Clinical Phenotype of Pediatric and Adult Patients With Spinal Muscular Atrophy With Four *SMN2* Copies: Are They Really All Stable?

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Objective: The aim of this study was to provide an overview of the clinical phenotypes associated with 4 *SMN2* copies.
Methods: Clinical phenotypes were analyzed in all the patients with 4 *SMN2* copies as part of a nationwide effort including all the Italian pediatric and adult reference centers for spinal muscular atrophy (SMA).
Results: The cohort includes 169 patients (102 men and 67 women) with confirmed 4 *SMN2* copies (mean age at last follow-up = 36.9 ± 19 years). Six of the 169 patients were presymptomatic, 8 were classified as type II, 145 as type III (38 type IIIA and 107 type IIIB), and 8 as type IV. The remaining 2 patients were asymptomatic adults identified because of a familial case. The cross-sectional functional data showed a reduction of scores with increasing age. Over 35% of the type III and 25% of the type IV lost ambulation (mean age = 26.8 years ± 16.3 SD). The risk of loss of ambulation was significantly associated with SMA type ($p < 0.0001$), with patients with IIIB and IV less likely to lose ambulation compared to type IIIA. There was an overall gender effect with a smaller number of women and a lower risk for women to lose ambulation. This was significant in the adult ($p = 0.009$) but not in the pediatric cohort ($p = 0.43$).
Interpretation: Our results expand the existing literature on natural history of 4 *SMN2* copies confirming the variability of phenotypes in untreated patients, ranging from type II to type IV and an overall reduction of functional scores with increasing age.

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Spinal muscular atrophy (SMA) is a severe genetic motor neuron disease, caused by a defect on the survival motor neuron-1 (*SMN1*) gene located on chromosome 5q, leading to progressive weakness and muscle atrophy. *SMN2* is a highly homologous paralogous gene, which only partially compensates for the absence of *SMN1*, by producing mainly transcripts lacking exon 7, related to a functionally compromised and unstable SMN protein.

The updated recommendations on standards of care have reported consensus on the need to routinely assess *SMN2* copies at the time of diagnosis as the number of *SMN2* copies is currently the main SMA phenotype modifier.[1] The number can vary from 1 to 5 or more, with higher *SMN2* copies generally leading to milder clinical expression of the disease even if this, however, does not always hold true for individual cases.[2]

The need to assess *SMN2* copies has been further highlighted by the evidence of different therapeutical responses in patients with different number of *SMN2* copies.[3–6] Recent clinical trials have confirmed that this also holds true for presymptomatic patients, as infants with 3 copies have better outcome than those with 2.[7–9] So far none of the clinical trials in presymptomatic patients has included infants with 4 *SMN2* copies.

On the other hand, there is increasing real world evidence of infants with 4 *SMN2* treated after their identification on screening,[10–12] but a general consensus on the need to treat them has not been yet formulated. In 2018 a US based working group developed a treatment algorithm for infants identified by newborn screening (NBS) suggesting to treat as early as possible all infants with 3 or fewer *SMN2* copies.[13] Their position was subsequently updated to include also the infants with 4 copies of *SMN2*.[14] This suggestion has not been shared in other countries and the need to treat infants with 4 copies is still controversial. The main concern is generated by the relative paucity of information on the natural history and by the variable phenotypes and SMA progression associated with 4 copies. Whereas studies focusing on or including pediatric patients report early signs of clinical involvement,[15] those in adult cohorts report a high percentage of late onset of relatively mild clinical signs.[16–19] Because of this, even in countries where treatment is available for all the infants identified by NBS, irrespective of the copy number, not all clinicians or families opt for early treatment.[20]

The difficulties in predicting the severity of the phenotype in patients with 4 copies is further complicated by the lack of validation and reproducibility data on *SMN2* copy number determination among different laboratories. This issue is even more relevant for patients with higher number of *SMN2* copies, with increasing evidence of discordances between different laboratories in cases of retesting.[21]

The aim of our study was to provide an overview of the clinical phenotypes associated with confirmed 4 *SMN2* copies as part of a nationwide effort including all the Italian pediatric and adult reference centers for SMA.

Methods

The study includes all the 36 centers/units identified by the Italian government as referral centers for SMA

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throughout the whole national territory.[22] All these centers have recently been involved in a nation-based network, ITASMAc, established in 2021 as an extension of an academic registry, International Spinal Muscular Atrophy Registry (iSMAR),[23] originally including only 5 Italian academic centers in collaboration with UK and US networks. Approval was granted by the Ethics Committee of Fondazione Policlinico Universitario Agostino Gemelli IRCCS (coordinating center) (Date = 26/05/2020 and No. = 1894) and by all the other participating centers. Written informed consent was obtained in all participants.

This study was performed in line with the principles of the Declaration of Helsinki.

As part of the first activities of the national network, in 2021, we performed a survey to investigate prevalence of 5q SMA in Italy,[22] including details on *SMN2* copy numbers. In August 2022, we asked all centers to update data on patients with 4 *SMN2* and to provide details on a number of variables, including age, sex, time of symptoms onset, SMA type, treatment, actual motor function, clarifying eventual loss of ambulation, last motor assessment performed (Hammersmith Functional Motor Scale – Expanded [HFMSE] and Revised Upper Limb Module [RULM])[24,25] and overall nutritional and respiratory status. The centers were also asked to specify if patients were detected by NBS.

Because some patients are sometimes followed in more than one center, in order to avoid case duplications, a system allowing to generate unique global identifier numbers was provided to each center. The results from each center were centrally reviewed to check for possible duplicates. If found, the patient was counted only once.

Genetic Analysis

The determination of *SMN2* copies had originally been performed over the years in several laboratories by multiplex ligation-dependent probe amplification (MLPA) assay (SALSA MLPA Probemix P021 or P060, MRC-Holland) or, in the Gemelli laboratory, by quantitative polymerase chain reaction (qPCR; author Francesco Danilo Tiziano). The original DNA samples were extracted according to different protocols, manuals, or automatized.

For patients previously classified as having 4 copies by using MLPA, DNA samples were sent to a central laboratory (author F.D.T.) to confirm the number of *SMN2* copies. If the samples were found to be inadequate, when possible, resampling was requested. The confirmation was performed by qPCR, as reported elsewhere.[26] In case of discrepancy, an independent laboratory (author Dario Ronchi) was asked to retest the sample by MLPA (P021 assay).

For patients previously classified as having 4 copies by using qPCR in the Francesco Danilo Tiziano (F.D.T.) laboratory, the confirmation was performed in the same independent laboratory (author D.R.).

The presence of the c.859G>C (p.Gly287Arg, rs121909192) variant was also evaluated.

Patients with 5 or more copies identified in the previous survey were retested to confirm that they had more than 4 copies but were not included in this study.

Statistical Analysis

A Cox proportional hazards model was used to investigate the association between gender and loss at ambulation time and between SMA type and loss at ambulation. The cohort was also classified in pediatric and adult subgroups if their age was < or ≥ 18 years.

Results

DNA samples were available for 194 of the 206 patients reported in the survey and from additional recent new diagnoses. In 5 of 194 patients, the DNA sample was insufficient or inadequate and no resampling could be obtained.

Retesting

Of the 189 patients with adequate DNA sample, *SMN2* copy number was confirmed in 169 of 189 patients. Of the remaining 20 patients, 1 had 2 copies, 3 had 2 copies and the c.859G>C variant, and 16 had 3 copies. The discordant results, analyzed in the independent laboratory, showed a full concordance between the qPCR and the new MLPA assessment.

With the exception of the discordant cases, none of the other patients had the c.859G>C (p.Gly287Arg, rs121909192) variant.

Of the 169 patients with confirmed 4 copies, 102 (60%) were men and 67 (40%) were women. The mean age at last follow-up was 36.9 ± 19 (±SD; range = 1–81); 6 of the 169 were presymptomatic; and 2 of the 169 were asymptomatic (Fig 1).

Presymptomatic Patients

Of the 6 presymptomatic patients, 4 were identified through NBS and 2 were siblings of previously diagnosed patients with SMA. The mean age at last follow-up was 2.5 ± 1 years (range = 1–4). All infants had no symptoms and all had achieved the expected motor function for age (5 walker and 1 sitter) at the time of the last follow-up available. Two of the 6 had started treatment within the first year of age, one with nusinersen and one with risdiplam.

Symptomatic Patients

In the cohort for whom diagnosis was prompted by clinical symptoms, the onset of symptoms ranged between 8 months and 60 years. According to the clinical classification, 8 (5%) were type II and 145 (90%) were type III, including 38 of 145 that were type IIIa and 107 of 145 that were type IIIb. Another 8 patients (5%) were classified as type IV.

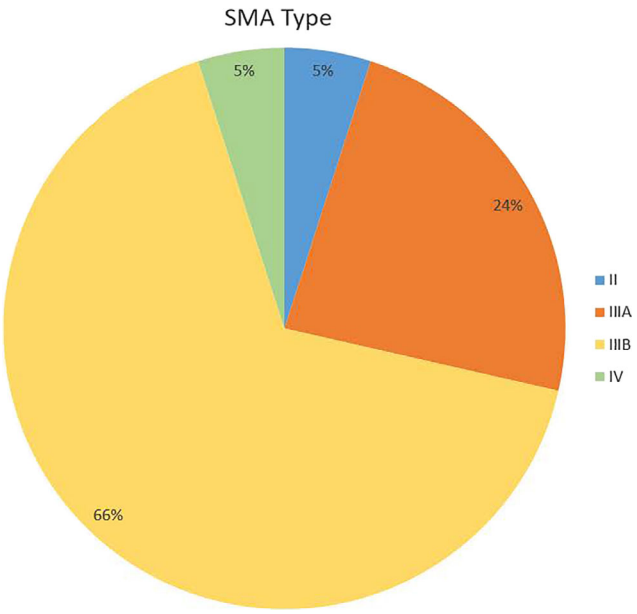

Figure 1: Details of the type of SMA in the symptomatic population (n = 161). SMA = spinal muscular atrophy. [Color figure can be viewed at www.annalsofneurology.org]

Type II

The 8 patients classified as type II never acquired the ability to walk independently and 3 of 8 patients also lost the ability to sit independently. Two of the 8 (25%) patients required noninvasive ventilation (NIV) ≤ 12 h/day and 4 of 8 (50%) patients showed mild swallowing problems not requiring tube feeding. At the last follow-up (mean age = 31.5 + 18.5 years, range = 7–56), 8 of 8 (100%) patients were treated with disease modifying therapies (3 with nusinersen and 5 with risdiplam).

Type III

Of the 145 patients classified as type III (38 as type IIIa and 107 and type IIIb), 52 (36%; 18 type IIIa and 34 type IIIb) lost ambulation at a mean age of 26.8 ± 16.3 (±SD; range = 3.5–69). The remaining 93 (64%) patients (20 type IIIa and 73 type IIIb) were still ambulant at a mean age at last follow-up of 32.5 ± 19.5 years (±SD; range = 3–78).

Seventeen of the 145 (12%) patients classified as type III required NIV ≤ 12 h/day after the age of 18 years

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❍ female untreated ambulant; ⊙ female treated ambulant; ● female untreated non-ambulant; ⊙ female treated non-ambulant
□ male untreated ambulant; ⊡ male treated ambulant; ■ male untreated non-ambulant; ◘ male treated non-ambulant

Figure 2: Representation of SMA progression in individual patients. Patients were grouped according to the age of onset (Y-axis) and, when available, subsequent follow-up visits using predefined age groups selected on the basis of their natural history data. Each individual was represented in the same position inside each cell and can be followed across the different cells (horizontally). Square = male patients; circles = female patients; blank symbols = ambulant patients; black symbols = non-ambulant patients; and dotted symbols = treatment. SMA = spinal muscular atrophy.

(range = 34–64 years): these included 6 of the 38 patients classified as type IIIa (16%) and 11 of the 107 (10%) patients classified as type IIIb.

Six of the 145 (4%) patients showed swallowing problems (3 patients classified as type IIIa and 3 patients classified as type IIIb) with 2 of them requiring a gastrostomy tube (G-tube).

One hundred seventeen of 145 (81%) patients were treated, 102 (87%) patients treated with nusinersen, 11 (10%) patients treated with risdiplam, and 4 (3%) patients switched from nusinersen to risdiplam.

Type IV

Eight patients were classified as type IV (mean age at last follow-up = 59.0 ± 18.2, range = 35–81), 2 of the 8 (25%) patients had lost ambulation at 60 and 70 years, respectively, at the time of the last follow-up available.

None of the 8 patients with SMA type IV showed bulbar function impairment. Only 1 of the 8 (1.2%) patients was treated with nusinersen (Fig 2 shows details of the whole symptomatic cohort).

Additional Patients

Two patients of 32 and 26 years (siblings), were identified because of a positive family history (2 first cousins were affected by type III SMA). The 2 sisters have been regularly followed since 2017 and are still asymptomatic.

Disease Progression

HFMSE results were available in 139 of the 161 symptomatic patients. The scores ranged between 0 and 66 (Fig 3 shows details of the HFMSE scores in ambulant and nonambulant patients).

SMA Type and Loss of Ambulation. When analyzing the population of patients with SMA III (a/b) and SMA IV, the Cox proportional hazards model results indicate that SMA type is significantly associated with the risk of loss of ambulation ($p < 0.0001$). The hazard ratios for SMA type IIIb and SMA type IV, compared to the reference group of individuals with SMA type IIIa, are 0.26 (95% confidence interval [CI] = 0.14 to 0.48) and 0.08 (95% CI = 0.018 to 0.0.38), respectively, indicating that patients with SMA IIIb and IV were less likely to lose ambulation (74% had a decreased risk for SMA IIIb and 92% for type IV) compared to the reference group of SMA type IIIa (Fig 4).

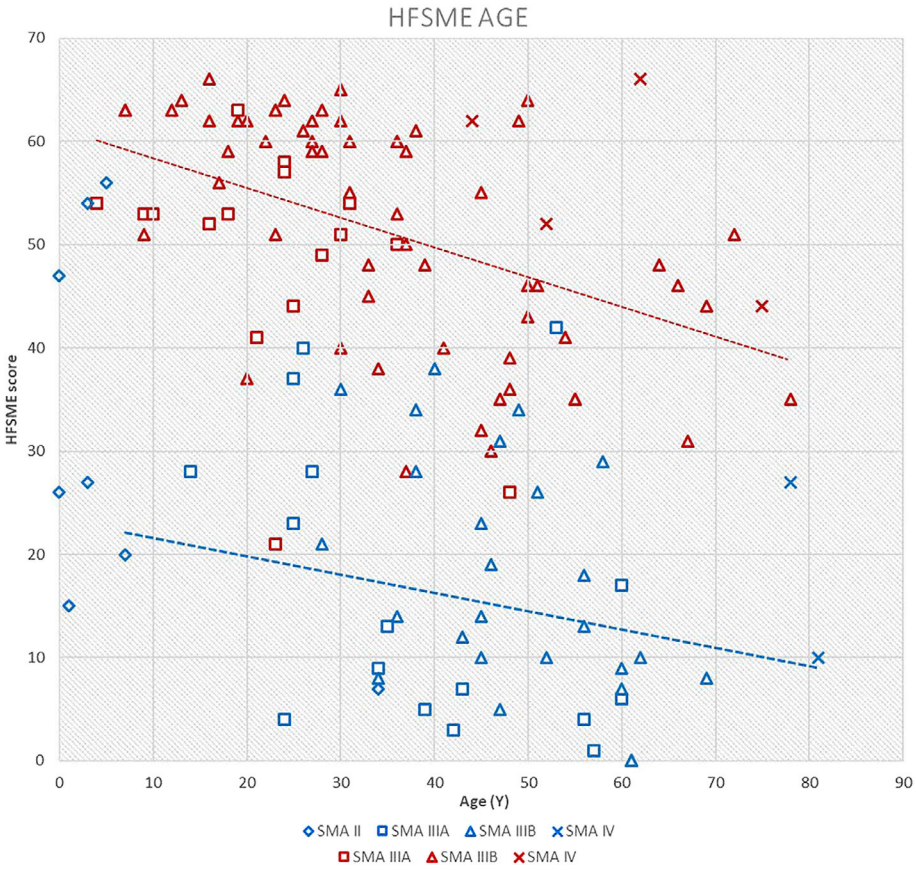

Figure 3: Details of the HFMSE scores in ambulant and nonambulant patients. HFMSE = Hammersmith Functional Motor Scale – Expanded; SMA = spinal muscular atrophy. [Color figure can be viewed at www.annalsofneurology.org]

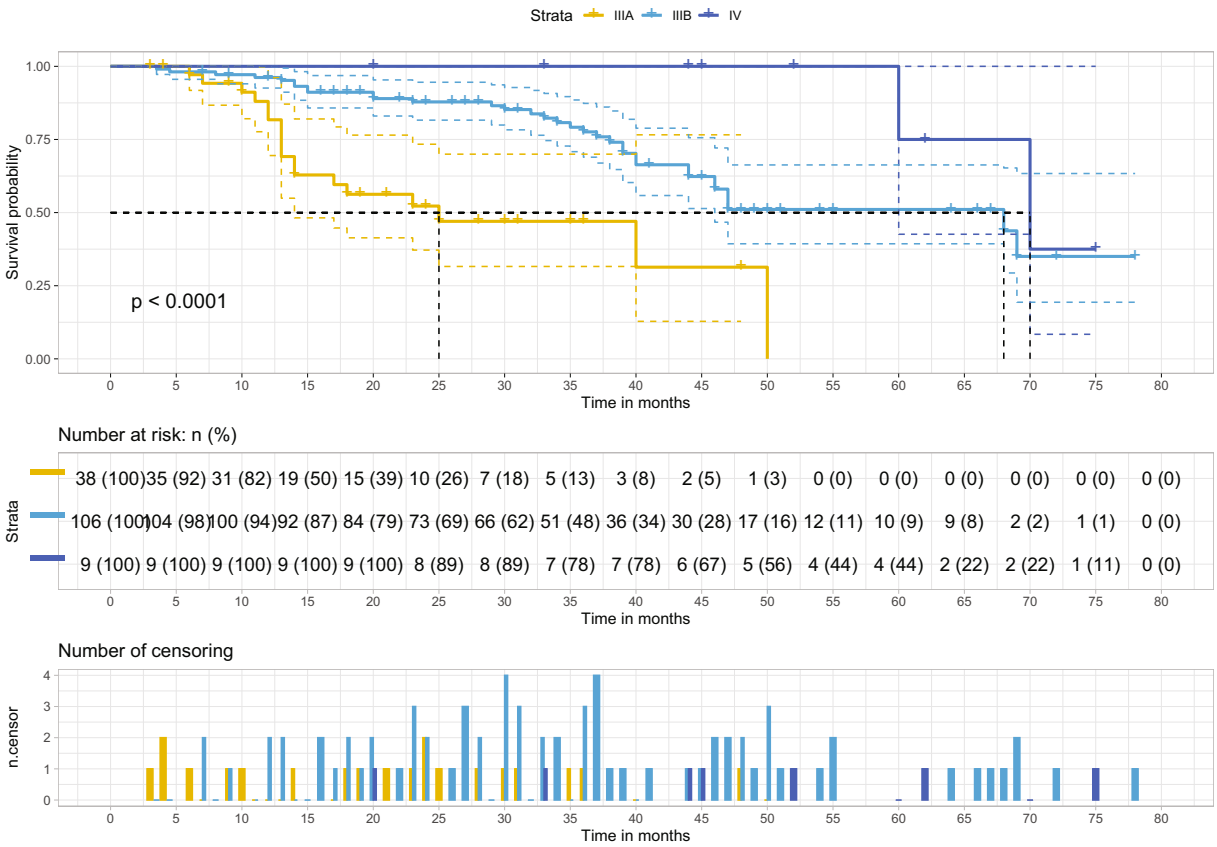

Figure 4: Hazard ratios for loss of ambulation in patients with SMA type IIIb and SMA type IV, compared to the reference group of individuals with SMA type IIIa. SMA = spinal muscular atrophy. [Color figure can be viewed at www.annalsofneurology.org]

Gender and Loss of Ambulation. Including the population of presymptomatic, SMA III, and SMA IV, the Cox proportional hazards model results indicate that the hazard ratio for male patients compared to female patients was 1.95 (95% CI = 1.05 to 3.61, $p = 0.032$; Fig 5).

Pediatric Population

In the pediatric population, the results of the Cox proportional hazards model analysis suggest that there is no statistically significant association between gender and loss of ambulation ($p = 0.43$). The hazard ratio for male patients compared to female patients was 0.67 (95% CI = 0.28 to 1.624; Fig 6).

Adult Population

When including only the adult population, the results of the Cox proportional hazards model analysis indicate that there is a statistically significant association between gender and loss of ambulation in the study population ($p = 0.009$). The hazard ratio for male patients compared to female patients was 3.16 (95% CI = 1.27 to 7.85), indicating that male patients have a higher hazard rate of losing ambulation than female patients (Fig 7).

Discussion

The ongoing discussion on if and when treatment should be started in patients with 4 *SMN2* copies identified through neonatal screening has highlighted the need to better understand the variability of phenotypes associated with 4 *SMN2* copies and the risk of developing a severe phenotype over the years. The phenotypic variability associated with 4 copies in the literature[16–19] partly reflects the cohorts studied, with studies focusing on adults more often reporting milder phenotypes and studies including pediatric cohorts also showing more severe phenotypes.

The definition of the phenotypic spectrum reported over years has been further complicated by the accuracy of *SMN2* copy number assessment, with recent evidence of discrepancies in the detection of *SMN2* copies among laboratories.[21] In our cohort, approximately 10% of the samples available for review were found to have a discrepancy. The reason for discrepancies was not assessable for all cases; in some cases, the poor quality of the available DNA samples, leading to the need for new samplings, may suggest that the discrepancy may be related to quantification and qualification of DNA samples. An additional reason

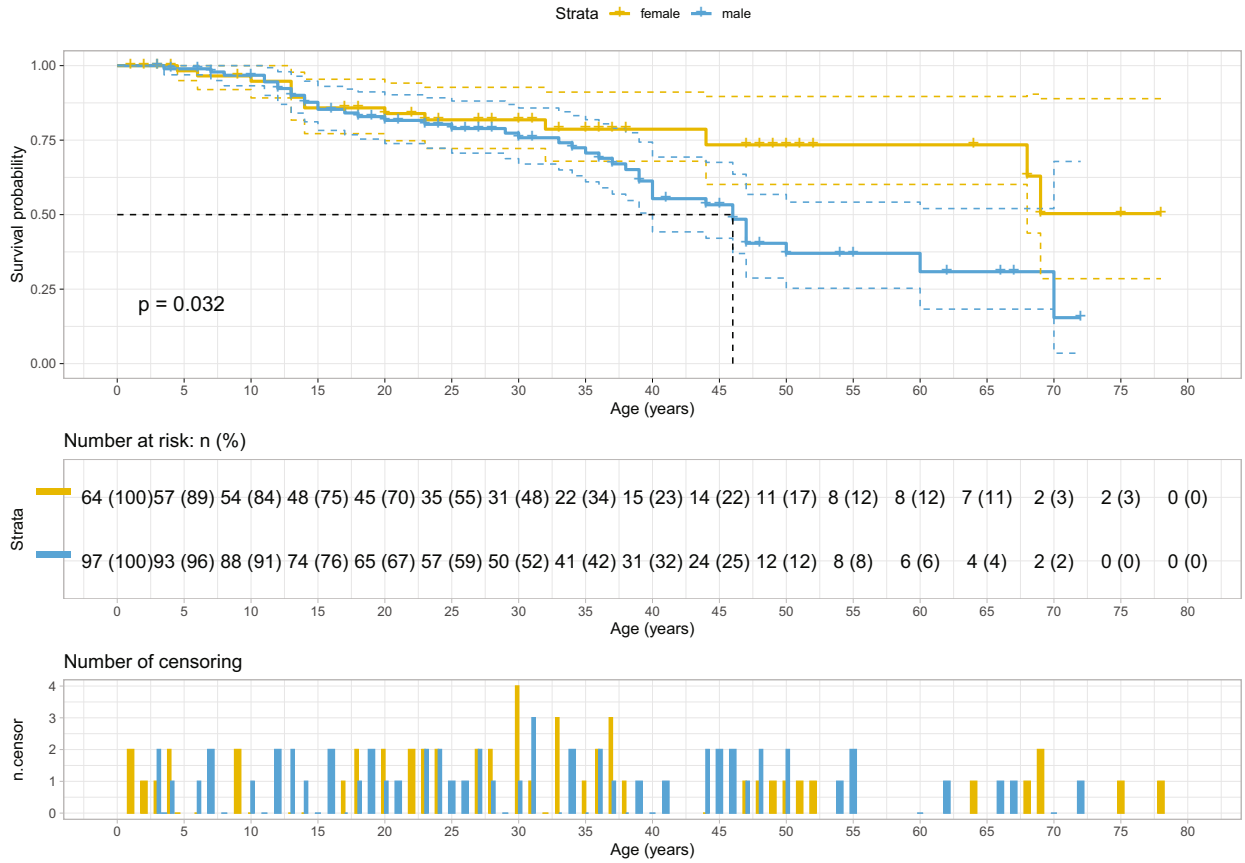

Figure 5: Hazard ratios for loss of ambulation in male patients compared to female patients in the whole cohort. [Color figure can be viewed at www.annalsofneurology.org]

may relate to the choice of control sample when setting semiquantitative assays.

The discrepant cases included 2 patients with a typical type I SMA found to have 4 copies on NGS who effectively had 2 and 3 copies, respectively, when retested on both qPCR and MLPA. These findings are in agreement with previous papers also reporting that the number of *SMN2* copies was not always confirmed on second testing in cases with severe phenotypes.[21] A more detailed multicentric study including patients with all copy numbers is in progress to further understand the frequency of discrepant cases and the possible reasons behind it. Our results, even if limited, suggest that accurate testing of optimal DNA samples for quality and quantity and the choice of appropriate controls is a crucial strategy independently of the technique used. Furthermore, they highlight the need for standard operating procedures to be shared to reduce the bias in *SMN2* copy number assessment.

The retesting of the samples and the possibility to include all patients with confirmed 4 *SMN2* copies from a nationwide survey, with an age ranging from birth to over 80 years, allowed us to better define the variability of phenotypes in a large cohort including all pediatric and adult patients. Approximately 5% of the patients with confirmed 4 *SMN2* copies had type II SMA and never acquired the ability to walk independently, confirming that 4 *SMN2* copies are generally associated with type III or IV SMA.[2,19] Interestingly, the type III SMA with onset before the age of 3 years, classified as IIIa, were also relatively few (26%), with over 74% of the type III individuals having onset after the age of 3 years and 47% of them after the age of 9 years.

The overall risk of losing ambulation in the whole cohort of type III and IV was 35% and was significantly associated with SMA type ($p < 0.0001$). Performing the Cox proportional hazards model, patients with SMA IIIb and IV were found to be less likely to lose ambulation (74% for patients with SMA IIIb and 92% for patients with type IV) compared to the reference group of patients with SMA type IIIa.

These findings have to be interpreted with caution as they do not reflect possible changes in loss of ambulation related to the advent of the new therapies. In our cohort, all patients but one lost ambulation before the new disease modifying therapies became available. A number of patients who are still ambulant have now been treated and although the post-treatment follow-up is too

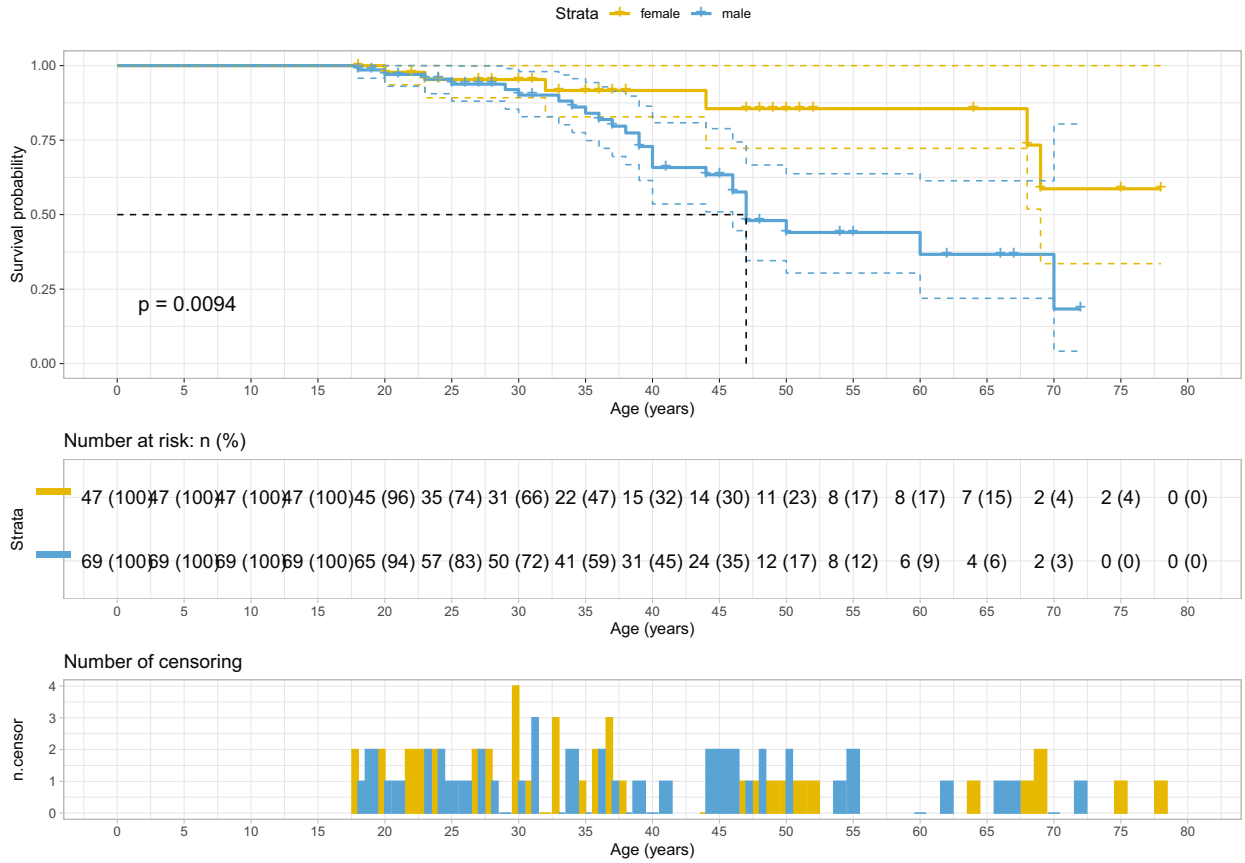

Figure 6: Hazard ratios for loss of ambulation in male patients compared to female patients in the pediatric population. [Color figure can be viewed at www.annalsofneurology.org]

short to draw any conclusion because none of the treated ambulant patients has so far lost ambulation.

Our findings also confirmed an overall gender[16,27,28] effect with a smaller number of female patients in the whole cohort, having an associated lower risk of losing ambulation, as has recently been reported in adult cohorts.[16] The availability of a larger dataset, including all ages, allowed further considerations. Our data suggest that the gender effect on risk of losing ambulation is significant in the adult population ($p = 0.009$), but not in the pediatric cohort ($p = 0.43$).

We also observed that the imbalance in gender representation was more obvious in patients with onset of clinical signs after puberty than in those with earlier onset. These findings are in agreement with the hypothesis that there may be other female patients who do not come to our observation because of a milder phenotype and that the number of overall 4 or more *SMN2* copies may be therefore underestimated.[16] The relatively high incidence of 4 *SMN2* copies found in some neonatal screening programs appears to support this hypothesis.[20]

In our cohort, we identified 4 patients through neonatal screening in addition to another 2 because of their family history. The relatively low number of patients identified at birth in our nationwide survey is justified by the fact that neonatal screening was available only in 2 of the 20 Italian regions at the time of data collection. All 6 patients were asymptomatic at birth with 2 of the 6 patients opting for treatment at the age of approximately 6 months. These numbers are similar to what has been reported in larger studies of neonatal screening also showing that even when the therapies are available, not all families opt for early treatment.[20,29]

The possibility to have a large cohort including all pediatric and adult patients also allowed to better characterize other aspects of the progression of the disease, showing that the need for respiratory or nutritional support was low and mainly limited to type II and IIIa patients.

Our results, with a nationwide approach, expand the existing literature on 4 *SMN2* copies, confirm the variability of phenotypes ranging from type II to type IV and provide details on the risk of having more severe forms with early onset (types II and IIIa). Our results also give evidence of the risk of progression and loss of ambulation in untreated populations. Despite the great majority of the patients with 4 *SMN2* copies, in the absence of early treatment, they had developed a type III or IV phenotype, approximately 1 out of 4 lost ambulation. The risk of

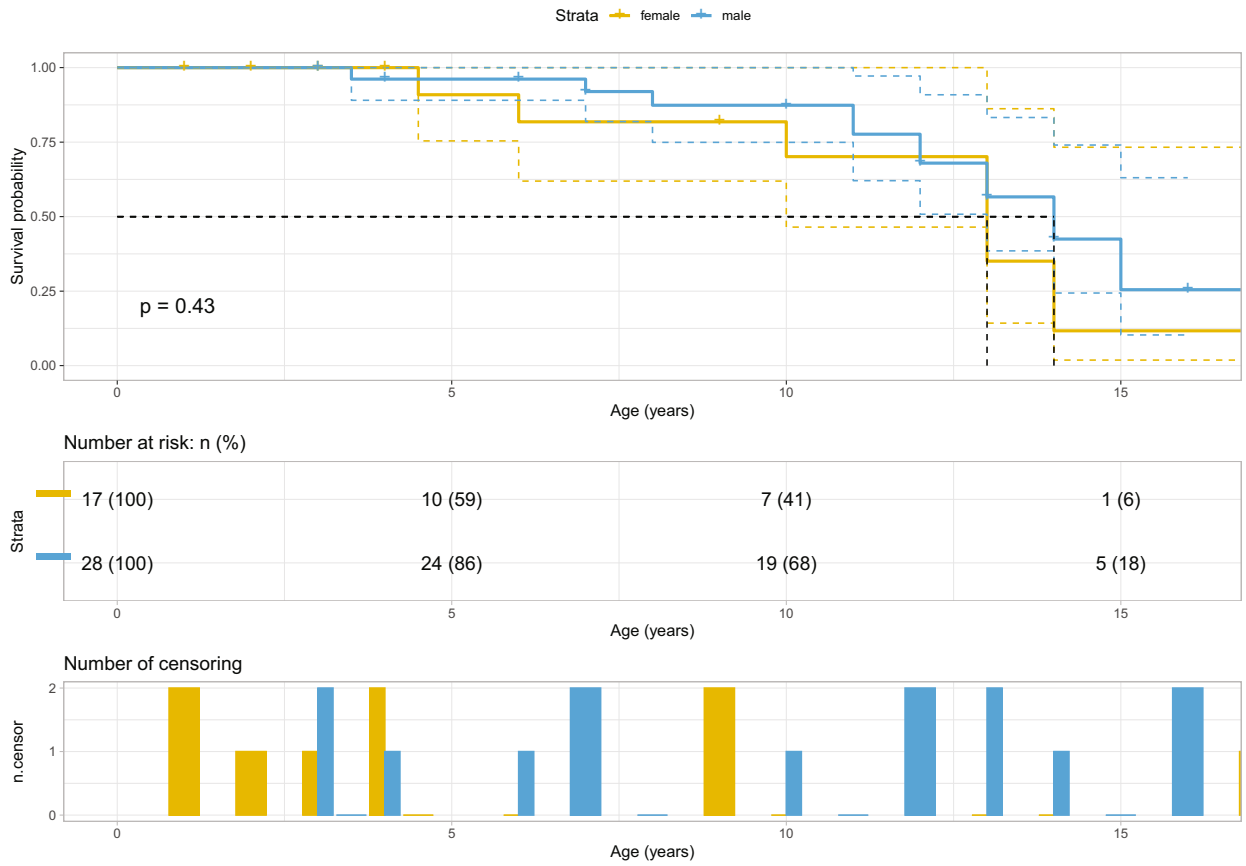

Figure 7: Hazard ratios for loss of ambulation in male patients compared to female patients in the adult population. [Color figure can be viewed at www.annalsofneurology.org]

losing ambulation was higher in male patients compared to female patients. Even if this was not a prospective study, the cross-sectional results of the HFMSE scores showed that there was a reduction of scores with increasing age even in the ambulant patients.

The evidence of progression and the relatively high number of patients with pediatric onset raised a discussion among the authors of this paper about the possibility to treat infants with 4 *SMN2* copies identified by neonatal screening. Whereas most of the authors felt that this was appropriate, there was no full consensus. All authors, however, felt that the availability of detailed information from a large cohort, including the risk of developing more severe phenotypes, will help families and clinicians to make a more informed decision on early therapeutic intervention.

Further studies assessing the structure of the SMN2 genes may help to detect differences or equivalencies beyond the SMN2 copy number, especially in the cases with more severe phenotype than expected.

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Author Contributions

M.R., G.C., A.C., M.P., E.M., and F.D.T. contributed to the conception and design of the study. MR, GPC, AC, MP, G.Coratti, E.M., and F.D.T. contributed to drafting the text or preparing the figures. V.A.S., C.B., S.M., T.M., M.C., G.S., E.P., R.M., M.F., L.M., M.G.D., R.L., C.T., L.R., L.V., F.S.R., A.B., M.A.M., M.G., V.N., S.C.P., G.Coratti, E.M., and M.P. and the ITASMAC working group (Table S1) participated in the acquisition and analysis of data.

Potential Conflict of Interest

M.R., C.T., A.C., M.C.P., G.Coratti, R.M., G.S., A.D., E.P., T.E.M., G.C., M.C., L.M., S.M., A.L.B., M.G.,

and F.S.R. report personal fees from BIOGEN S.R.L., ROCHE and NOVARTIS outside the submitted work. M.P. and V.A.S. are part of the advisory boards for BIOGEN S.R.L., ROCHE, AVEXIS, and NOVARTIS. E.M. is part of advisory board for BIOGEN S.R.L., ROCHE, AVEXIS, and NOVARTIS, Scholar ROCK, EPIRIUM, CYTOKINETICS, and NMD PHARMA. All remaining authors have nothing to disclose.

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