-expert laboratories with specific guidelines and standard operating procedures for the molecular diagnosis of SMA; and finally, (6) the issue of patients with small *SMN1* pathogenic variants, missed on the SMA-NBS, remains open.

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Acknowledgements We are very grateful to Famiglie SMA (in particular Daniela Lauro) for the continuous support. The authors acknowledge Alessio D'Amato, Alessandra Barca and Bruna Villani from Regione Lazio; Simone Bezzini, Stefania Saccardi and Cecilia Berni from Regione Toscana; Teresa Petrangolini from ALTEMS. We are grateful for the continuous support to the following current and former Biogen employees: Giuseppe Banfi, Fabio Buelli, Luca Bonvissuto, Federico Bressa, Patrizia Costa, Barbara De Cristofano, Laura Iannuzzi, Chiara Marchesi and Marco Pacifici.

Collaborators Italian SMA-NBS group: Maria Accardo, Beatrice Gambi, Rino Agostiniani, Simonetta Giampaoli, Ubah Farah Ahmed, Carlo Giannini, Concetta Alecci, Valerio Giardini, Antonella Amendolea, Monica Guarguagli, Monica Bartolucci, Cristina Haass, Ivana Belisario, Isabella Innocenti, Paola Belluomini, Maria Cristina Innocenti Berti, Claudia Bernardini, Claudia Maggi, Roberto Bernardini, Letizia Magi, Luisa Bertò, Roccuccia Malorgio, Rita Bini, Giovanna Maragliano, Leonardo Bolgia, Vita Maraglino, Mario Boscioni, Anna Marotta, Giorgio Bracaglia, Cristina Martini, Elisa Brachini, Palmina Martufi, Elisa Bruschi, Stefania Mastropasqua, Monica Campa, Maria Rosaria Matera, Monica Caprilli, Graziano Memmini, Simona Carcione, Angela Mendicino, Angelo Cardiello, Sabrina Mercurio, Francesco Cartolano, Alessandra Meucci, Alessandra Casati, Osvaldo Milita, Sandra Ceccarelli, Zeudi Morano, Marzia Chiellini, Eleonora Nardi, Rossella Ciccotti, Sandra Novelli, Elena Cioli, Luigi Padovano, Flavio Civitelli, Maria Gabriella Palermo, Maria Giovanna Colella, Serena Pascucci, Luigina Coppotelli, Caterini Patrizia, Francesco Crescenzi, Assunta Percoco, Carlo Dani, Stefania Petrolati, Roberto Danieli, Rosanna Pitaro, Tamara De Angelis, Marilena

Raponi, Mauro De Martinis, Francesco Riccobene, Elena degl'Innocenti, Giovanni Rosa, Laura Del Sette, Amanda Roversi, Ambrogio Di Paolo, Sabrina Sadocco, Luigi Di Ruzza, Emanuela Santarelli, Rosalia Di Silvio, Luca Tafi, Dituri Francesco, Barbara Tomasini, Gwenna Egho, Martha Traupe, Daniela Emili, Angelina Vaccaro, Luca Filippo, Pierluigi Vasarri, Claudia Foglia, Valentina Ventura, Francesco Gabriellini, Stefano Vitale, Luigi Gagliardi, Anna Maria Zingoni, Annamaria Gallo.

Contributors Conceptualisation: FDT, EM, MAD and EB; data curation: SS, EA, AN and AV: formal analysis: FDT, EA and AV; funding acquisition: FDT; investigation: TG, AD'A, GV, RS, FG, AD'A, SC, AP, MP, CT, MD, MS, IB, AL, GL, MG and MP; methodology: SF, AN, SS and MVF; project administration and writing (original draft): FDT, EA and AV; software: SS; supervision: FDT and MAD; writing (review and editing): any authors; informed consent from families and neonates' enrolment: Italian SMA-NBS group; guarantor and responsible for the entire content of the study: FDT.

Funding This study was supported by Biogen Italia (IIT #ITA-NBS-17-11262).

Competing interests None declared.

Patient consent for publication Consent obtained from parent(s)/guardian(s).

Ethics approval This study involves human participants and has been approved for the whole Lazio perinatal network by the ethics committee of Fondazione Policlinico Universitario - IRCCS 'Agostino Gemelli' (protocol #16790/18, study ID:2043) and for the Tuscanian network by the paediatric ethics committees of the Azienda Ospedaliera Universitaria 'Anna Meyer' (protocol #43/2020). The participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data sharing not applicable as no datasets generated and/or analysed for this study.

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