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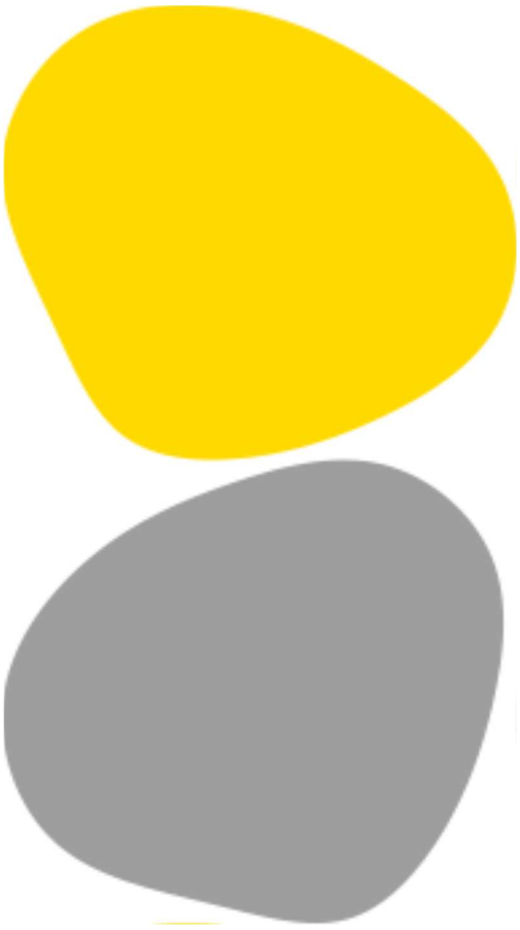
Screening for two *SMN1* variants associated with spinal muscular atrophy carriers of 2+0 genotype

Alberto Pessoa



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Resumo

A atrofia muscular espinhal (SMA) é a principal causa genética de mortalidade infantil e caracteriza-se pela perda de neurónios motores na medula espinhal e no tronco cerebral inferior, fraqueza muscular e atrofia. A deleção homozigótica do gene *SMN1*, o ortólogo totalmente funcional, é a causa genética mais prevalente da SMA. O gene *SMN2*, que partilha uma elevada homologia de sequência com o *SMN1*, produz sobretudo RNA mensageiro (mRNA) instável e truncado e, consequentemente, uma quantidade limitada de proteína totalmente funcional. O número de cópias do gene *SMN2* constitui um biomarcador prognóstico valioso para avaliar a gravidade clínica da SMA, ainda que não absoluto. Os portadores heterozigóticos de SMA possuem tipicamente uma cópia de *SMN1* num dos alelos (genótipo 1+0). Alguns portadores apresentam deficiência de *SMN1* num cromossoma, mas possuem duas cópias de *SMN1* em *cis* no outro (genótipo 2+0), tornando-se portadores "silenciosos", indistinguíveis de indivíduos não-portadores (genótipo 1+1) quando avaliados com as técnicas quantitativas atualmente disponíveis. Importa referir que as variantes de *SMN1* c.*3+80T>G e c.*211_*212del foram associadas a portadores 2+0 em populações judaicas asquenazes e espanholas. Neste estudo, utilizamos um método de rastreio adequado para identificar estas variantes e avaliar a sua relação com o estatuto de portador 2+0 na população portuguesa. Dado que o CGM-ULSSA funciona como laboratório de referência nacional para o diagnóstico genético da SMA, a implementação de métodos de rastreio custo-efetivos para estas variantes poderá ser determinante para reforçar o aconselhamento genético.

Palavras-chave: Proteína de Sobrevivência do Neurónio Motor 1 / genética; Atrofia Muscular Espinhal / diagnóstico; Atrofia Muscular Espinhal / genética; Rastreio Genético de Portadores; Aconselhamento Genético

Abstract

Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality and is characterized by motor neuron loss in the spinal cord and lower brainstem, muscle weakness, and atrophy. The homozygous deletion of the *SMN1* gene, the fully functional orthologue, is the most prevalent genetic cause of SMA. The *SMN2* gene, which shares a high sequence homology with *SMN1*, primarily produces unstable, truncated messenger RNA (mRNA), and consequently a limited amount of fully functional protein. *SMN2* gene copy number serves as a valuable prognostic biomarker for assessing the clinical severity of SMA, albeit not absolute. Heterozygous carriers of SMA typically possess one *SMN1* copy on one allele (1+0 genotype). Some carriers exhibit *SMN1* deficiency on one chromosome but have two *SMN1* copies in *cis* on the other (2+0 genotype), rendering them "silent" carriers, indistinguishable from non-carrier individuals (1+1 genotype) using current quantitative techniques. Notably, the *SMN1* variants c.*3+80T>G and c.*211_*212del were associated with 2+0 carriers in Ashkenazi Jews and Spanish populations. In this study, we employ a suitable screening method to identify these variants, and evaluate their relationship with 2+0 carrier status within the Portuguese population. As the CGM-ULSSA serves as a national reference laboratory for SMA genetic diagnosis, implementing cost-effective screening methods for these variants could prove instrumental in enhancing genetic counselling.

Keywords: Survival of Motor Neuron 1 Protein / genetics; Muscular Atrophy, Spinal / diagnosis; Muscular Atrophy, Spinal / genetics; Genetic Carrier Screening; Genetic Counselling

Index

1. Introduction

Spinal Muscular Atrophy (SMA) stands as the leading genetic cause of infant mortality, with a profound impact in the lives of individuals and their families (1). Its progression, characterized by motor neuron loss in the spinal cord and lower brainstem, leads to profound muscle weakness and atrophy, often beginning in infancy or early childhood. The consequences of SMA extend far beyond muscle function, influencing daily activities, mobility, and, in severe cases, even basic survival. The SMA classification system, based on the severity of the symptoms, reflects the complexity of the condition (Figure 1). The clinical presentation of SMA is classified from type 0 to type 4, in which the lowest number representing the most severe phenotype. Types 0 and 4 types the rarest (2,3):

1. Type 0 (often described as 1A): This is the most severe form, typically presenting in the prenatal or neonatal period. Newborns with SMA type 0 have profound muscle weakness and sever respiratory insufficiency. The prognosis for Type 0 SMA is very poor, and with survival often limited to the perinatal period.
2. Type 1 (Werdnig-Hoffmann disease, OMIM #253300) sometimes designated as 1B and 1C depending on age of onset): Symptoms appear within the first few months of life. Infants with SMA type 1 have marked muscle weakness and may struggle with basic functions like breathing and swallowing. Prognosis varies but generally indicates severe motor impairment and a limited life expectancy without intervention.
3. Type 2 (OMIM #253550): Characterized by muscle weakness that typically arise in early childhood. While individuals with type 2 SMA may never achieve the ability to walk independently, they often have a longer life expectancy and improved quality of life than those with type 1.
4. Type 3 (Kugelberg-Welander disease, OMIM #253400): Presents with milder symptoms, typically emerging in adolescence or adulthood. Patients with type 3 SMA may experience muscle weakness and motor difficulties but often retain the ability to walk and maintain independence in daily activities.
5. Type 4 (Adult-Onset SMA, OMIM #271150): This is the mildest form, with symptoms appearing in adulthood. Muscle weakness is usually less pronounced, and individuals with type 4 SMA can often lead relatively normal lives.

SMA classification is not merely academic but carries significant implications for prognosis, treatment options, and therapeutic strategies (4).

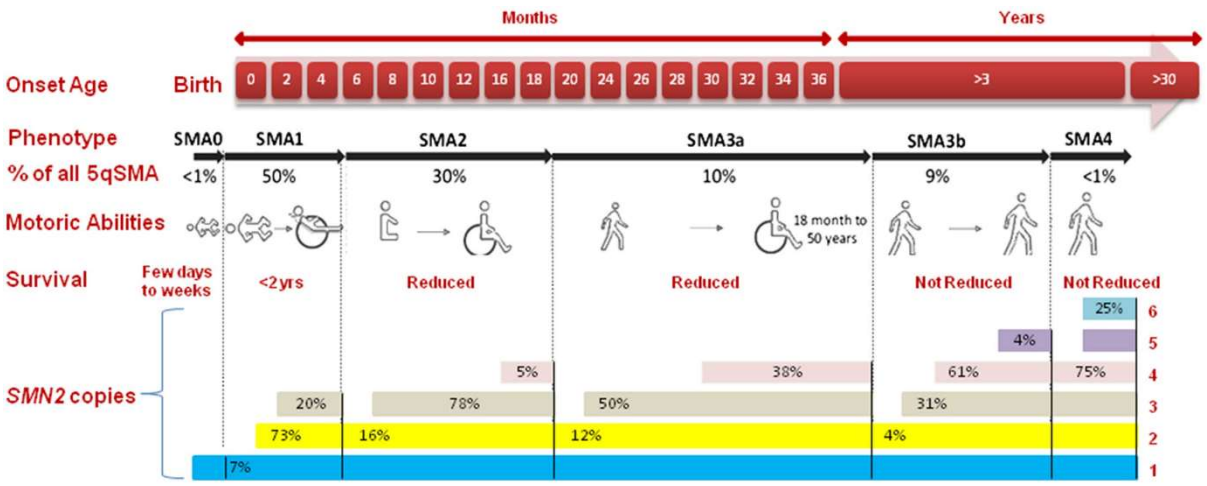

Figure 1 – SMA classification and progression. The figure depicts the five SMA types (0–4), organized by age of onset and motor function milestones. Type 3 is subdivided into 3a (<3 years) and 3b (>3 years). The distribution of *SMN2* gene copy number across SMA types is also presented, reflecting its association with clinical severity Adapted from Bagga *et al.* (5).

1.1. The vital role of Survival Motor Neuron protein

The Survival Motor Neuron (SMN) protein is an essential component for the functioning of organisms across the animal kingdom (6). SMA, as a result of the absence or deficiency of the SMN protein, gives rise to the wide range of symptoms that can vary in severity, as mentioned before. Because motor neurons are particularly dependent on SMN for maintaining axonal transport and neuromuscular junction integrity, its deficiency disrupts motor neuron survival and communication with muscle, giving rise to the progressive weakness characteristic of SMA. Understanding this crucial role played by the SMN protein has led to significant advances in SMA research, particularly on identifying ways to restore SMN protein levels, thereby aiming to halt or slow down the progression of the disease: innovative treatments, such as gene therapy and targeted drug interventions, have been developed (4,7,8). These approaches aim to address the underlying cause of SMA by boosting the production of the SMN protein, potentially offering a lifeline to those affected by this devastating disease.

Moreover, the significance of the SMN protein extends beyond SMA. This multifunctional protein is involved in various cellular processes, making it a crucial player in the intricate web of molecular activities that sustain life.

One of the key roles of the SMN protein is in RNA metabolism: it participates in the assembly of ribonucleoprotein complexes, which are essential for the proper functioning of RNA molecules within cells. SMN interacts with small nuclear ribonucleoproteins (snRNPs), critical components of the spliceosome machinery hat mediates pre-mRNA splicing (9). Beyond its role in RNA metabolism, the SMN protein is also implicated in DNA recombination and repair, signal transduction, intracellular trafficking, endocytosis, and autophagy (6).

Emerging evidence links SMN dysfunction to other pathological conditions such as inclusion body myositis (IBM), amyotrophic lateral sclerosis (ALS), osteoarthritis, and male infertility/testicular underdevelopment. While still under investigation, these findings suggest that the functions of the SMN protein may have broader implications for the health and development of various tissues and systems within the body (10).

The multifunctional nature of the SMN protein underscores its significance in a wide range of cellular processes, making it a focal point for ongoing research into both SMA and other pathological conditions. A deeper understanding of the diverse roles of SMN holds the potential to unveil new therapeutic avenues for addressing not only SMA but also other disorders where SMN dysfunction is implicated.

1.2. Genetic underpinnings of SMA

At the core of SMA's genetic basis is the homozygous disruption of the *SMN1* gene (OMIM *600354), located on chromosome 5q13. This gene encodes the SMN protein, which, as previously discussed, is an essential multifunctional protein. Homozygous disruption of *SMN1* genes is the most frequent genetic cause of SMA (11). The autosomal recessive inheritance pattern means that an individual needs to inherit two defective copies of the *SMN1* gene (one from each parent) to develop disease. Individuals who inherit only one copy of the gene with a variant (carrier status) do not typically exhibit symptoms of the disorder but can pass the variant on to their offspring (12).

The prevalence of carrier status for SMA has been estimated in various populations. In populations of European ancestry, the carrier frequency is estimated to be approximately 1 in 30 to 1 in 60 individuals (13). Interestingly, the carrier frequency is lower in populations of African ancestry and even lower in North American Hispanics, with an estimated carrier frequency of 1 in 110 (14). In Portugal,carrier frequency is around 1 in 52 (15). These variations in carrier frequencies between populations are significant when considering the global impact of SMA and the necessity for targeted awareness and screening programs based on the genetic makeup of different regions.

In a small percentage of SMA cases (about 2%), affected individuals harbor a *de novo* variant on one allele (16). A *de novo* variant refers to a genetic alteration that occurs for the first time in the affected individual rather than being inherited from either parent. These sporadic cases highlight the complexity of genetic variants and the potential for new ones to arise in the absence of a family history of SMA. The occurrence of *de novo* events adds an additional layer of variability to the genetics of SMA and emphasizes the need for ongoing genetic research to uncover the factors contributing to these sporadic cases.

1.3. The paralogous gene *SMN2*

The genetics of SMA becomes more complex when we delve into the genomic organization of the *SMN* genes, particularly *SMN1* and its paralogous counterpart *SMN2* (OMIM *601627), also located at chromosome 5q13 . This unique genomic arrangement contributes significantly to SMA pathogenesis and has profound implications for the regulation of SMN protein production.

SMN1 and *SMN2* are highly similar and closely located within the genome, but their subtle differences have far-reaching consequences. One of the most critical disparities between these two genes lies in five nucleotide differences, and one specific variation holds particular significance: a cytosine (C) to thymine (T) transition in exon 7 of *SMN2* and leads to altered splicing patterns of the resulting mRNA (Figure 2).

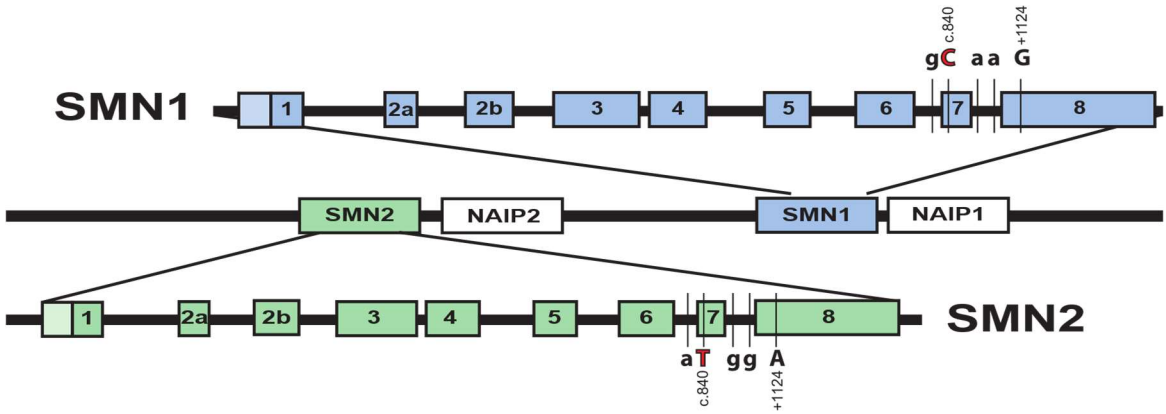

Figure 2 – SMN locus and *SMN1/ SMN2* gene organization. *SMN1* and *SMN2* are located on chromosome 5q13, which contains a large inverted duplication. *SMN1* lies on the telomeric side and *SMN2* on the centromeric side of this region. Although these two genes are nearly identical, they differ by a small number of base pairs, including a critical nucleotide change in exon 7. The precise arrangement of the *SMN* and NAIP genes within this locus remains to be elucidated. Adapted from Wadman *et al.* (17).

Splicing is the process by which introns (non-coding regions) are removed from pre-mRNA, and exons (coding regions) are joined to generate mature mRNA. In the case of *SMN2*, this nucleotide change primarily results in the exclusion of exon 7 from approximately 90% of the transcripts. Exon 7 encodes a critical portion of the SMN protein, and its exclusion leads to a truncated and less stable form of the protein that is promptly degraded (18).

This unique genetic characteristic is a major factor in the clinical presentation of SMA. Individuals with SMA typically carry variants in both copies of the *SMN1* gene, resulting in a deficiency of functional SMN protein. However, the presence of the *SMN2* gene provides a degree of compensation. Although most of the protein produced by *SMN2* lacks the amino-acid residues encoded by exon 7, some functional full-length SMN protein is still produced from *SMN2*, albeit at lower levels than from *SMN1*. This is why *SMN2* is often referred to as a "modifier" gene in SMA (19) (Figure 3).

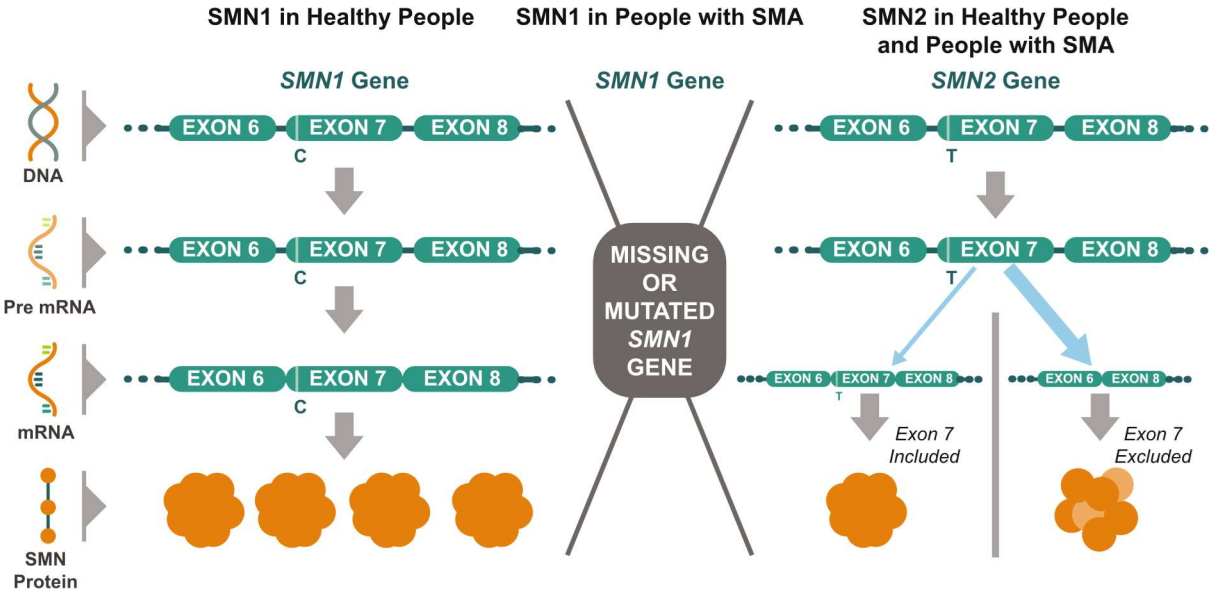

Figure 3 – Normal SMN protein expression and alterations in spinal muscular atrophy. *SMN1* is the primary source of full-length SMN protein required for motor neuron survival and muscle function. *SMN2*, nearly identical to *SMN1*, predominantly produces transcripts lacking exon 7, leading to unstable and rapidly degraded protein. A small fraction of *SMN2* transcripts generate full-length functional SMN protein, providing partial compensation. In patients with SMA, the loss of *SMN1* eliminates the main protein source, leaving *SMN2* as an insufficient backup. The number of SMN2 gene copies influences disease severity, with higher copy numbers associated with milder phenotypes. Adapted from Day *et al.* (20).

The ratio of functional SMN protein derived from *SMN1* and *SMN2* plays a critical role in determining the severity of SMA. Individuals with more copies of *SMN2* tend to have milder forms of the disease due to the increased production of partially functional SMN protein from *SMN2*. Conversely, those with fewer copies of *SMN2* experience a more severe phenotype due to the limited compensatory effect of SMN2 (10).

Although *SMN2* copy number alone isn't enough for a reliable prognostic prediction in SMA pacients, understanding the interplay between *SMN1* and *SMN2*, particularly the impact of the C>T transition in exon 7, and the resulting alteration in protein production, has been essential for developing targeted therapeutic strategies (7).

1.4. Carrier status in SMA

Similar to the variations in *SMN2* copy numbers, individuals can have varying copy numbers of *SMN1*. While having multiple copies of *SMN2* can mitigate the severity of SMA, the number of *SMN1* copies is also of great significance. Typically, healthy individuals inherit at least one functional copy of *SMN1* from each parent (e.g., 1+1, 2+1, 2+2, etc.). Because SMA is an autosomal recessive disease, affected

individuals will have no functional *SMN1* genes (0+0), while carriers possess only one functional copy of *SMN1* (1+0) (21).

However, there exists another category in SMA genetics, known as "2+0 carriers" or "silent carriers", in which two *SMN1* copies in the genome are in *cis* (22) (Figure 4). The individuals carrying this genetic configuration are phenotypically indistinguishable from the ones with two copies of *SMN1* from each parent (in *trans*) (1+1). Traditional genetic testing methods, such as sequencing and copy number analysis, struggle to distinguish between these two genotypes accurately, necessitating the use of familial haplotyping.

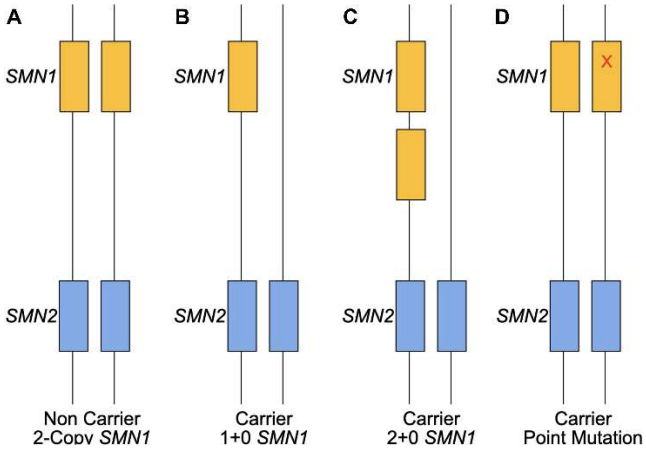

Figure 4 – Representative configurations of the *SMN1*/*SMN2* loci. (A) Wild-type individual carrying two copies of *SMN1* and *SMN2* on each chromosome. (B) Classical SMA carrier with one intact *SMN1* allele and complete loss of *SMN1* on the homologous chromosome. (C) Silent (2+0) carrier with both *SMN1* copies located on the same chromosome and absence of *SMN1* on the other. (D) Carrier with one functional *SMN1* copy and a second *SMN1* allele harboring a pathogenic point mutation. Adapted from Prior *et al.* (23)

Familial haplotyping involves analyzing of the genetic markers surrounding the *SMN* genes (upstream, downstream and one intragenic marker shared by both genes) to phase the alleles, tracing the origin of the functional and non-functional copies within a family. This technique can identify risk haplotypes for the disease and provide crucial insights into whether a patient is a 2+0 carrier, as the patterns of *SMN1* copies may differ between relatives within a family (24). However, familial haplotyping is not without its challenges as it requires extensive genetic data from family members, which may not always be available or easily obtained.

Determining carrier status of individuals with family history of SMA is invaluable for prenatal planning decisions and genetic counselling, as it can have significant implications for their offspring.

In 2014, two *SMN1* variants c.*3+80T>G and c.*211_*212del were identified as associated with the 2+0 carrier status, particularly in Ashkenazi Jews (25). In 2018, a study of 270 Spanish individuals revealed a

similar association of these variants with carriers possessing two copies of *SMN1* in *cis* compared to those with one copy (17.9 vs. 0.7%; $p < 0.001$) (26).

Although these variants do not produce clinical symptoms, they may serve as biomarkers for carrier status, providing a useful tool in genetic counselling, particularly in cases where familiar haplotyping is not feasable.

1.5. Objectives

In this study, we will implement an appropriate method for screening each of the *SMN1* variants c.*3+80T>G and c.*211_*212del, associated with SMA silent carriers, and use such methods to characterize Portuguese individuals. Although the absence of these variants does not exclude that a given individual is a carrier of SMA 2+0, their presence substantially increases the risk of being a carrier. As CGM-ULSSA (Centro de Genética Médica – Unidade Local de Saúde de Santo António) is a national reference laboratory for the genetic diagnosis of SMA, the implementation of cost-effective screening methods for these variants may be a valuable tool to improve genetic counselling. It is expected to obtain results in line with those published for the Spanish and Ashkenazi Jews populations.

In order to achieve these goals, our study will comprise of:

- Development and validation of a PCR-based methodology for screening of the c.*3+80T>G and c.*211_*212del variants;
- Frequency determination of these variants in individuals with genotype 2+0 and 1+1.
- Assessment of a statystical association between both variants and genotypes, in a portuguese cohort.

2. Methods

The following methods describe the molecular workflow used to detect and assign the *SMN1/SMN2*-associated variants c.*3+80T>G and c.*211_*212del. The overall experimental workflow and decision points are summarized in a decision tree (Figure 5). Briefly, fluorescence PCR (F-PCR) assays were used as primary screens for the c.*211_*212del and c.*3+80T>G variants as described in Section 2.3. Positive outcomes for c.*3+80T>G prompted re-amplification using the alternative primer set described in Section 2.7 and targeted digestions (*Dra*I alone and *Dra*I+*Hpy*188I) to localize the variant to *SMN1* specifically.

To ensure the robustness of the developed workflow, methodology validation was performed by Sanger sequencing of representative PCR products, confirming the accuracy of the variant calls derived from restriction digestions and capillary electrophoresis (Section 2.5). Furthermore, statistical analysis was undertaken to evaluate the distribution of the c.*3+80T>G and c.*211_*212del variants across 2+0 SMA carriers and 1+1 non-carriers. Differences in prevalence were tested using chi-squared analysis, with a threshold of $p < 0.05$ considered statistically significant (Section 2.6).

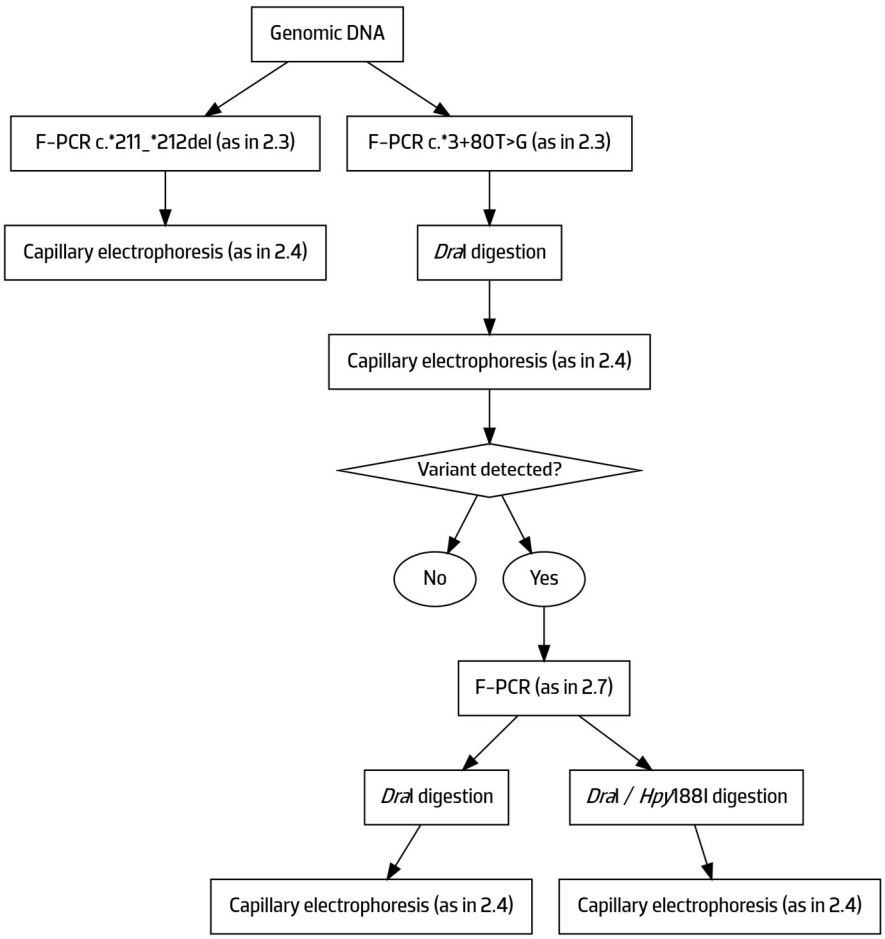

Figure 5 – Decision tree summarizing the molecular workflow for the detection and assignment of *SMN1/SMN2*-associated variants.

2.1. Biological samples

For this study, the selection of the individuals took into account their *SMN1* genotype, which had previously been confirmed using Multiplex Ligation-dependent Probe Amplification (MLPA), for copy number determination, and haplotyping analysis for genotype characterization. Genomic DNA samples, previously extracted from peripheral blood,from a cohort of anonymously selected individuals, were used.

The sampling comprised 2+0 carriers (n = 10) and 1+1 non-carriers (n = 28), both confirmed through familial haplotyping. In addition, individuals with three (n = 73), four (n = 3), and five (n = 1) *SMN1* copies were included. For these cases, since haplotyping was not available, the observed copy numbers were interpreted under the assumption that they most likely represent 2+1, 2+2, and 3+2 configurations, respectively. Genotypes such as 3+0, 4+0, or 5+0 were not considered to be present in this cohort, as reports of such haplotype arrangements are scarce in the literature, with only isolated descriptions of individuals carrying multiple *SMN1* copies on a single allele confirmed by familial haplotyping (26).

This study has been approved by DEFI – Departamento de Ensino, Formação e Investigação – Unidade Local de Saúde de Santo António (ULSSA), as well as the Hospital's Ethical Committee – N/REF. 2023-013(011-DEFI/012-CE).

2.2. Primer Design

Appropriate primer sets were designed to specifically amplify the regions containing the c.*3+80T>G and c.*211_*212del variants (NM_000344.4).

The primer design process began with the retrieval of the sequence region in GRCh37/hg19 version of human genome for the *SMN1* gene from UCSC Genome Browser (https://genome.ucsc.edu/) (27), harbouring the variants of interest, c.*3+80T>G and c.*211_*212del.

These primers were designed in Primer3Plus (28) using the recommended settings: primer length, melting temperature (Tm), and GC content were kept within recommended values and the size of the amplicons generated by the primers was kept within a defined range (200 to 400 bp) to facilitate ease of detection and downstream analysis, particularly in capillary electrophoresis.

The designed primers were subjected to *in-silico* validation to assess their specificity, sensitivity, and potential for primer dimer formation. This validation process involved UCSC Genome Browser BLAT and

Primer-BLAST analysis against the human genome to confirm the absence of significant off-target matches.

SNPCheck (https://genetools.org/SNPCheck/snpcheck.htm) was used to ensure no relevant Single Nucleotide Polimorphisms (SNPs) would disrupt proper primer annealing.

For the screening of the variant c.*3+80T>G, an internal restriction control was incorporated in the reverse primer. Forward primers were labeled with FAM.

2.3. Fluorescence PCR and Restriction Fragment Length Analysis

The amplification of the genomic DNA samples,by fluorescence PCR (F-PCR), was performed using the PCR Master Mix (Promega®, Madison, WI, USA), with the following thermal cycling conditions: denaturation of 10 min at 95 ºC, 35 cycles of 1 min at 95 ºC, 1 min at 56 ºC, and 1 min at 72 ºC, with a final extension of 10 min at 72 ºC. Each reaction of 20 µL included 50 ng of genomic DNA sample and 5 pmol of both primers. Distinct PCR assays were prepared for each variant using the appropriate primer pairs designed for them.

Restriction fragment length analysis (RFLA) was conducted for the variant c.*3+80T>G using 5 µL of the corresponding F-PCR product digested with the restriction enzyme *Dra*I, *Hpy*188I and both (New England BioLabs®, Ipswich, Massachusetts, EUA),in distinct assays, and incubated overnight at 37 ºC.

2.4. Capillary Electrophoresis of PCR Products

The F-PCR (for the c.*211_*212del variant) and the digestion products (for the c.*3+80T>G variant) were subjected to capillary electrophoresis and resolved on the ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems™, Foster City, CA, USA) using 500 ROX™ size standard (Gene Scan™, Warrington, UK). Electropherograms were analysed using GeneMapper® software version 4.0 (Applied Biosystems™) to determine the presence or absence of the c.*3+80T>G and c.*211_*212del variants.

2.5. Methodology Validation

To validate the methodology, the genomic DNA samples used in the development phase were subjected to Sanger sequencing. This approach consisted in symmetric amplification performed using the PCR Master Mix (Promega®, Madison, WI, USA), with the following thermal cycling conditions: denaturation of 10 min at 95 ºC, 40 cycles of 1 min at 95 ºC, 1 min at 56 ºC, and 2 min at 72 ºC, with a final extension of 10 min at 72 ºC. Each reaction of 20 µL included 100 ng of genomic DNA sample and 10 pmol of the primers 5′-TGTAAAACGACGGCCAGTTGAGCCACTGCAAGAAAA-3′ (forward) and 5′-

ACTACAACACCCTTCTCACAGC-3′ (reverse). The PCR products were purified using the Illustra ExoStar 1-Step (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions, and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The asymmetric PCR for the sequencing reaction used 0,85 pmol of previously mentioned reverse primer and consisted of an initial denaturing step at 96 °C for 1 min, followed by 27 cycles (96 °C for 10 s; 50 °C for 5 s; and 60 °C for 1 min and 15 s), and a final extension at 60 °C for 5 min. Products were resolved on ABI PRISM 3130xl Genetic Analyser (Applied Biosystems) and the results analysed using SeqScape Software version 2.5 (Applied Biosystems).

2.6. Statistical analysis

Differences in the prevalence of the c.*3+80T>G and c.*211_*212del variants between 2+0 SMA carriers and 1+1 non-carriers were tested using the chi-squared test, with significance levels set at $p < 0.05$. Statistical analysis with SigmaPlot version 14.0 (Systat Software®Inc., Chicago, IL, USA).

2.7. Uncover the gene loaction of the variant c.*3+80T>C

To determine whether the c.*3+80T>G variant is present on *SMN1*, an additional PCR-based approach was implemented. A distinct forward primer (5′- FAM·TTTGTTGAATAAAATAAGTAAAATGT-3′) was combined with the previously designed reverse primer for this variant to amplify a genomic region encompassing both the *Dra*I and *Hpy*118I restriction sites.

Following amplification, the PCR products were subjected to separate digestions with *Dra*I alone and a combination of *Dra*I and *Hpy*118I (New England BioLabs®, Ipswich, MA, USA), under conditions described in Section 2.3. The resulting restriction fragments were analysed by capillary electrophoresis on the ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems™, Foster City, CA, USA) using the 500 ROX™ size standard (Gene Scan™, Warrington, UK), as previously described in Section 2.4.

Fragment sizes generated from the different digestions allowed differentiation between *SMN1*- and *SMN2*-derived alleles, facilitating the precise localization of the c.*3+80T>G variant to the corresponding gene. Electropherograms were interpreted using GeneMapper® software version 4.0 (Applied Biosystems™) to assign the variant to either *SMN1* or both, based on the presence or absence of specific restriction fragments.

3. Results

3.1. Primer design strategy

Primer pairs were designed to specifically amplify the target regions harbouring the variants individually, while minimizing the potential for nonspecific amplification of closely related genes or sequences. This was achieved by strategically positioning primer binding sites to span the variant positions, enabling variant-specific amplification (Figure 6).

>NC_000005.9:70247460-70248929 Homo sapiens chromosome 5, GRCh37.p13 Primary Assembly
TGCCCAGGGTGGTGTCAAGCTCCAGGTCTCAAGTGATCCCCCTACCTCCGCCTCCCAAAGTTGTGGGATT
GTAGGCATGAGCCACTGCAAGAAAACCTTAACTGCAGCCTAATAATTGTTTTCTTTGGGATAACTTTTAA
AGTACATTAAAAGACTATCAACTTAATTTCTGATCATATTTTGTTGAATAAAATAAGTAAAATGTCTTGT
GAAACAAAATGCTTTTTAACATCCATATAAAGCTATCTATATATAGCTATCTATGTCTATATAGCTATTT
TTTTTAACTTCCTTTATTTTCCTTACAGGGTTTCAGACAAAATCAAAAAGAAGGAAGGTGCTCACATTCC
TTAAATTAAGGAGTAAGTCTGCCAGCATTATGAAAGTGAATCTTACTTTTGTAAAACTTTATGGTTTGTG
GAAAACAAATGTTTTTGAACATTTAAAAAGTTCAGATGTTAAAAAGTTGAAAGGTTAATGTAAAACAATC
AATATTAAAGAATTTTGATGCCAAAACTATTAGATAAAAGGTTAATCTACATCCCTACTAGAATTCTCAT
ACTTAACTGGTTGGTTATGTGGAAGAAACATACTTTCACAATAAAGAGCTTTAGGATATGATGCCATTTT
ATATCACTAGTAGGCAGACCAGCAGACTTTTTTTTATTGTGATATGGGATAACCTAGGCATACTGCACTG
TACACTCTGACATATGAAGTGCTCTAGTCAAGTTTAACTGGTGTCCACAGAGGACATGGTTTAACTGGAA
TTCGTCAAGCCTCTGGTTCTAATTTCTCATTTGCAGGAAATGCTGGCATAGAGCAGCACTAAATGACACC
ACTAAAGAAACGATCAGACAGATCTGGAATGTGAAGCGTTATAGAAGATAACTGGCCTCATTTCTTCAAA
ATATCAAGTGTTGGGAAAGAAAAAAGGAAGTGGAATGGGTAACTCTTCTTGATTAAAAGTTATGTAATAA
CCAAATGCAATGTGAAATATTTTACTGGACTCTATTTTGAAAAACCATCTGTAAAAGACTGGGGTGGGGG
TGGGAGGCCAGCACGGTGGTGAGGCAGTTGAGAAAATTTGAATGTGGATTAGATTTTGAATGATATTGGA
TAATTATTGGTAATTTTATGAGCTGTGAGAAGGGTGTTGTAGTTTATAAAAGACTGTCTTAATTTGCATA
CTTAAGCATTTAGGAATGAAGTGTTAGAGTGTCTTAAAATGTTTCAAATGGTTTAACAAAATGTATGTGA
GGCGTATGTGGCAAAATGTTACAGAATCTAACTGGTGGACATGGCTGTTCATTGTACTGTTTTTTTCTAT
CTTCTATATGTTTAAAAGTATATAATAAAAATATTTAATTTTTTTTTAAATTAGCTGTATCTGTGATTGT
ATTTCTTTTTTGCATATTATTTTGCCCTTTGGCCCATATTTTGATATGGATGCCACCATAGCATTTTGTG

Figure 6 - Region of interest for the *SMN1* genomic sequence on chromosome 5, NCBI accession number NC_000005.9. Designed primers are in bold and underlined. Location of c.*3+80T>G is highlighted in yellow; location of c.*211_*212del is highlighted in green; *Dra*I restriction sites are highlighted in violet.

The c.*3+80T>G variant is located within a *Dra*I restriction site in the wild-type allele. This enables the use of RFLA for the detection of this variant, since the restriction site is lost if the variant is present. To ensure the reliability of the RFLA assay, an internal restriction control was incorporated into the reverse primer designed for this variant. This was accomplished by adding a *Dra*I restriction site (5′-TTTAAA-3′) followed by an M13 adapter (5′-CAGGAAACAGCTATGA-3′).

The primers designed for each variant were, therefore, as follows:

- c.*211_*212del:
  - Forward - 5′-FAM·GTGGAATGGGTAACTCTTCTTG-3′
  - Reverse - 5′-ACTACAACACCCTTCTCACAGC-3′
- c.*3+80T>G:
  - Forward - 5′-FAM·AGTCTGCCAGCATTATGAAAG-3′
  - Reverse - 5′-TCATAGCTGTTTCCTGTTTAAAGTTAAACCATGTCCTCTGTGG-3′

3.2. RFLA for c.*3+80T>G

To detect the c.*3+80T>G variant within the *SMN* genes, RFLA was performed using the *Dra*I restriction enzyme, which recognizes the wild-type sequence. The presence of the variant abolishes this restriction site and results in larger undigested fragments. The previously designed primers amplified the target region encompassing the c.*3+80T>G variant. Subsequent digestion of the PCR products with *Dra*I generated distinct fragment patterns for wild-type and variant alleles when resolved in capillary electrophoresis (79bp and 403bp peaks, respectively). A run of the non-digested PCR product showed a peak at the expected value of 422bp, which corresponds to the full PCR product size (Figure 7). Table 1 summarizes the expected fragment sizes and interpretation for each sample.

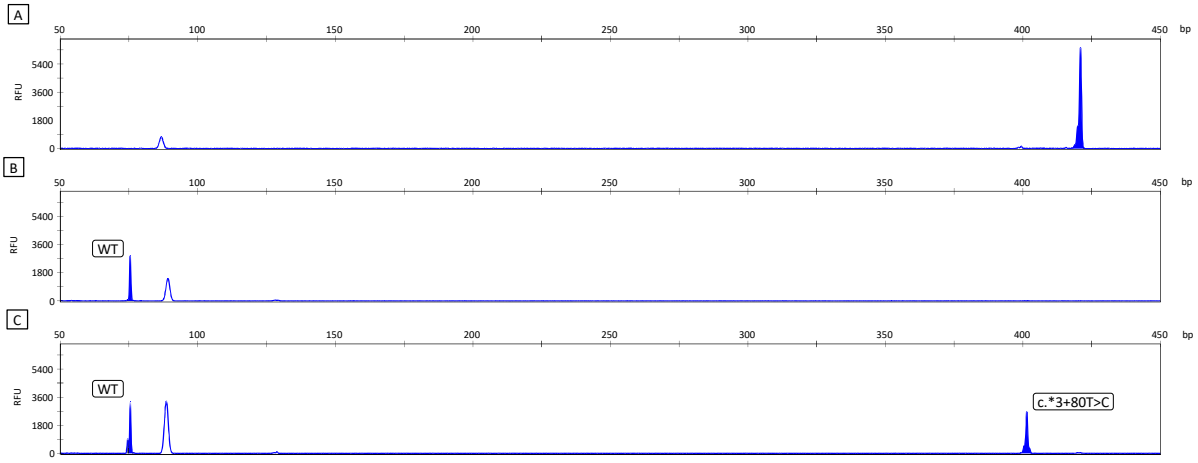

Figure 7 - Typical electropherogram for the c.*3+80T>G variant detection assay. A – Non-digested PCR product; B - wildtype sample after *Dra*I digestion; C - heterozygote sample for the c.*3+80T>G variant after *Dra*I digestion; RFU - Relative Fluorescence Units; bp - base pairs.

Table 1 – Expected fragment sizes in the c.*3+80T>G variant detection assay using *DraI* digestion and capillary electrophoresis.

Sample	Expected fragment size(s) (bp)	Interpretation
Non-digested PCR product	422 bp	Full-length PCR product
Wild-type	79 bp	Wild-type allele pattern after *DraI* cleavage
Homozygous for c.*3+80T>G	403 bp	Variant allele pattern after *DraI* cleavage
Heterozygous for c.*3+80T>G	79 bp and 403 bp	Both alleles present; both fragment peaks observed

3.3. F-PCR for c.*211_*212del

Detection of the c.*211_*212del variant on the *SMN* genes, was performed via F-PCR using the designed primers. PCR products representing the wild-type and variant alleles were generated and resolved with capillary electrophoresis. The electrophoretic analysis provided distinct profiles for the two alleles, allowing precise discrimination.

Individuals carrying the wild-type allele exhibited a specific signal peak at the expected size value of 224bp, while those harbouring the c.*211_*212del variant displayed a size shift due to two nucleotides deletion (Figure 8). Table 2 summarizes the expected fragment sizes and interpretation for each genotype.

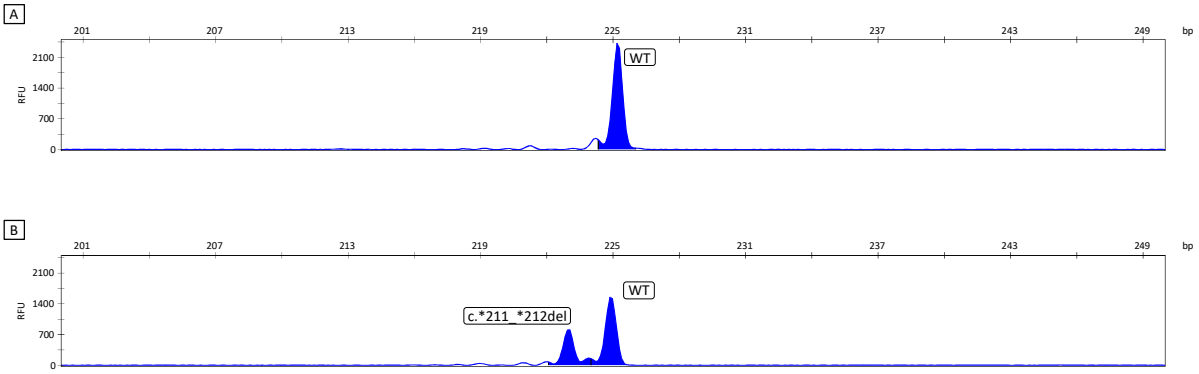

Figure 8 – Typical electropherogram for the c.*211_*212del variant detection assay. A – wildtype sample; B – heterozygote sample for the c.*211_*212del variant; RFU – Relative Fluorescence Units; bp – base pairs.

Table 2 – Expected fragment sizes in the c.211_212del variant detection assay using F-PCR and capillary electrophoresis.

Sample	Expected fragment size(s) (bp)	Interpretation
Wild-type	224	Normal allele profile
Homozygous for c.*211_*212del	222	Two-nucleotide deletion relative to wild-type
Heterozygous for c.*211_*212del	224 and 222	Presence of both alleles (wild-type + deletion)

3.4. Methodology validation

To validate the accuracy and reliability of the methodologies previously described for detecting *SMN1* variants c.*3+80T>G and c.*211_*212del associated with SMA silent carriers, all analysed samples were subjected to Sanger sequencing. results were consistent with earlier findings, confirming the presence or absence of the variants in all tested samples. The sequencing chromatograms clearly depicted the heterozygote deletion at the specified genomic position expected for the samples with the c.*211_*212del variant, which appeared as a 2bp frameshift (Figure 9). Similarly, the sequencing chromatograms for the samples with the c.*3+80T>G variant exhibited the specific nucleotide change at the designated position (Figure 10). Both results matched the profiles obtained by RFLA and F-PCR.

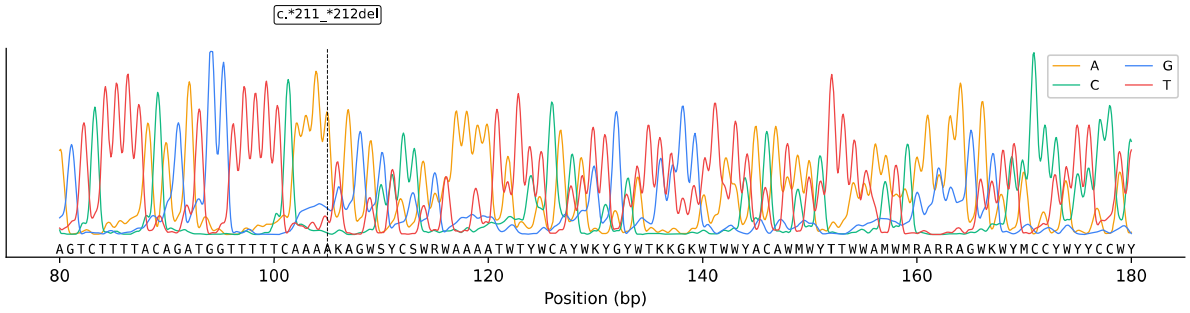

Figure 9 – Sanger sequencing chromatogram from a sample heterozygous for the c.*211_*212del 2-bp deletion. The deletion site is annotated, and the overlapping peaks following the variant position illustrate the frameshift characteristic of heterozygous indels.

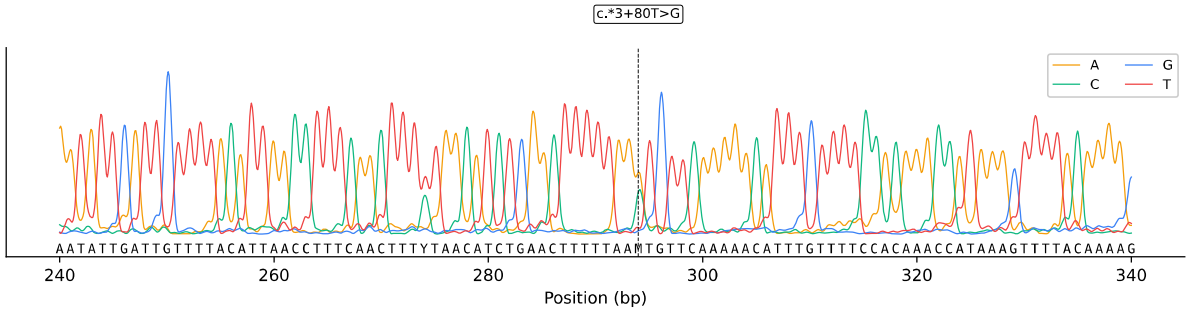

Figure 10 – Sanger sequencing chromatogram from a sample heterozygous for the c.*3+80T>G substitution. The variant position is indicated by a callout, with clear trace separation confirming the presence of both T and G alleles.

3.5. Characterization of the Portuguese cohort

The selected cohort comprised a diverse group of individuals, including known 2+0 carriers, as well as individuals with known 1+1 genotype or with 3 or more copies of *SMN1*. DNA samples from each participant were screened using the methods previously described, including F-PCR and capillary electrophoresis for c.*211_*212del detection, as well as RFLA with *Dra*I for c.*3+80T>G detection, followed by capillary electrophoresis (Table 3).

Table 3 – Distribution of carriers and normal individuals in relation to the presence or absence of the studied variants c.*3+80T>G and c.*211_*212del

	SMA carriers	**Non-SMA carriers**			
	2+0 carriers (n=10)	2 *SMN1* copies (1+1 genotype) (n=28)	3 *SMN1* copies (n=73)	4 *SMN1* copies (n=3)	5 *SMN1* copies (n=1)
c.*3+80T>G; c.*211_*212del	2	0	21	2	0
c.*3+80T>G; [=]	0	0	1	0	0
c.*211_*212del; [=]	1	0	3	0	0
	2+0 carriers (%)	2 *SMN1* copies (1+1 genotype) (%)	3 *SMN1* copies (%)	4 *SMN1* copies (%)	5 *SMN1* copies (%)
c.*3+80T>G; c.*211_*212del	20,00	0,00	28,77	66,67	0,00
c.*3+80T>G; [=]	0,00	0,00	1,37	0,00	0,00
c.*211_*212del; [=]	10,00	0,00	4,11	0,00	0,00

Of the 10 individuals classified as 2+0 carriers, 2 carried both variants simultaneously (20%). Additionally, one 2+0 carrier presented only the c.*211_*212del variant (10%). Among the 73 individuals identified with three *SMN1* copies (2+1 genotype), 21 carried both variants (28.77%), while three carried only the c.*211_*212del variant (4.11%) and one carried only the c.*3+80T>G variant (1.37%). Interestingly, two of the three individuals tested with four *SMN1* copies carried both variants (66.67%), whereas the single individual with five *SMN1* copies (3+2 genotype) did not carry either of the studied variants. In contrast, all 28 individuals with the 1+1 genotype tested negative for the studied variants. Furthermore, a statistical comparison between 2+0 and 2+1 individuals against 1+1 controls revealed a significant difference (Chi-square value = 8,880; p = 0,003) in the prevalence of the studied variants. It should be noted that all variants detected were present in the heterozygous state in every individual in whom they were found.

3.6. Uncovering the gene location of the c.*3+80T>G variant

To determine whether the c.*3+80T>G occurs in *SMN2* a different RFLA approach was taken, involving two restriction enzymes: *Dra*I and *Hpy*188I. The latter is able to differentiate both *SMN* genes due to the presence of a *Hpy*188I restriction site in *SMN1* that doesn't exist in *SMN2*. This restriction site lies upstream of the c.*3+80T>G variant (Figure 11).

```
>NC_000005.9:70247460-70248929 Homo sapiens chromosome 5, GRCh37.p13 Primary Assembly
TGCCCAGGGTGGTGTCAAGCTCCAGGTCTCAAGTGATCCCCCTACCTCCGCCTCCCAAAGTTGTGGGATT
GTAGGCATGAGCCACTGCAAGAAAACCTTAACTGCAGCCTAATAATTGTTTTCTTTGGGATAACTTTTAA
AGTACATTAAAAGACTATCAACTTAATTTCTGATCATATTTTGTTGAATAAAATAAGTAAAATGTCTTGT
GAAACAAAATGCTTTTTAACATCCATATAAAGCTATCTATATATAGCTATCTATGTCTATATAGCTATTT
TTTTTAACTTCCTTTATTTTCCTTACAGGGTTTCAGACAAAATCAAAAAGAAGGAAGGTGCTCACATTCC
TTAAATTAAGGAGTAAGTCTGCCAGCATTATGAAAGTGAATCTTACTTTTGTAAAACTTTATGGTTTGTG
GAAAACAAATGTTTTTGAACATTTAAAAAGTTCAGATGTTAAAAAGTTGAAAGGTTAATGTAAAACAATC
AATATTAAAGAATTTTGATGCCAAAACTATTAGATAAAAGGTTAATCTACATCCCTACTAGAATTCTCAT
ACTTAACTGGTTGGTTATGTGGAAGAAACATACTTTCACAATAAAGAGCTTTAGGATATGATGCCATTTT
ATATCACTAGTAGGCAGACCAGCAGACTTTTTTTTATTGTGATATGGGATAACCTAGGCATACTGCACTG
TACACTCTGACATATGAAGTGCTCTAGTCAAGTTTAACTGGTGTCCACAGAGGACATGGTTTAACTGGAA
TTCGTCAAGCCTCTGGTTCTAATTTCTCATTTGCAGGAAATGCTGGCATAGAGCAGCACTAAATGACACC
ACTAAAGAAACGATCAGACAGATCTGGAATGTGAAGCGTTATAGAAGATAACTGGCCTCATTTCTTCAAA
ATATCAAGTGTTGGGAAAGAAAAAAGGAAGTGGAATGGGTAACTCTTCTTGATTAAAAGTTATGTAATAA
CCAAATGCAATGTGAAATATTTTACTGGACTCTATTTTGAAAAACCATCTGTAAAAGACTGGGGTGGGGG
TGGGAGGCCAGCACGGTGGTGAGGCAGTTGAGAAAATTTGAATGTGGATTAGATTTTGAATGATATTGGA
TAATTATTGGTAATTTTATGAGCTGTGAGAAGGGTGTTGTAGTTTATAAAAGACTGTCTTAATTTGCATA
CTTAAGCATTTAGGAATGAAGTGTTAGAGTGTCTTAAAATGTTTCAAATGGTTTAACAAAATGTATGTGA
GGCGTATGTGGCAAAATGTTACAGAATCTAACTGGTGGACATGGCTGTTCATTGTACTGTTTTTTTCTAT
CTTCTATATGTTTAAAAGTATATAATAAAAATATTTAATTTTTTTTTAAATTAGCTGTATCTGTGATTGT
ATTTCTTTTTTGCATATTATTTTGCCCTTTGGCCCATATTTTGATATGGATGCCACCATAGCATTTTGTG
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Figure 11 - Region of interest for the *SMN1* genomic sequence on chromosome 5, NCBI accession number NC_000005.9. Primers are underlined and in bold. Location of c.*3+80T>G is highlighted in yellow; *Dra*I restriction sites are highlighted in violet; *Hpy*188I restriction sites are highlighted in red, underlined site enables the distinction between *SMN1* and *SMN2* genes.

Using a distinct forward primer already available in the lab (5'-TTTGTTGAATAAAATAAGTAAAATGT-3') in combination with the previously designed reverse primer for this variant, it was possible to generate a PCR product that included both restriction sites of interest, which was digested separately with *Dra*I and with a combination of *Dra*I/*Hpy*188I. This approach generated different fragment patterns for both wild-type and variant alleles for the *SMN2* gene.

The *Dra*I digestion yields a 586bp fragment for *SMN* genes with the c.*3+80T>G variant, as well as 265bp fragment for the wild-type allele. However, it is important to note that *Dra*I digestion alone does not distinguish between *SMN1* and *SMN2*, as both genes share this restriction site. The resulting 265 bp wild-type fragment is therefore identical in size to the wild-type *SMN2* fragment obtained in the *Dra*I/*Hpy*188I restriction assay (Figure 12).

If the variant c.*3+80T>G is present in the *SMN2* gene, then the *Dra*I/*Hpy*118I assay produces a 273bp fragment instead of the 265 bp wild-type fragment. This size shift occurs because the variant disrupts the *Dra*I restriction site in *SMN2*, leading to incomplete cleavage and retention of 8 extra base pairs, hence the 273 bp fragment (Figure 12).

In contrast, the fragment corresponding *SMN1* gene is always 136bp for the *Dra*I/*Hpy*118I restriction assay, whether the c.*3+80T>G variant is present or not, due to the *Hpy*118I site exclusive to *SMN1*. For a sample with no copies of the *SMN2* gene, the *Dra*I/*Hpy*118I restriction assay will show no fragments with the size 265bp or 273bp (Figure 13). Table 4 summarizes the expected fragment sizes and interpretation for each sample.

Table 4 – Expected fragment sizes in *Dra*I and *Dra*I/*Hpy*118I assays for *SMN1* and *SMN2* alleles

Gene	Assay	Variant status	Expected fragment size(s) (bp)	Interpretation
SMN	*Dra*I only	Wild-type	265	Cannot distinguish *SMN1* vs *SMN2*
SMN	*Dra*I only	c.*3+80T>G	586	Variant fragment, but still not gene-specific
SMN2	*Dra*I + *Hpy*118I	Wild-type	265	Confirms wild-type *SMN2* allele
SMN2	*Dra*I + *Hpy*118I	c.*3+80T>G	273	Size-shifted fragment due to disrupted *Dra*I site
SMN1	*Dra*I + *Hpy*118I	Wild-type or variant	136	*SMN1* fragment size, independent of variant
Absent *SMN2*	*Dra*I + *Hpy*118I	–	Absence of: 265 or 273	Confirms lack of *SMN2* copies

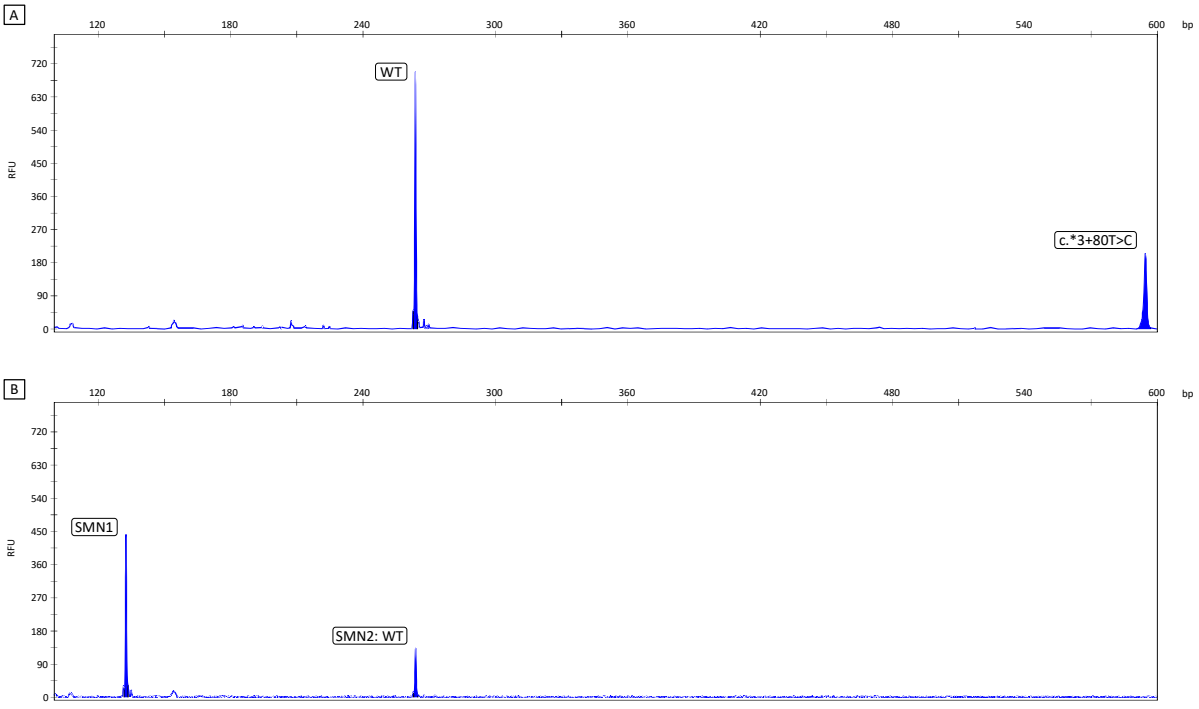

Figure 12 – Electropherogram for a sample with 3 copies of *SMN1*, 2 copies of *SMN2* and heterozygote for the c.*3+80T>G variant. A – Digestion with *Dra*I; B – Digestion with *Dra*I and *Hpy*188I; RFU – Relative Fluorescence Units; bp – base pairs

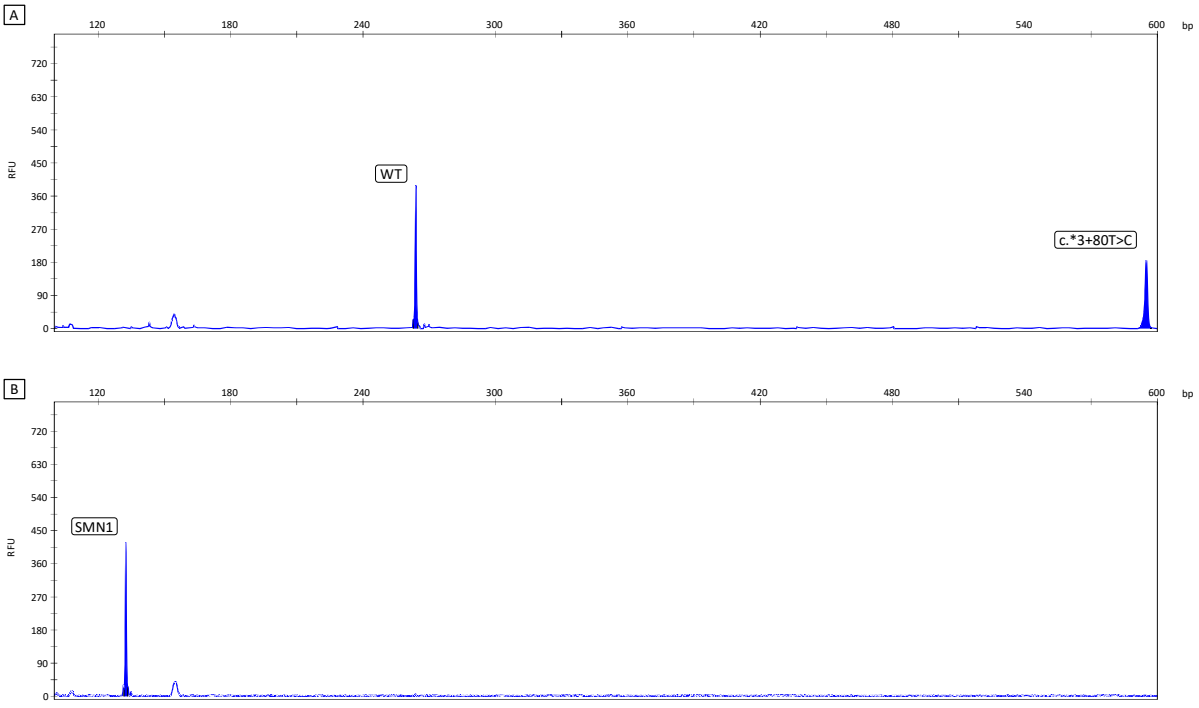

Figure 13 – Electropherogram for a sample with 3 copies of *SMN1*, no copies of *SMN2 and heterozygote for the c.*3+80T>G variant.* A – Digestion with *Dra*I; B – Digestion with *Dra*I and *Hpy*188I; RFU – Relative Fluorescence Units; bp – base pair

4. Discussion

In the pursuit of improved genetic counselling for silent carriers of SMA, a cost-effective approach for the detection of two key variants within the *SMN1* gene, specifically c.*3+80T>G and c.*211_*212del, was developed and validated. This methodology leverages common laboratory techniques, including F-PCR and RFLA, which are well-established within the genetics diagnosis realm. The advantage of this approach lies in the simplicity of setup, as these techniques are accessible and easily integrated into existing laboratory protocols. Additionally, this methodology is compatible with established software workflows commonly utilized for genetic data analysis. While commercially available alternatives such as the AmplideX® SMA Plus kit (Asuragen®, Austin, TX, USA) exist, proprietary products often come with additional costs, potentially restricting their use in laboratories with budget constraints. Moreover, proprietary kits may be associated with software licensing agreements that limit flexibility and control over diagnostic processes. In contrast, the present methodology is cost-effective, avoids restrictive licensing, minimizes the need for specialized equipment, and therefore offers accessibility to a broader range of laboratories

The variants c.*3 +80T>G and c.*211_*212del have previously been associated with silent carriers within the Ashkenazi population (25), as well as in the Spanish population (26). The previously developed and validated method for detecting these variants was used in a selected cohort of the Portuguese population comprising of a diverse group of individuals, including known 2+0 carriers, as well as individuals with a 1+1 genotype and those possessing three or more copies of *SMN1*. The prevalence of the variants within the cohort was in line with what had been described in prior reports. The incidence of both variants was 20% for 2+0 carriers and 30.14% for individuals with three *SMN1* copies (2+1 genotype). Among individuals with four *SMN1* copies, one individual was found to carry the variants, and the sole individual with five *SMN1* copies (3+2 genotype) did not carry either of the studied variants. In contrast, all 28 individuals with the 1+1 genotype tested negative for the studied variants, confirming the absence of these variants in non-carrier individuals.

Statistical analysis comparing 2+0 and 2+1 individuals against 1+1 controls showed a significant difference in the prevalence of the studied variants ($p=0.003$). This means that presence of one or both variants significantly elevate the risk of 2+0 carrier status. However, an important point to consider is that the absence of these variants in individuals with two *SMN1* copies does not necessarily exclude the

possibility of an individual being a 2+0 carrier, emphasizing the need for familial haplotyping analysis to confirm the carrier status particularly in such cases.

Alías et al. (26) had previously suggested the exclusivity of c.*3+80T>G c.*211_*212del variants to *SMN1* out of their research for the Spanish population, based on these variants not being detected in patients with a complete absence of the *SMN1* gene, as well as the presence of both variants in individuals possessing three *SMN1* copies but lacking the *SMN2* gene. Furthermore, in subjects with *SMN2-SMN1* hybrid genes, the presence of the c.*211_*212del variant was observed in the portion of the hybrid corresponding to *SMN1*.

Our RFLA approach corroborates these findings for the c.*3+80T>G variant by directly showing, for the first time, that this variant is not in the *SMN2* gene for all the samples we tested. This direct confirmation represents a relevant advance, as previous studies had only inferred the exclusivity of this variant to *SMN1* indirectly through absence in *SMN1*-deleted individuals or presence within hybrid alleles. By providing molecular evidence that unambiguously rules out the occurrence of c.*3+80T>G in *SMN2*, our results strengthen the interpretation of this variant as a reliable marker for increased risk of 2+0 carrier status. Furthermore, this insight contributes to refining diagnostic strategies by reducing ambiguity in variant assignment and adds robustness to carrier screening methodologies.

5. Conclusion

The development and validation of a cost-effective methodology for the detection of the *SMN1* variants c.*3+80T>G and c.*211_*212del represents an improvement in the realm of genetic counselling for silent carriers of SMA. The prevalence of these variants within the Portuguese cohort and their relation with 2+0 carrier status aligns with previous findings in other populations, emphasizing the reproducibility and reliability of this approach. Importantly, this methodology also enabled the first direct confirmation that the c.*3+80T>G variant is not associated with the *SMN2* gene, thereby strengthening its role as an informative marker for silent carrier detection. The insights gained from this study pave the way for more informed genetic counselling, broader accessibility of carrier screening protocols in the context of SMA and underscore the importance of continued research in this field.

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P.PORTO

ESCOLA SUPERIOR DE SAÚDE

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MESTRADO

ANÁLISES CLÍNICAS E SAÚDE PÚBLICA