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Implementation of molecular techniques for the Neonatal Screening of Spinal Muscular Atrophy

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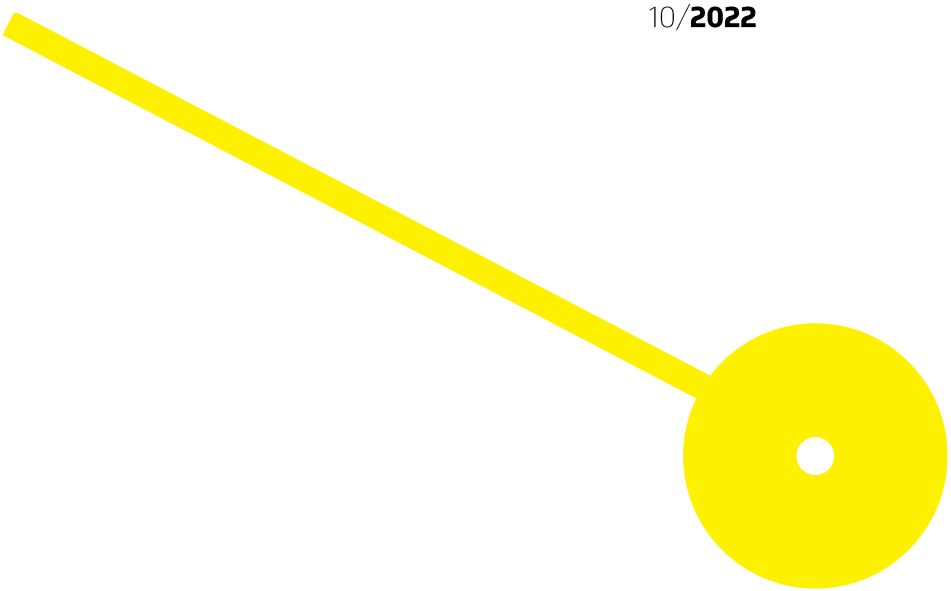

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Implementation of molecular techniques for the Neonatal Screening of Spinal Muscular Atrophy

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Resumo

A atrofia muscular espinhal (AME) é uma doença neurodegenerativa causada principalmente pela deleção homozigótica do exão 7 do gene funcional telomérico survival motor neuron 1 (*SMN1*). Esta ausência leva à falta da proteína ubíqua SMN que, por sua vez, leva à destruição seletiva dos neurónios motores alfa. A gravidade da doença torna-a na principal causa genética de morte infantil. O número de cópias do gene centromérico *SMN2* tem uma correlação inversa com o fenótipo da doença. A SMA é atualmente classificada em 5 tipos – Tipo 0 (letal no útero ou nas primeiras semanas de vida), Tipo 1 (responsável pela maioria dos casos existentes e com sintomas que aparecerem ao longo do primeiro mês de vida), Tipo 2 (crianças que adquirem a capacidade de se sentar sozinhas), Tipo 3 (crianças conseguem andar sem auxílio) e Tipo 4 (forma mais ligeira que surge normalmente na idade adulta). O diagnóstico só é feito quando surgem sintomas e, por essa altura, os neurónios motores já estão irreversivelmente perdidos. Três terapias estão agora disponíveis e são mais eficazes na fase pré-sintomática. Para se conseguir este objetivo, a estratégia passa por incluir a AME no painel de doenças rastreadas nos Programas de Rastreio Neonatal. Nesse sentido, descreve-se a implementação de um ensaio, baseado em reagentes preparados *in house*, que deteta a ausência do exão 7 do gene *SMN1* através da técnica reação em cadeia da polimerase em tempo real (qPCR), adaptado ao Rastreio Neonatal. Usou-se como amostras os círculos de sangue seco dos cartões de Guthrie que já são usados no dia a dia do rastreio neonatal. Este método é uma forma fiável, simples e acessível de rastrear esta doença letal.

Palavras-chave: Atrofia Muscular Espinhal; Rastreio neonatal; Reação em cadeia da polimerase em tempo real em tempo real

Abstract

Spinal muscular atrophy (SMA) is neurodegenerative disease mainly caused by the homozygous deletion of the functional telomeric survival motor neuron 1 gene (*SMN1*) exon 7. This absence causes a lack of the ubiquitous SMN protein which, in turn, selectively destroys alfa motor neurons. Due to the disease severity, it is the leading genetic cause of infant death. The copy number of the centromeric *SMN2* gene has an inverse correlation with the phenotype. SMA is currently classified in 5 types – Type 0 (lethal in womb or in the first weeks of life), Type I (that counts for the majority of cases), Type 2 (children can sit alone), Type 3 (children can walk independently) and Type 4 (mildest form that appears in adults). Diagnosis is only made when symptoms arise and by that time motor neurons are already irreversibly loss. Three therapies are now available but they are most effective in the asymptomatic phase. To achieve this objective, the strategy is to include SMA in the panel of diseases screened in the Neonatal Screening Programs. In this sense, we describe the implementation of an assay, based on reagents prepared *in house*, that detects the absence of exon 7 of the *SMN1* gene through the real-time polymerase chain reaction (qPCR) technique, adapted to Neonatal Screening. Samples used were from the dried blood spots of the already implemented Guthrie cards on the newborn screening. This method is a reliable, simple and affordable way to screen for this lethal disease.

Keywords: Spinal Muscular Atrophy; Newborn Screening; Real-time Polimerase Chain Reaction

Index

Abreviations:

ASO – Antisense oligonucleotide

AAV9 – Adeno-associated virus serotype 9

CHP1 – Calcineurin-like EF-hand protein 1

CMAP – Compound muscle actions potential

CNS – Central nervous system

Cq – Quantification cycles

DBS – Dried blood spots

dsDNA – Double-stranded-DNA

EDTA – Ethylenediamine tetraacetic acid

ESE – Exonic splicing enhancer

ESS – Exonic splicing silencer

hnRNP – Heterogeneous nuclear ribonucleoprotein

ISS – Intronic splicing silencer

LNA – Locked nuclei acids

MNJ – Motor neuron junctions

NBS – Newborn screening

NCALD – Neurocalcin delta

NICU – Neonatal intensive care unit

OMIM – Online mendelian inheritance in man

PLS3 – Plastin 3

PKU – Phenylketonuria

PCR – Polimerase chain reaction

PHP – Primary health provider

qPCR – Real Time polimerase chain reaction

RPP30 – Ribonuclease P protein subunit p30 gene

SCID – Severe combined immunodeficiency

SMA – Spinal muscular atrophy

SMN – Survival motor neuron

1. Introduction

1.1. What is Spinal Muscular Atrophy (SMA)?

SMA is a rare neurodegenerative disease that, due to the lack of Survival Motor Neuron (SMN) protein, selectively destroys the alfa neurons of the anterior horn of the grey matter in the spinal cord (lower motor neurons). This leads to weakness and atrophy of the skeletal muscles that they enervate (1–9). The SMN protein has 38 kDa and 294 amino acid and is ubiquitously expressed between species (4–6, 10–13), and it is localized in gems – a nuclear structure that is colocalized and interacts with coiled bodies in nucleus and cytoplasm (14). This protein is involved in the biogenesis and function of Small Ribonuclear Proteins (15–17) and affects both pre-mRNA splicing and interactions between splicing components (11, 18). It also regulates self-oligomerization (19, 20), stress granular formation, apoptosis (21–24), pancreatic/metabolic (25, 26), neural and ubiquitin homeostasis (27), among other which gives it a housekeeping role (12, 28, 29). The lack of SMN protein is incompatible with life since it leads to cell death in early embryos. (10, 30).

Epidemiologically, SMA is the leading genetic cause of death in infants and the most common autosomal recessive disease after cystic fibrosis (31–33). The mortality rate at two years old is 68% for the most severe form (34, 35). The median incidence of this disease in Europe is 11,9 in 100000 (1 in 3900 to 16000 births) and it is the most affected continent (33, 36, 37). The average carrier frequency is 1 in 50 people and it is more frequent in the Caucasian population (33, 36, 38). In Europe carrier frequency is around 1:40 (26, 38, 39). The gender distribution is equitable (33).

1.2. History of SMA

SMA was first reported in 1891 by the Austrian neurologist Guido Werdnig from the Pathological-Anatomical Institute of Graz (40). In his exposition, Guido described two brothers with progressive loss of strength and early death. The patients were born from healthy parents and had one healthy brother. He also affirmed that the symptoms were due to a primary degeneration of the motor pathways of the spinal cord. In the same year, Johann Hoffmann from the Heidelberg University, described patients with similar symptoms and introduced, for the first time, the term "Spinale Muskelatrophie" (Spinal Muscular Atrophy). Dr. Hoffmann also pointed out that the children had healthy parents and the brothers were also affected by the disease (41). Later, in 1956, Kugelberg and Welander described the milder forms of the disease and, for the first time, suggested a recessive transmission (42). The first attempt to classify SMA was made in 1961 by Byers e Banker. They divided the illness into 3 groups according to the age of onset. Group I had onset in utero or in the

first two months after birth and early death. The second Group were those with onset between 2 and 12 months of age with better survival than the first group. Group III symptoms onset would be in the first year of life and these patients could live for several years (1). In 1980, John Pearn from the Department of Child Health in Royal Children's Hospital in Brisbane, suggested a classification divided into 7 classes but it has never been used, although some parts where later used (43). In 1991, at the fourth meeting of the International SMA Consortium, scientists have agreed in a classification with 3 categories (Type I, II and III) based on age of onset and motor milestones acquisition (sit, stand and walk independently) (34, 44). Finally, the 90's were the decade of genetic discoveries for SMA. It started with the discovery of the location of the gene that is responsible for the disease by Brzustowicz and his colleagues but also by Melki *et al.* (2, 3).

1.3. Clinical presentation

Today the classification is based in five Types according to the criteria of the former classification and adding life expectancy (45):

- Type 0 or Neonatal/Congenital Form – It is used to describe fetus with very low or no movement in uterus. Normally they don't survive birth but, when they do, extreme muscular weakness and hypotonia, weak cries, joint contractures and difficult in swallowing and breathing are the most frequent symptoms (39, 45–48). Areflexia and facial diplegia are some of other characteristics of these patients (1, 39, 45). Because of all these serious conditions, life expectancy is just of a few weeks and the patient becomes a Type Ia case (39, 45, 48, 49).
- Type I / Werdnig–Hoffmann disease / Infantile SMA / Severe Form (online mendelian inheritance in man [OMIM] 253300) – It is the more common form (approximately 50% of the cases) (35, 50, 51). Like in Type 0, this one is also, sometimes, subdivided in Type Ib (age of onset of 3 months) and Ic (age of onset between 3 and 6 months) (39, 45). Patients are born normal but start to present hypotonia, weak or none control over the head and reduced or inexistent reflexes (areflexia) before 6 months of life (Fig. 1). The cry of these babies is weak and they are never able to sit (non–sitters) (1, 39, 45, 51–53). Due to the severe hypotonic weakness of the lower limbs, patients present spread legs also known has "frog–legs" (1, 39, 49). The weakness of the intercostals muscles, without the involvement of the diaphragm, leads to the formation of bell–shaped chest and a pattern of paradoxical breathing (Hoover's sign – asynchrony between thoracic and abdominal breathing where the thorax moves inward and the abdomen moves outward during inspiration) (1, 45, 52–54). Also characteristic, are the tongue fasciculations and weakness due to bulbar

denervation (1, 45, 52, 53, 55). This leads to difficulty in breathing and swallowing thus eventually leading to nasal feeding (52). The purpose is to nurture the patient but also prevent potential aspirations and thus pneumonia (49, 52, 56). These patients aren't able to achieve any motor milestone and only achieve 2 years if life supportive care is present (28, 35, 39, 52, 53).

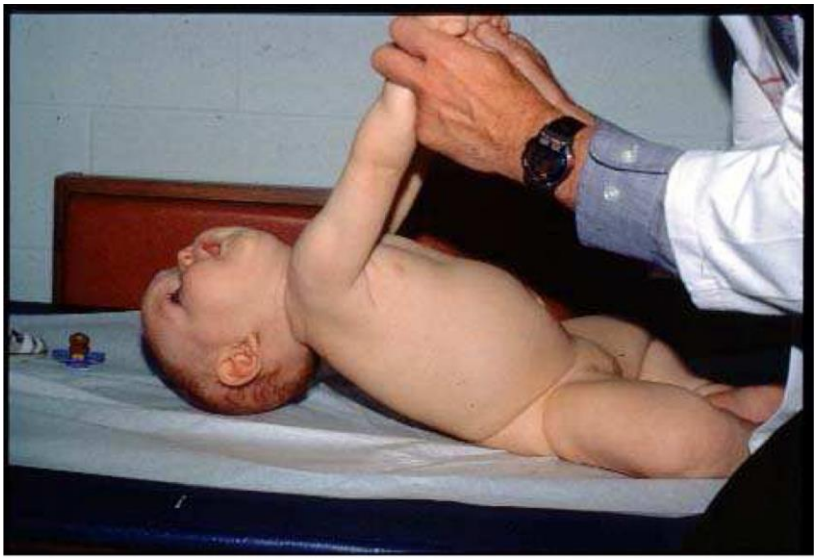

Figure 1 – Baby showing the typical symptoms for Type I SMA (non-sitters). Retrieved from Kolb *et al.* (57).

- Type II / Dubowitz disease / Intermediate form (OMIM 253550) – Represents around 20% of the cases (49). Manifests between 6 and 18 months (39, 45). Patients with this type can sit unaided but never develop the ability of stand or walk independently (sitters) (Fig. 2) (35, 39, 45, 52, 53). Usually, progressive proximal weakness of the lower limbs arises before the upper limbs (45, 52). Hypotonia and areflexia are common as are the fine tremors in the distal limbs (34, 39, 45, 53, 58). Tongue fasciculations are also present in this type as are its consequences (1, 34, 49). The comorbidities associated with this type are due to complications in the bone and tendons growth which, alongside with the muscular weakness, lead to scoliosis and articular contractures (39, 49, 52). This set of symptoms associated with the intercostal muscular weakness can result in restrictive pulmonary disease (49, 52, 53). Patients have a life expectancy of 10 to 40 years (39, 49).

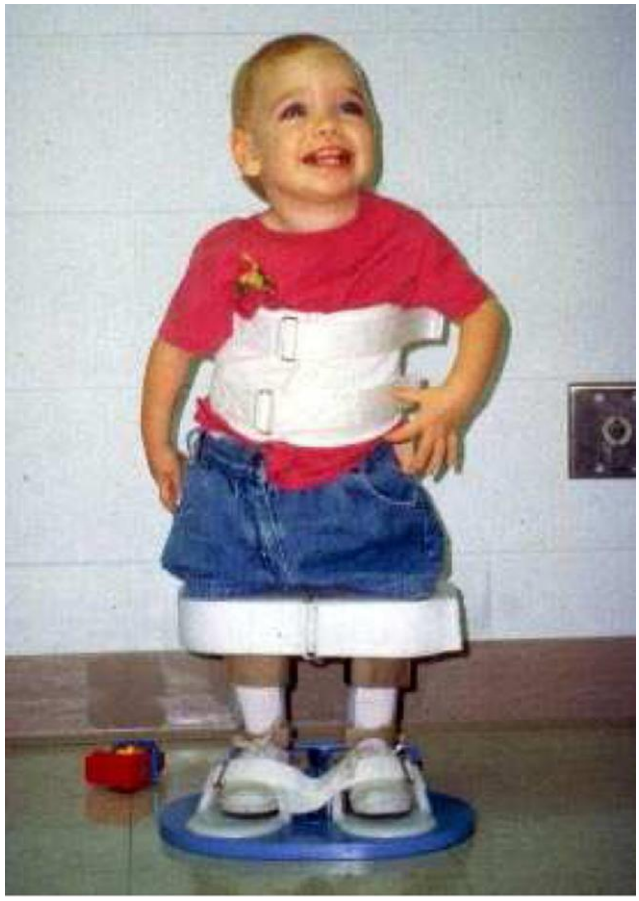

Figure 2 –Toddler standing with aid showing the major sign of Type II SMA (sitter). Retrieved from Kolb *et al.* (57).

- Type III / Kugelberg-Welander disease / Juvenile form (OMIM 253400) – Approximately 30% of the cases (49). The symptoms first appear after 18 months. There are those who divide this type into two subtypes: IIIa (age of onset between 18 months and 3 years) and IIIb (age on onset between 3 and 10 years) (35, 39, 45). Patients with this type develop the ability of walking unaided (walkers) (Fig. 3) but, with the progression of the disease, loose that capability leading to the necessity of using a wheelchair (28, 35, 39, 45, 52, 53, 59). Symptomatically it is a very heterogeneous type because there are patients who need wheelchair while some adults only present light muscle weakness (39, 45, 53). Due to this weakness (more present in lower than upper limbs) these patients have a history of falls and difficulty in climbing stairs. However, they do not have the tendency to develop the comorbidities present in patients with type II (49, 52). Joint overuse is frequently seen as is scoliosis (52, 53). The life expectancy for these patients is similar to the general population (49).

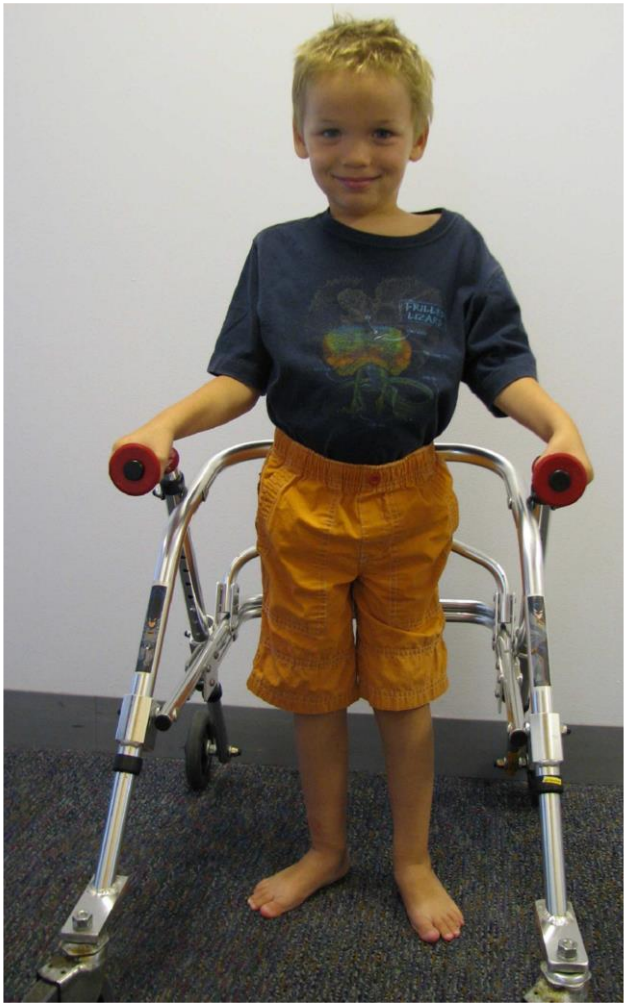

Figure 3 – Child walking with the aid of stroller that is the main characteristic of Type III SMA (walker) patients. Retrieved from Kolb *et al.* (57).

- Type IV / Finkel Type / Adult form (OMIM 271150) – It is the mildest form of SMA. The symptoms normally occur in the third decade of life (35, 45, 52, 53). It represents less than 5% of the cases (49). Patients reach the adulthood with the capability of autonomous mobility. Motor difficulty is low and they do not present respiratory or nutritional problems (45, 52, 53, 60). Life expectancy is normal (39).

Cognitive functions of SMA patients are never affected regardless of the type. In fact, these patients are reported as quite bright and sociable when compared with patients with other types of muscular atrophies (34, 39, 61, 62).

Other non-motor symptoms have been described in the most severe form of SMA (Type 0) which can be related to the susceptibility of other tissues to the low levels of SMN protein. These include congenital heart defects (46–48, 63–65), vasculopathy (66, 67) and sensory nerves problems (68) and gastrointestinal dysfunctions (69, 70).

However, there are many cases of overlapping/variation between types. So, it is consensual in the scientific community that it is important to have a new classification or subdivision of the existent types (35, 38).

The diagnosis of SMA has evolved throughout the times. Muscle biopsies were classically used for SMA diagnosis (40–42, 50) but the histological alterations are very heterogeneous (50, 71). In the majority of the cases, a neurogenic picture is represented by hiperatrophy of the fibers (50, 71–73), reduction in the number of the motor neurons of the anterior horn and migration of the motor neurons throughout the axonal paths (heterotopy) (73–76). Motor neuron junctions (MNJ) show ultra-structural abnormalities such acetylcholine agglomeration, synaptic vesicle transport defects and abnormal terminal nerves (73). In Type I we can also see features of immaturity in some cells (small myoblast, myotube-like cells and abundance of satellite cells) and denervation (73, 77). In Type III is more common to see fatty acids infiltration and clustering of the same fibers type. Some studies suggest that secondary degenerative alterations not specific to SMA patients, can also be detected. In these findings they observed gliosis, chromatolysis and balloon neurons (47, 55, 74, 76). An increase in empty bed cells and involvement of the dorsal, cortical or thalamic roots neurons have also been documented (74, 76). Nevertheless, with the increase of the efficiency and availability of genetic testing, muscle biopsies are no longer needed for the SMA diagnostic (38, 78).

Neurophysiological function is also used in SMA diagnosis. This type of evaluation is made by electromyography and nerve conduction studies (1, 28, 50, 71). In these tests, motor unit action potentials of healthy people are regular and show bi or triphasic morphology with normal duration and amplitude for age and muscle quantity of the patient. In SMA's patients, this morphology is lost and an increase of the spontaneous charges generates a polyphasic pattern with higher amplitudes and duration (38, 50, 71, 76). Fibrillation, positive Sharp waves and motor neuron loss are signs of active denervation in SMA Type 0 and I (47, 55, 72, 79, 80). Fasciculations are found more often in Type III and are a strong indicator of chronic denervation (70–72, 80). Conduction studies show normal conduction speeds for SMA patients and compound muscle action potential (CMAP) amplitudes are mainly normal (50, 70, 71). But, if the number of functional motor units drops substantially, we could see a reduction in CMAP (80). These features are not exclusive of SMA so other means are needed to perform a credible diagnostic (80).

1.4. Molecular basis

The gene responsible for producing the complete transcript that originates the functional SMN protein is the telomeric gene *SMN1* (OMIM 600354) (4, 44, 81, 82). It is found in a 500kb inverted duplication in the long arm (q13) of chromosome 5 (Fig. 4) (2–4, 81). This region is prone to unequal

crossing-over between homologous copies and intrachromosomal deletions (4, 30, 81, 83–85). Also in this region, we can find Alu repeats which have been shown to be prompt to recombination (86). Methylation of the CpG islands of the *SMN1* gene promoter can also cause the disruption of the SMN protein (87). The disease-causing gene *SMN1* have 9 exons (1, 2a, 2b, 3, 4, 5, 6, 7 e 8) and 8 introns (4, 88). The first exon is mainly the 5' UTR of the gene and only 1/3 serves has coding region (89). Exon 2a and 2b codes for a peptide responsible for binding DNA, RNA and p53 (11, 13, 24). Exon 2b is also a self-association site (19). Bertrandy *et al* showed that exon 3 encodes the protein part that is responsible for modulating RNA-binding activity (11). The exon 4 encodes for the protein parcel that acts as scaffold for the exon 2a and 2b encoded monomers. This allows them to fold together in the reformation of the epitope of the protein portion encoded by exon 2 (19). The region encoded by exon 6 is responsible for binding Bcl-2, a family of pro-apoptotic molecules (23). Exon 6 is also the second self-association site of the *SMN1* gene (19, 20). The exon 7 encodes for a monomer that showed to enhance the ability of SMN protein to self-associate through exon 6 self-association site (19). The last exon encodes the 3' UTR (89). Exon 7, and sometimes exon 8, suffer, in SMA patients, homozygous deletion which leads to a truncated, non-functional SMN protein (4, 52, 90). These deletions are the main (95%) cause of SMA (4, 52, 86, 87, 91). The majority of missense variants occur in exons 6 and 7 (85). To date, over 200 variants have already been described in the Clinvar variant database. In Portugal, the most frequent pathogenic variant (other than exon 7 deletion) is c.770_780dup (p.Gly261fs) (92, 93). The remaining cases are triggered by variants in other chromosomes (non 5q SMA) such as SMA with arthrogryposis X-linked and 11q13 diaphragmatic SMA (4, 53, 75, 94, 95).

After humans diverged from primates, gene conversion events of the *SMN1* started to occur (4, 8, 30, 84, 86, 87, 96). These events gave birth to a paralog gene called *SMN2* (OMIM 601627) and, later, an increase in its copy number thus making them more common in the less severe types (4, 8, 30, 47, 59, 84, 85, 87). This centromeric SMN gene of the chromosome 5 is inverted and differs in only five nucleotides from its ancestral (Figure 4) (4, 82). Four of these nucleotides are located in non-coding zones: c.835-44G>A (rs1454173648); c.888+100A>G (rs212214); c.888+215A>G (rs1244569826); c.1155G>A (rs1208416968) (4, 88, 91). The only one localized in a coding region is c.840C>T (rs1164325688) and it is a silent variant not having any effect on the final amino acid (Phe280=). Nevertheless, this last variant changes the C-terminal end leading to a profound effect on the amount of full-length protein (4, 20, 82, 91).

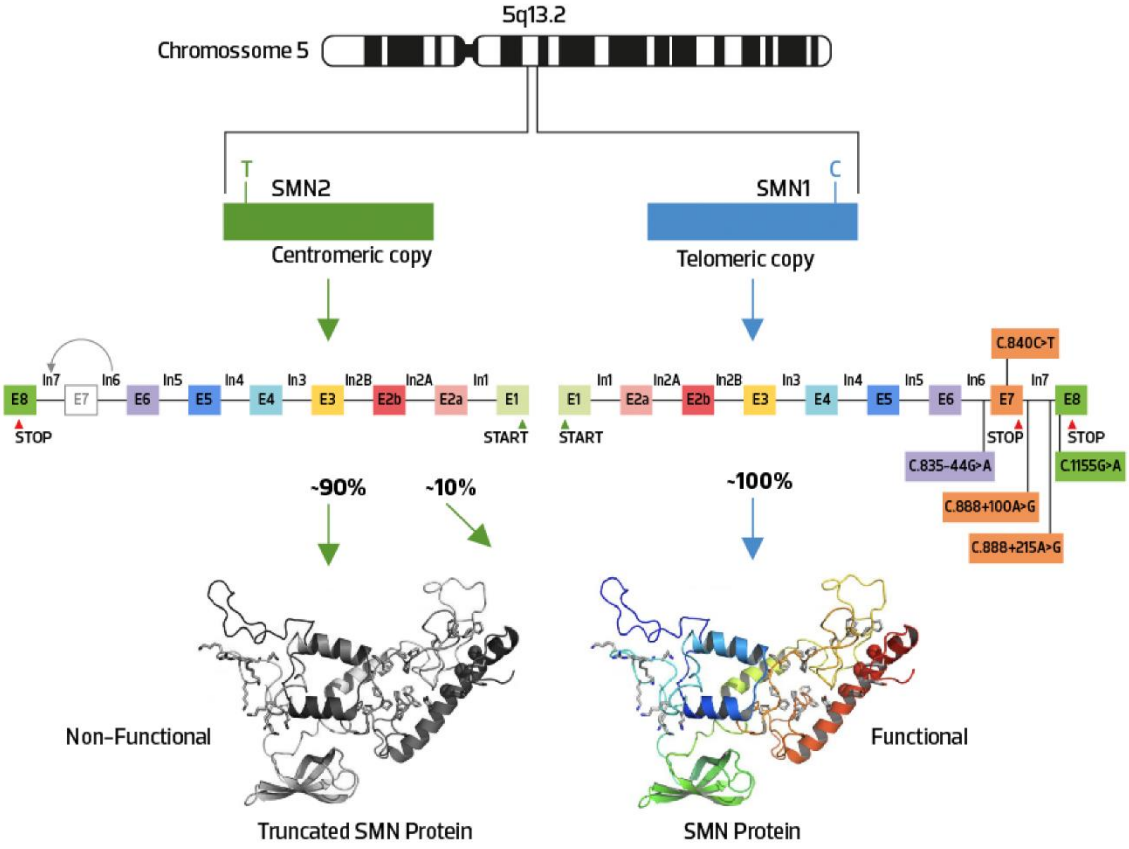

Figure 4 – Organization of *SMN1* and *SMN2* genes. *SMN1* and *SMN2* are localized in the long arm of chromosome 5 at position 13.2. They have the 3' and 5' sites reversed (in mirror). *SMN2* suffers exon 7 skipping leading to a truncated form in 90% of the cases and thus non-functional. The other 10% does not undergo alternative splicing leading to a functional protein. Adapted from Singh *et al.* and Kolb *et al.* (57, 89).

The putative AG-rich Exonic Splicing Enhancer (ESE) region 2 of exon 7, a cis element that binds with the splicing regulatory factor serine/arginine-rich protein SF2/ASF, promotes inclusion of this suboptimal flanking splice signals region (7, 20, 97, 98). SF2/ASF motif binds very specifically to *SMN1* RNA but not to the *SMN2* RNA (98). The C>T (C6U) substitution is, then, responsible for disrupting the activity of this SF2/ASF dependent AG-rich ESE leading to an alternative splicing of the pre-RNA (Figure 5) (44, 82, 97, 98). The consequence is the skipping of *SMN2* exon 7 in the majority of the transcripts (20, 44, 82, 97, 98). Since exon 7 is fundamental for the oligomerization and function of the SMN protein, the resulting protein (SMNΔ7) is truncated and non-functional in the majority of the cases (90%) resulting in its rapid degradation (4, 7, 82, 97, 99). The other 10% does not undergo through alternative splicing, originating normal and functional protein (Figure 4) (4, 7, 82, 97). This small amount of protein produced by *SMN2* allows fetal development but it is not

enough to sustain the survival of the motor neurons after birth unless it has multiple copies of the *SMN2* gene, so it is considered to be a disease modifying gene (4, 7, 30, 59, 82, 100).

Several other hypotheses have been proposed to explain the inhibitory effect of the C6U substitution in SMN exon 7 splicing. While studying the C6U substitution, Kashima *et al.* in 2003 found that the depletion of heterogeneous nuclear Ribonucleoprotein (hnRNP) A1/A2 dependent Exonic Splicing Silencer (ESS), markedly increased *SMN2* exon 7 inclusion (Figure 5) (101). Five years later, Chen and colleagues showed that low extracellular pH increased the concentrations of hnRNP A1 and Sam68 (a RNA binding protein recruited by splice sites that influences alternative splicing through interactions with other proteins or RNA) thus promoting *SMN2* exon 7 exclusion (102). Pedrotti *et al.* found later that Sam68 interacts with hnRNP A1 favoring exon 7 skipping and, therefore, suggested a modification of the initial model (Figure 5) (103). In 2003, Singh and colleagues found that a 16 nucleotides extended inhibitory context which they called "Extinct" was able to abrogate exon 7 splicing (Figure 5). They also showed that this complex could compensate the effects of the ESE SF2/ASF and the ESS hnRNP A1 (104).

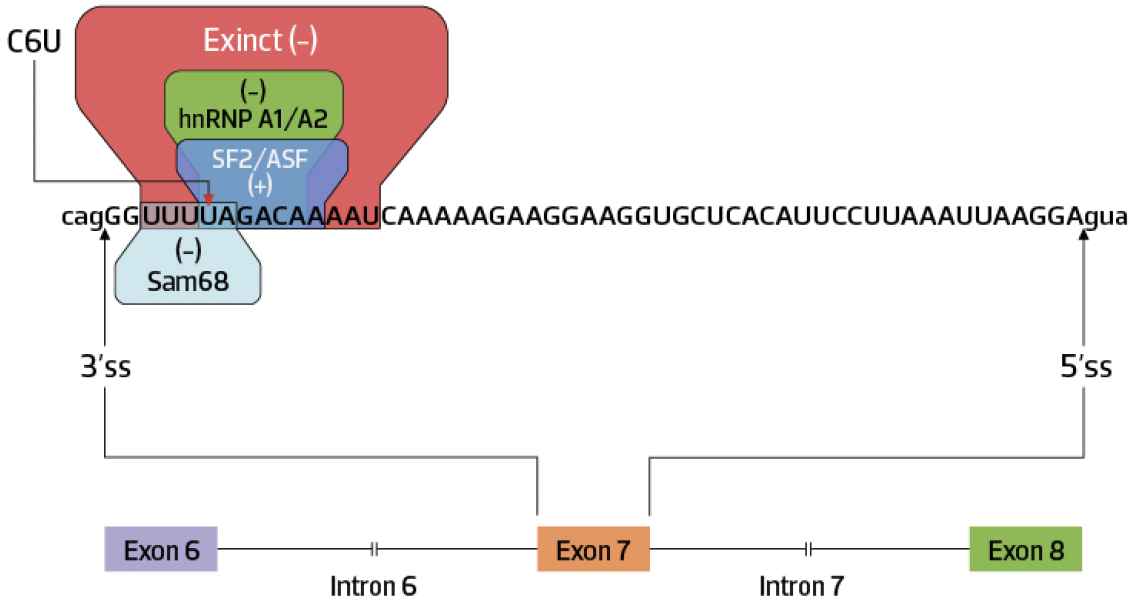

Figure 5 – Schematic representation of exon 7 splicing regulation in the region of the C6U substitution. Positive and negative elements are indicated by (+) and (-), respectively. Upper-case letters represent exonic sequences and intronic sequences are shown in lower-case letters. The 3′ and 5′ss are indicated by arrows. Adapted from Singh *et al.* (89).

A few years later, the same team showed that an Antisense Oligonucleotide (ASO) could bind to an Intronic Splicing Silencer (ISS) N1 (ISS-N1), localized immediately downstream of the splice site in intron 7 of *SMN2*, displacing inhibitory splicing factors and consequently increasing the proportion

of exon 7 inclusion and the amount of full-length SMN protein (105). These regulatory elements are not mutually exclusive despite their contradictory functions.

Normal population have zero to four copies of *SMN2* (78, 90). Severe SMA patients have less copies than patients with milder disease but at least one copy is necessary to ensure the survival in SMA patients (4, 5, 30, 47, 78, 90). Patients with Type I have 2 or 3 copies and Type II usually has 3 copies (48, 78, 90). Type III patients show 3 to 4 copies and type IV is associated with 4 or more copies (78, 90, 106). The more gene conversions occur the less severe is the phenotype (Types III and IV) (30, 85, 87, 106). Therefore, there is an inverse proportion between the severity of the disease and number of copies of *SMN2* so it is considered a prognostic biomarker that predicts possible outcomes (5, 30, 78, 86, 99).

Other phenotypic modulators exist since persons without clinical symptoms, with the homozygous deletions of *SMN1* and sibs with identical haplotypes have been described (107–109). Prior et al found that the transition c.859 G>C (G287R) creates a new ESE with the presence of the high-score SF2/ASF motif that subsequentially increases the amount of full-length transcripts and thus leads to a milder phenotype (110). Plastin 3 (PLS3) (OMIM – 300131) is a calcium-dependent protein known to be a well-studied protective modifier in woman (109, 111). It binds with actin and SMN stabilizing growth cones in axonogenesis. When overexpressed and in the presence of SMN, PLS3 can restore axons length, increase life spans and improve muscle and MNJ function (109, 111). It has also been shown that, in milder forms of SMA, increasing PLS3 levels alone can reduce the severity of the disease (111). Unlike PLS3, Neurocalcin Delta (NCALD) (OMIM – 606722) is a negative phenotypic modifier and acts as a genetic suppressor of SMA. NCALD is a neuronal calcium (Ca^{2+}) sensor protein that interacts with clathrin (an essential protein for the coating of endocytic vesicles) preventing its function in endocytosis. When levels of the SMN protein are low, influx of voltage-dependent Ca^{2+} is reduced. NCALD binds with clathrin at these low or null Ca^{2+} concentrations, thus inhibiting endocytosis. By himself NCALD can facilitate endocytosis but, when the levels of NCALD are reduced and there is sufficient SMN protein, axonal growth and synaptic maturation are restored and maturation and neural circuit function of MNJs is rescued (112). In 2018, Janzen *et al.* found a direct interacting partner of PLS3 called Calcineurin-like EF-hand protein 1 (CHP1) (OMIM - 606988) a protein that binds to Ca^{2+} and co-localizes with PLS3. It inhibits Calcineurin, a major regulator of proteins required for presynaptic endocytosis, thus leading to an increase of hyperphosphorylated Dynamin 1. The consequence is the impairment of the endocytosis. CHP1 knockdown was found to restored neurite growth, prolonged survival, alleviated electrophysiological defects and improved major SMA hallmarks (113).

1.5. Treatments

For several decades, treatment of SMA was based on symptoms management (52, 114, 115). On Table 1 is summarized the main concerns and respective approaches to address them according to the Standard of Care of 2007.

Table 1 – Clinical management of the main manifestations of SMA patients.

Clinical Problem	Manifestations	Treatment	References
Pulmonary	Restrictive lung disease	Noninvasive ventilation with bilevel or mechanical ventilator	(52, 54, 115)
	Weak cough and mucus	Airway-secretion mobilization	
	Pulmonary infections	Antibiotics – only in acute illness	
	Respiratory failure	Invasive ventilation – when everything fails	
Gastrointestinal	Difficulty feeding and swallowing	Gastrostomy and laparoscopic Nissen fundoplication	(52, 56, 114, 116)
	Reflux	Acid neutralizers / Inhibitors of acid secretion	(52, 56, 114)
	Delayed gastric emptying	Prokinetic agents	
	Constipation		
Nutritional	Overweight	Nutritionist prescribed diet	(52, 114, 116)
	Malnutrition		
Orthopedic and musculoskeletal	Scoliosis	Spinal surgery and bracing	(52, 114, 117)
	Fatigue and contractures	Physical therapy	(114)
	Osteopenia and fractures	Vitamin D and bone supplements	(114, 118)

In most inherited diseases, incorrect or no protein is produced. In SMA patients, an ineffective protein is produced by *SMN1*, so there is a therapeutic benefit in up-regulating *SMN2* gene expression and thus increasing the amount of full-length protein (5, 59, 85, 97-99, 110). So far, 3 molecularly treatments have been approved.

Spinraza™ (Nusinersen) is an 18-mermodified 2'-O-2-methoxyethyl phosphorothioate ASO that promotes exon 7 inclusion by blocking the binding of splicing factors to the ISS-N1 motif and in 2017 it became the first worldwide approved treatment to SMA patients. It is administered intrathecally by lumbar puncture, traveling through the cerebrospinal fluid to the central nervous system (CNS)

and also into systemic circulation (119, 120). The drug is administered every 14 days in the first 3 doses, a fourth loading dose 30 days later and maintenance doses once every 4 months (119, 121).

In 2019, Zolgensma® (Onasemnogene abeparvovec) became the first approved gene therapy for SMA (122). It delivers, through an Adeno-Associated Virus serotype 9 (AAV9) vector, a functional copy of *SMN1* gene to motor neuron cells providing the necessary full length SMN protein. The virus allows for the drug to pass the brain blood barrier so an intrathecal administration is not necessary (123). Zolgensma® is a one-time intravenous infusion of 1.1×10^{14} vg/kg vector genomes that takes 60 minutes to be administered via a peripheral vein (123).

More recently, Evrysdi (Risdiplam) was approved. Its composition consists in a brain penetrant small molecule (RG7916) that function as a RNA splice modifier promoting the inclusion of exon 7. This drug increases the expression of full-length SMN protein in CNS and peripheral tissues and a single oral pill per day is enough to increase this expression (124, 125).

Non-SMN treatments are also being tested and show promising results in combination with the already approved treatments (126, 127). These drugs downregulate myostatin (126) or upregulate troponin (127) to improve muscle function and mass thus ameliorating the symptoms of SMA patients.

Therapy follow-up biomarkers are presently being studied because there is a need to monitor patients that have been submitted to these new treatments. Some have already shown promising results, including SMN protein and mRNA and some serum proteins such has cadherin-13 and peptidase D (128). From all, the most promising molecular biomarker to date is the phosphorylated neurofilament heavy chain levels in serum, a protein that is specific to axon injury (129). These levels are quite elevated in SMA patients comparing to controls and during the course of treatment with Nusinersen the levels decreased (129).

The levels of SMN protein are higher during fetal development when they are most needed. 3 months after birth these levels show significant decline (73, 130). Treatments are most effective in the asymptomatic phases and before the irreversible loss of the motor neurons (37, 51, 120, 129, 131, 132). Unfortunately, diagnostic is always made after the onset of symptoms and sometimes the delay is considerably large (51). Therefore, the solution lies in the implementation of newborn screening for SMA (28, 37, 51, 53, 129, 131-133).

1.6. Neonatal Screening

Newborn screening (NBS) are public health programs that have as main goal the detection of newborns affected by a disorder in the pre-symptomatic period. This way treatment can be initiated in a timely manner and be more effective resulting in huge heath gains. NBS started with the work of Robert Guthrie, back in the 60's of last century in the USA. He developed an easy and cost-effective method for the semi-quantification of phenylalanine in newborns, so they could be screened for phenylketonuria (PKU) (134). Guthrie was also the first to use dried blood spots (DBS) as sample matrix for NBS. They are easy to collect and to send by mail to the laboratory, after dried. The junction of an effective laboratory method, and easy sample collection and transportation, alongside with huge clinical achievements for screened and early treated PKU patients, were the basis of the expansion and success of NBS programs.

Figure 6 – Guthrie Card used currently in the Portuguese Newborn Screening.

In order to introduce some regulation to screening programs, World Health Organization published some principles that must be achieved, so a disorder could be screened, the so-called Wilson and Jungner criteria. The 10 principles state that (135):

a) The condition sought should be an important health problem.
b) There should be an accepted treatment for patients with recognized disease.
c) Facilities for diagnosis and treatment should be available.
d) There should be a recognizable latent or early symptomatic stage.
e) There should be a suitable test or examination.
f) The test should be acceptable to the population.
g) The natural history of the condition, including development from latent to declared disease, should be adequately understood.

h) There should be an agreed policy on whom to treat as patients.

i) The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.

j) Case-finding should be a continuing process and not a "once and for all" project.

Mostly based on these principles and supported by technical and treatment developments, several disorders have been added to NBS panels throughout the years, with many of the developed countries (including Portugal) screening for more than 26 treatable disorders (136). Usually, the sample collection is made between the first 24 hours of life and the 6th day (depending on the country/region) on Guthrie cards. The samples are then sent to the laboratory to be processed.

In Portugal the NBS program started in 1979 with the screening of PKU. It is a non-mandatory program with a very successfully implementation and screens for over 99,9% of Portuguese newborns (137). Sample collection is made on health centers/hospitals and is advised to be made at 3rd day and preferentially until the 6th. The laboratory activities of the Portuguese NBS program are centralized in one single laboratory – the Newborn Screening, Metabolism and Genetics Unit, at Porto delegation of the National Institute of Heath Doutor Ricardo Jorge. Nowadays, a total of 26 disorders are included in the screening panel (24 metabolic disorders, congenital hypothyroidism and cystic fibrosis) which are described below on table 2.

NBS has the potential to increase the benefit of therapies without increasing their cost and even may substantially decrease the cost of support needed to help patients with functional impairment (133, 138). With the appearance of effective treatments for SMA in these recent years, that are more effective if started in the pre-symptomatic period, alongside with a feasible methodology to detect the patients in the neonatal period, SMA accomplishes Wilson and Jungner criteria and become in the radar of several NBS programs worldwide. Numerous countries like Taiwan, United States of America, Canada, Australia, Japan, Belgium, Slovakia, Germany, Italy, Spain and The Netherlands have performed or have ongoing pilot studies to implement SMA in their NBS programs (9, 37, 91, 136, 139-144).

Table 2 – Panel of the screened diseases in the Portuguese NBS (137).

I. Congenital Hypothyroidism		
II. Cystic Fibrosis		
III. Hereditary Metabolic disorders	Aminoacidopathies	Phenylketonuria / Hyperphenylalaninemia
		Type I tyrosinemia
		Type II/III tyrosinemia
		Leucinosis
		Classical homocystinuria (cystathionine β-synthetase deficiency)
		Hypermethioninemia
	Diseases of urea cycle	Type I Citrulinemia
		Argininosuccinic aciduria
		Hyperargininemia
	Organic aciduria	Propionic acidemia
		Methylmalonic aciduria (deficiency in methylmalonyl-CoA mutase/cobalamins)
		Isovaleric acidemia
		3-Hydroxy-3-Methylglutaric aciduria
		Type I glutaric aciduria
		3-methylcrotonyl-CoA carboxylase deficiency (3-MCC deficiency) / multiple carboxylase deficiency
		Malonic Aciduria
	Mitochondrial β-Oxidation Diseases of Fatty Acids	Short chain 3-hydroxyacyl-coa dehydrogenase deficiency
		Medium chain fatty acid dehydrogenase deficiency
		Long chain 3-hydroxyacyl-coa dehydrogenase deficiency
		Very long chain fatty acid dehydrogenase deficiency
		Carnitine palmitoyltransferase I deficiency
		Carnitine palmitoyltransferase II deficiency
		Multiple acyl-CoA dehydrogenase deficiency
		Primary carnitine deficiency

SMA doesn't have a metabolite/protein marker that could be easily used for patient detection (132). Considering that the great majority of SMA patients are homozygous for the same variant, molecular testing emerged as a good option for NBS. Because SMA's differential diagnosis is very complex,

even to experts, DNA testing becomes a valuable diagnostic tool since it can reliably detect the homozygous deletion of *SMN1* exon 7 (63, 64, 79).

1.7. NBS consensual technic – quantitative real time Polimerase Chain Reaction (qPCR)

Since mid 90's, several groups demonstrated that PCR is a valuable tool to study the molecular basis of SMA (8, 83, 84, 86, 87, 145, 146). The molecular hallmark of SMA is the homozygous deletion of exon 7 in *SMN1* and Wilson and Jungner advised not to report unaffected carriers (135) therefore a quantitative qPCR methodology is necessary to accurately distinguish patients from carriers (146, 147). This technique allows for high throughput screening while maintaining very high sensitivity and specificity (9). It also allows for multiplex assays with other genetic conditions such as Severe Combined Immunodeficiency (SCID) (148, 149) making it more affordable. Despite this we have to be careful when designing an assay because false positives/negatives can occur due to the conversion's events or primers/probes binding site variants (9). From a NBS point of view it is also important to alert all intervenients in the process that this screening approach only allow to identify patients with homozygous deletion of *SMN1* exon 7, so about 5% of the SMA cases with other missense variants on *SMN1* or other chromosomes will not be identified.

There is a need for a simple, inexpensive, reliable and stable method to transport the neonate samples and DBS are already used in almost all NBS. They don't need anticoagulants and the only added thing to the sample is cellulose from the card paper. Previous studies have already shown efficiency of DNA extraction with DBS from Guthrie Cards used in the NBS (9, 131, 132, 148–150).

There are companies making kits for SMA NBS based on qPCR detection of *SMN1* exon 7 deletion. Our main objective is to implement and test an *in house* methodology that is cheaper than the commercial kits but, hopefully, still feasible, reliable and quick. We will access its efficiency by testing normal and pathological samples.

2. Methods

2.1. Samples

Anonymized peripheral blood samples and Guthrie cards were provided by Instituto Ricardo Jorge. Each sample was labeled as negative (presence of *SMN1* exon 7) or positive (absence of *SMN1* exon 7) for SMA. There was a total of 33 samples used (29 negative and 4 positive).

2.2. DNA Isolation

Peripheral blood samples DNA extraction was made automatically with Bio Robot EZ1 (Quiagen, Hilden, Germany) using EZ1&2 DNA Blood Kit and card (Quiagen, Hilden, Germany) according to manufacturer's instructions. Concerning DBS DNA extraction, two different methods were used, both starting from a DBS with 3,2mm diameter. One was an automated extraction procedure with Quiagen's Bio Robot EZ1 using EZ1&2 DNA Tissue Kit (Quiagen, Hilden, Germany). Manufacturer's protocol was again respected. The other method, a manual one, used the solution Extracta DBS (Quantabio, Massachusetts, USA). The first step of the manual method was to punch the 3,2 mm DBS to a sterile 96 well plate and then add 90µL of the Extracta DBS solution. Homogenization was then made by flushing with the pipette 10 times. The plate was covered with a sealing film (ThermalSeal RTS™ Sealing Films, Thermo Fisher Scientific, Massachusetts, USA) to avoid cross contamination. Centrifugation was made at 3500 rpm for 5 seconds. Supernatant was discarded and another 54µL of the extraction solution was added and briefly spanned. An incubation period of 25 minutes at 96°C was made on a thermal block. Solution was spanned again and finally was ready to use. All the procedures were performed on a clean area, using DNA free filter tips and sterile material, complying with all good laboratory practices to avoid cross contamination.

Measure of DNA concentration and 260/280nm ratio to assess purity of the DNA of the samples were made on NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

To simulate carriers (heterozygous samples) we mixed 15µL of DNA solution extracted from a DBS negative sample with another 15µL of DNA solution extracted from a DBS positive sample, using samples with similar DNA yields.

2.3. qPCR assay to acess *SMN1* exon 7 deletion

Primers and probes used were previously described by Mei Baker and her team (151). Primers were order from Invitrogen (Invitrogen, Massachusetts, USA) and TaqMan probes were from Eurogentec

(Eurogentec, Seraing, Belgium). Both are described on table 3. qPCR master mix used was TaqMan™ Fast Advanced Master Mix (Applied Biosystems, Massachusetts, USA). The total volume of the reaction mixture was 20 µL. It contained 10µL of master mix, SMN primers (46,875 nM each), *RPP30* primers (25 nM each), *SMN1* probe (56,25 nM), *SMN2* blocker (56,25nM), *RPP30* probe (75 nM) and 6 µL of DNA extracted from the DBS with the Extracta DBS protocol. The qPCR conditions were 94°C for 5 minutes, 40 cycles of melting at 94°C for 15 seconds, annealing at 60°C for 33 seconds and extension at 68°C for 40 seconds. Reactions were carried out in a Bio Rad's CFX Touch 96. Quantification cycles (Cq) were informed by instrument software.

Table 3 – Sequences of primers, probes and blocker used on the qPCR assay.

Oligo Name	Sequence
SMN1 Forward Primer	5'- CTT GTG AAA CAA AAT GCT TTT TAA CAT CCA T -3'
SMN1 Reverse Primer	5'- GAA TGT GAG CAC CTT CCT TCT TTT T -3'
RPP30 Forward Primer	5'- AGA TTT GGA CCT GCG AGC G -3'
RPP30 Reverse Primer	5'- GAG CGG CTG TCT CCA CAA GT -3'
SMN1 Probe	5'- FAM - AGG GTT TCA GAC – BHQ -3'
RPP30 Probe	5'- HEX - TTC TGA CCT GAA GGC TCT GCG CG – EDQ -3'
SMN2 Blocker	5'- AGG GTT TTA GAC -3'

Bases in red are LNA nucleotides.

Underlined nucleotides symbolize the location of the C>T transition in exon 7.

FAM represent the location of the 6-FAM reporter dye on the 5' end of the *SMN1* probe.

HEX denote the region where the Hexachlorofluorescein reporter dye is on the 5' end of the *RPP30* probe.

BHQ and EDQ designate the locations of the dark quenchers used on the 3' ends of *SMN1* and *RPP30* probes respectively.

An internal control is necessary to monitor the efficiency of the PCR reaction since SMA detection is based on the absence of amplification (78, 146). For this purpose, we used the now widely accepted reference gene ribonuclease P protein subunit p30 (*RPP30*) (91, 140, 149, 151, 152). Locked Nuclei Acids (LNA) were used to make the *SMN1* probe and *SMN2* blocker. The intent was to increase thermal stability, hybridization specificity and accuracy and allelic discrimination between *SMN1* and *SMN2* (91, 141, 153). *SMN2* blocker was used to reduce the possibility of annealing between the *SMN1* probe and *SMN2* sequence (151, 153).

The assay is designed to detect only *SMN1* and not *SMN2*, thus avoiding false negatives. It identifies the absence of *SMN1* exon 7 due to biallelic deletion or conversion, so a 100% sensitivity is expected. Other *SMN1* variants and non 5q SMA cases will not be detected with our assay. This is a qualitative assay so carriers will not be identified. Since we must detect the absence of a gene region, contaminations should be avoided and looked for in all steps.

2.4. Statistics

Cq values means ± standard deviation, histograms and percentiles were performed in Microsoft® Excel® 2019 MSO, version 2209 Build 16. 0. 15629. 20152 (Microsoft, Washington, EUA).

3. Results

3.1. DNA extraction

The choice for an DNA extraction method must take in consideration that it must not only deliver DNA in quantity and purity adequate for the procedure, but also to be adapted to high throughput. The goal is that all the system, from DNA extraction to detection, must be adapted to NBS laboratories, that typically process hundreds of samples a day. Considering all these prerequisites, the choice was to test Extracta DBS protocol from QuantaBio, already used by some NBS laboratories. In order to test initial primers and probes conditions, DNA extracted from peripheral blood with ethylenediamine tetraacetic acid (EDTA) samples were first used. Then we tested DNA extracted from DBS using an automated method that is known to deliver high quality DNA and, at last, the method was tested in samples extracted using the Extracta DBS protocol. When comparing all the extraction protocols, peripheral blood with EDTA samples had, as expected, the highest extracted DNA concentration and also the purest DNA from the three. The second higher DNA concentration was seen in samples from DBS extracted with the Extracta DBS protocol, nevertheless the ratio A260/A280 pointed to a low purity DNA solution. The NanoDrop used for DNA quantification identified the contamination as a possible protein contamination and corrected the DNA quantification accordingly. After this correction, the DNA concentrations were still higher than the amount obtained from DBS that were subjected to the automatized extraction protocol. DNA purity was closer to the desired ratio of 1,8 on samples made with the Extracta DBS protocol than the samples made with the automated extraction. These results are described below on table 4.

Table 4 - DNA quantification and purity of samples made with the three different extraction methods (peripheral blood with automatic extraction, DBS with automatic extraction and DBS with Extracta DBS extraction).

Sample	DNA concentration (ng/µL)	A260/A280	Contaminant	Corrected DNA concentration (ng/µL)	A260/A280
Peripheral Blood 1	85.1	1.84			
Peripheral Blood 2	81.2	1.83			
Peripheral Blood 3	59.5	1.8			
DBS Automatic Extraction 1	21.6	2.56			
DBS Automatic Extraction 2	19.9	2.43			
DBS Automatic Extraction 3	19	3.19			
Extracta DBS 1	65.4	1.08	Protein	32.4	0.59
Extracta DBS 2	63.3	1.08	Protein	31.0	0.58
Extracta DBS 3	80.6	1.09	Protein	47.0	0.67
Extracta DBS 4	55.7	1.13	Protein	30.9	0.53

3.2. qPCR assay

The amplification tests performed with the DNA extracted from peripheral blood and from DBS, by automated methods, result in good and reproducible amplification curves using the initial conditions (Figure 7).

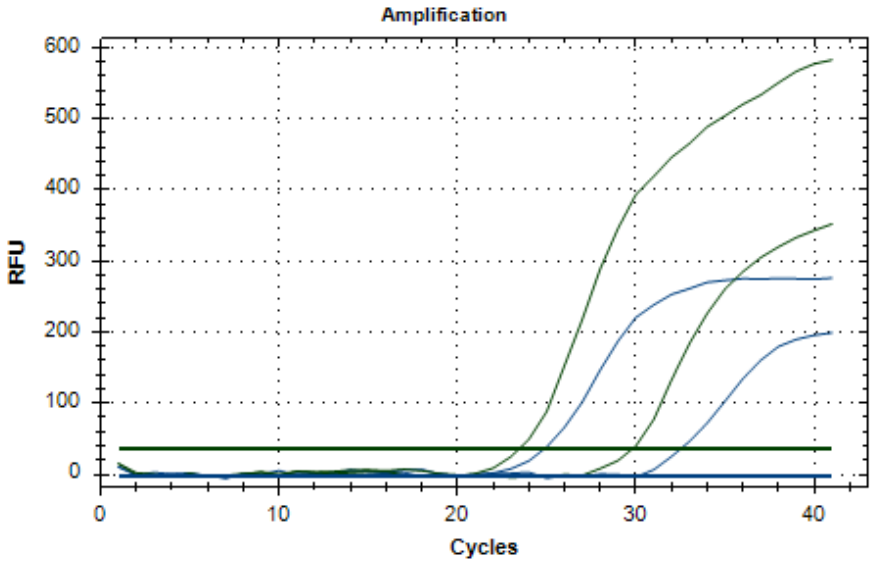

Figure 7 – Amplification curves of peripheral blood and DBS extracted by automated methods.

So, these same conditions were replicated on the tests using the DNA extracted from DBS using the Extracta DBS protocol.

First the conditions were tested without multiplexing the primers/probes and three samples were tested each time. Regarding *RPP30*, amplification was obtained in all the three samples used on the first test. Two of the three samples with *RPP30* primers and probes had similar amplification curves and Cq. The third sample had a slightly lower Cq but still within the range of the other two (Figure 8).

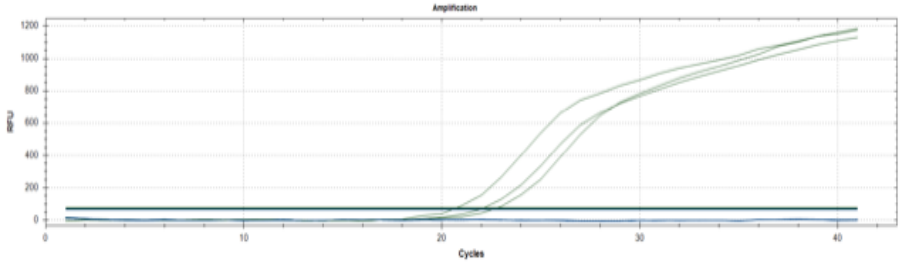

Figure 8 – Amplification of three normal DBS samples with only *RPP30* primers and probe.

Positive amplification was also obtained in the three samples with only *SMN1* primers and probe. Here we also observed one sample with lower Cq. The other two had very similar curves until the start of the plateau stage (Figure 9).

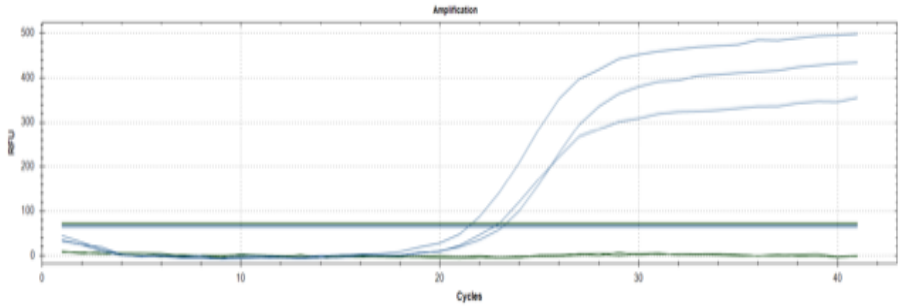

Figure 9 – Amplification of three normal DBS samples with only *SMN1* primers and probe.

The three samples were positively amplified when using primers and probes of both genes (*RPP30* and *SMN1*) and *SMN2* blocker. The *RPP30* curves (labeled with HEX probe) appear in green and show a similar behavior when compared with the previous samples that only had *RPP30* (Figure 10). The *SMN1* curves (samples labeled with FAM probe) are shown in blue and had a somewhat different Cq between them but they were still in the same range (Figure 10). Overall, the Cq of *RPP30* amplification were slightly lower than the Cq of the *SMN1* amplification.

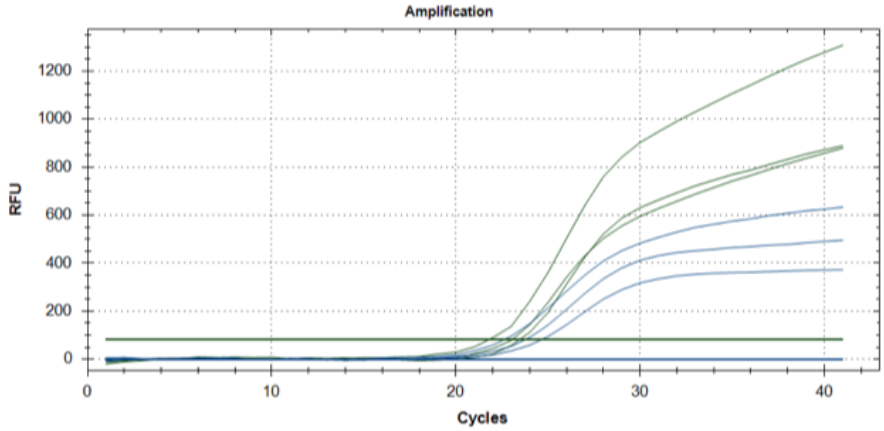

Figure 10 – Amplification of three normal DBS samples with *RPP30* and *SMN1* primers and probes on the reaction mixture. The green curves (HEX labeled probe) correspond to the amplification of the *RPP30* gene and the blue curves (FAM labeled probe) shows the amplification of the exon 7 of the *SMN1* gene.

3.3. qPCR detection *SMN1* exon 7 deletion

Our *SMN1* qPCR assay showed 100% concordance with the known genotypes of the 31 control samples and 4 positive controls (100% sensitivity and specificity). A summary of all Cq results is available on table 5.

Table 5 – qPCR Cq for *RPP30* and *SMN1* genes.

Sample	With blocker			Without blocker		
	RPP30	*SMN1*	*SMN1/RPP30*	*RPP30*	*SMN1*	*SMN1/RPP30*
N1	26.42	28.57	1.081			
N2	25.81	26.82	1.039			
N3	26.47	27.51	1.039			
N4	25.48	25.73	1.009			
N5	25.32	26.15	1.032			
N6	25.93	26.35	1.016			
N7	26.48	26.84	1.013			
N8	26.12	27.12	1.038			
N9	26.07	26.28	1.008			
N10	26.15	27.35	1.045			
N11	26.32	27.56	1.047			
N12	25.73	27.10	1.053			
N13	26.02	27.13	1.042	24.94	25.71	1.030
N14	25.98	27.02	1.040	25.84	27.23	1.053
N15	26.47	29.23	1.104	26.14	29.20	1.117
N16	25.99	27.15	1.044	25.95	28.32	1.091
N17	25.87	26.49	1.023	25.67	27.13	1.056
N18	26.51	27.21	1.026	26.31	27.99	1.063
N19	25.50	26.59	1.042	25.33	26.65	1.052
N20	26.80	28.03	1.045	26.40	28.55	1.081
N21	26.19	26.78	1.022	26.17	26.99	1.031
N22	26.22	27.38	1.044	26.13	28.03	1.072
N24	25.76	26.19	1.016	25.60	25.88	1.010
N25	24.90	25.24	1.013	24.18	24.67	1.020
N26	25.52	26.81	1.050	25.46	27.05	1.062
N27	25.14	25.70	1.022	25.07	25.88	1.032
N28	25.38	26.23	1.033	25.34	26.21	1.034
N29	25.46	26.34	1.034	25.31	26.07	1.030
N30	25.63	26.66	1.040	25.61	26.83	1.047

Table 5 – qPCR Cq for *RPP30* and *SMN1* genes (continuation).

Sample	With blocker			Without blocker		
	RPP30	*SMN1*	*SMN1/RPP30*	*RPP30*	*SMN1*	*SMN1/RPP30*
P1	24.53	0	0	25.16	0	0
P2	24.94	0	0	24.87	0	0
P3	26.61	0	0	26.51	0	0
P4	25.57	0	0	25.04	0	0
H1	25.67	30.35	1.182	23.98	29.28	1.221
H2	25.34	28.23	1.114	25.35	29.58	1.166

N is for negative samples;

P stands for positive samples;

H are the heterozygous samples.

All known normal and heterozygous (or carriers) samples produced amplification (presence of *SMN1* exon 7) (Figure 11) whether they had the *SMN2* blocker or not. The Cq values varied between 25 and 30 (Table 5). These samples would be considered the negative results in NBS. With or without *SMN2* blocker, no amplification was seen in samples belonging to known SMA patients with the homozygous deletion of *SMN1* exon 7 (Figure 11). These samples would be the positive results in NBS.

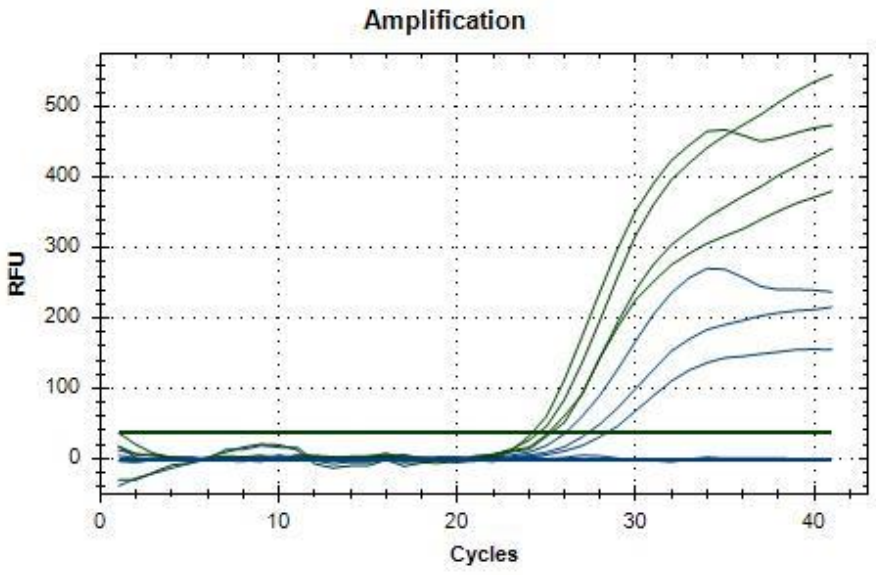

Figure 11 – Amplification curves of 3 negative DBS samples plus 1 positive sample.

On the other hand, when we compare the Cq values of normal samples (N13 to N30) with and without *SMN2* blocker, there is a tendency for a higher Cq dispersion in the reaction without the *SMN2* blocker. Amplification Cq for the exon 7 of the *SMN1* gene in normal samples with *SMN2* blocker present in the reaction mixture started at 25 and had a maximum of 29 (Figure 12A). The mean Cq value of such samples was 26,78 ± 0,90. When the *SMN2* blocker was not used, Cq values fluctuated between 25 and 30 (Figure 12B) and mean and standard deviation values raised (26,99 ± 1,18).

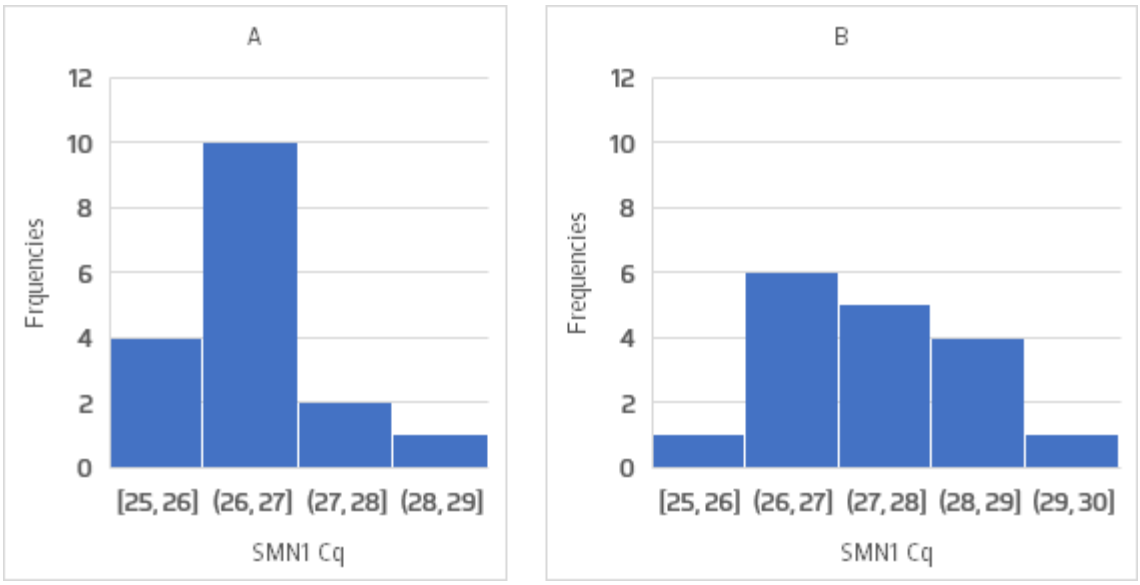

Figure 12 – Distribution of *SMN1* Cq results of 17 normal samples (N13 to N30). (A) *SMN1* Cq for samples with *SMN2* blocker in the reaction mixture. (B) *SMN1* Cq samples without *SMN2* blocker in the reaction mixture.

Regarding *RPP30*, however, there was no significant variation in Cq values on the two tests (with and without *SMN2* blocker).

As initial cut-offs, 99,5 percentiles were calculated for both *RPP30* and *SMN1*. P99,5 for *RPP30* is Cq 26,8 and for *SMN1* is Cq 29,1.

4. Discusion

The final goal of this work is to implement a qPCR method for the detection of *SMN1* exon 7 deletion, based on *in house* prepared reagents and on DNA extracted from DBS that fits the requisites of a NBS laboratory. It should be reliable, with high sensitivity and specificity and adapted to high throughput. Considering that there are several commercial kits on the market for SMA NBS (based on *SMN1* exon 7 deletion detection), to be considered for use, it should also be economically advantageous in comparison with those.

DBS are on the basis of the success of NBS programs, because it's easy to collect the sample, after dried the sample is very stable and they are easily sent by mail to NBS laboratories (9, 131, 132, 148–150). Portuguese NBS programs uses 3,2mm DBS and this seems to be within range with what most successful NBS use (91, 151, 153–155). Size of the DBS is important as smaller DBS have shown to be a source of inconclusive results because of lack of DNA material (152). When comparing the different extraction protocols, the automatic extraction protocol had the least DNA concentration. It is also a long method, not adapted to high throughput, so it is not the best choice for NBS. On the other hand, the Extracta DBS assay was able to provide a considerable amount of DNA, even when the value correction because of the contamination with proteins was made. Although not providing a very pure DNA, our results show that it did not compromise the efficiency of the qPCR assay tests. As shown by previous studies, this method is simple, reliable and fast, making it a valuable choice to include in the NBS routine (143, 151).

SMA has an early onset and rapidly declining clinical course which, alongside with the existence of effective treatment approaches that are more efficient when started earlier, makes NBS an essential need (37, 51, 120, 131–133). The history and biology of SMA have been defined since the discover of the its molecular cause in the 90's. Around 95% of the diagnosed SMA cases are caused by the homozygous absence of *SMN1* exon 7 either by deletion or gene conversion into *SMN2* (4, 52, 86, 87, 91). To date, there are several commercial solutions for NBS of SMA (testing the homozygous absence of *SMN1* exon 7), nevertheless these are solutions that can cost around 4,50€ per newborn tested (156). Our main goal was to choose and implement an *in house* method that could deliver hight sensitivy and specificity, adapted to high throughput and at a significant lower cost than the commercial kits. To choose the molecular assay, we have to consider only the most unexpensive, robust and replicable methods. We have to ponder as well, a protocol that can be adapted to multiplexing with other testing (such as SCID) in order to minimize costs and workflow. Several studies already shown that qPCR is the ideal candidate for the job (9, 91, 142, 148, 149, 151) so we choose to test this tecnhique. The designed and tested protocol was based on the one published by Mei Baker and collaborators in 2021 (151). Since this assay does not detect pathogenic

variants in other *SMN1* regions nor in different chromosomes, we estimate a final sensitivity of approximately 95%. For this reason, every baby that presents delayed motor development and/or SMA indicating symptoms should be evaluated by a specialist, even if they had a previous negative SMA NBS result.

SMN2 blocker is appearing in a crescent number of NBS qPCR protocols for detection of the homozygous deletion of *SMN1* exon 7 (151, 153). To see if there is a real need to use this type of blocker and its impact on the assay, we have made two tests: with and without the *SMN2* blocker incorporated in the qPCR reaction mixture. Standard deviation values increased when no blocker was added. These results suggest that the *SMN1* probe struggle to find the *SMN1* exon 7 given its similarity with *SMN2*. On the other hand, the results of the samples with the *SMN2* blocker are more robust advocating that the blocker does prevent the annealing of the *SMN1* probe with the *SMN2* sequence thus reducing the off-target probability (153).

Since the aim of the test is to detect an absence of a specific DNA region, contaminations are a major concern. Automated punching is an indispensable tool in NBS has it reduces costs and precious labor time. Czibere and his team showed that automated punching is a key source of contaminations and that these events are quite common (91). Despite this, there isn't any other method that can compete with automated punching. Incorrect pipetting during DNA extraction or qPCR setup and incomplete sealing of the qPCR plate are also steps where contamination can occur and lead to inconclusive/false negative results (91).

Georgia's NBS program reported 8 false positives results, being 6 of those samples from babies admitted in the neonatal intensive care unit (NICU) (152). In the NICU babies are often burdened with drugs and have several analytical alterations in the blood. Also, these babies' blood is mainly collected from a line rather than the traditional direct heel stick and that can bring PCR inhibitors to the test (that, at the limit, may affect the detection of *RPP30* and *SMN1* differentially) so, these samples should be carefully evaluated (157). Rare blood genetic disorders, such as Shwachman-Diamond Syndrome (that affects bone marrow and white blood cells production), could also be a source of false positives for these conditions often alter the amount and quality of the DNA extracted (143). In a Taiwan trial, an assay for NBS of SMA with the probe targeting the c.888 + 100A>C site was described. They had eight false positive results and had to implement a second-tier test to correctly detect SMA patients. Five of these specimens had hybrid *SMN1* thus their assay did not recognize them has negative results (9). We used a probe that targets the c.840C site of the *SMN1* instead because this is where the C>T transition of the conversion of *SMN1* into *SMN2* occurs. This allows the correct differentiation between *SMN1* and *SMN2*. We also used a *SMN1* probe with LNA at this region to lower the probability of off-target (Table 3). The *SMN2* blocker was also made

with LNA to increase the discrimination between the *SMN1* and *SMN2* loci (Table 3). To rise even more the sensitivity of the assay, *SMN1* and *RPP30* probes with a dark quencher at 3' were used (Table 3). Dark quenchers absorb the energy emitted by the fluorophore when they are near to each other not allowing the signal to spread. When the Taq polymerase reaches the zone where the probe is anchored (final of intron 7 and start of the exon 7 comprising the C6T transition region in our case) the probe breaks, thus allowing the separation of the fluorophore from the quencher and the emission of the signal. Therefore, when we see an amplification signal, we are positively sure that it corresponds to the *SMN1* with its exon 7 incorporated and we have a true negative result. Despite targeting the exon 7 region, the New York NBS had an inconclusive result. The specimen had a rare heterozygous variant on the exon 7 region (c.842 G>C) that resulted in partial allelic dropout (154). Variants in the region where primers or probes bind will hinder them from anchor thus increasing *SMN1* Cq and creating inconclusive results. These variants can even be an unsurpassable obstacle therefore preventing the annealing and turning the sample into a false negative.

For ethical reasons, carrier detection should be avoided in NBS (135, 140, 141). When combined with the definition of positive (absence of the *SMN1* exon 7) or negative result, our assay does not identify carriers. Sample H1 (simulated carrier) had the highest *SMN1* Cq (30,35). With this in mind, we have set the cut-off for *SMN1* at Cq ≥ 31. Samples with this characteristic should be considered inconclusive and re-tested with new DNA extraction to avoid false negatives (Figure 13) (152). If the new test does come back again with a Cq ≥ 31 and with a validated *RPP30* Cq, it is not consensual what to report, therefore we opted to consider it a positive result for the exon *SMN1* exon 7 deletion. Retesting all samples with a Cq ≥ 31 will allow to minimize the risk of false negatives due to contamination during punching/DNA extraction, since in these cases, it is expected to *SMN1* Cq to be over 31. For the *RPP30* we suggest a final cut-off lower then 26,8 since we expect to have 99,5% of samples under this value. Every sample with higher values should be considered invalid and should also be re-tested, regardless of the *SMN1* Cq value (Figure 13). If the new test comes with the same Cq values, a new sample must be requested. These values are in range with other NBS cut-offs (151–153). If the *RPP30* is validated and there is no *SMN1* Cq, the specimen should also be re-tested for confirmation. If the re-test comes back validated for *RPP30* and with no *SMN1* Cq the sample can be considered positive for the NBS of SMA. These results should be reported as soon as possible to the primary health provider (PHP) and/or referred to a specialist (Figure 13). This flowchart and conservative cut-off values are an initial proposal and should be adjusted during the course of a pilot study.

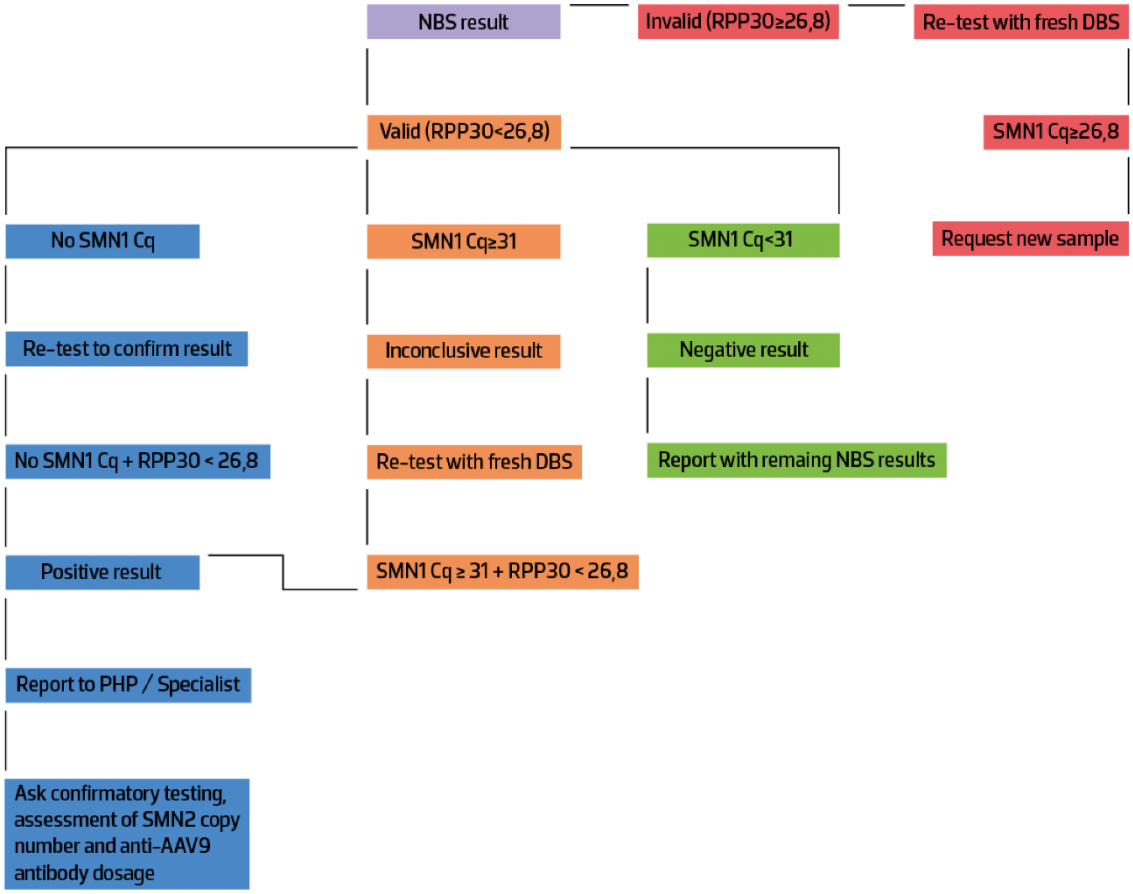

Figure 13 – Suggested SMA NBS flowchart, referral to specialist and confirmatory testing.

Digital PCR and Multiplex Ligation-dependent Probe Amplification have been used in other countries to confirm inconclusive results and/or determine *SMN2* copy number (9, 37, 142, 155) but, because of the severity of the disease and the expensive treatment, it is mandatory for NBS positive results to be subjected to confirmatory testing (133). In these cases, the test of a fresh blood sample is used to confirm the NBS result and, at the same time, determine *SMN2* copy number (Figure 13). It has also been found that NBS assessment of *SMN2* copy number has not been the most reliable (158). Thus, in our opinion, for the time being, the determination of *SMN2* copy number should be done in a second phase, during diagnostic confirmation, and not alongside with NBS first tier. To reduce even more the time between diagnosis and treatment, anti-AAV9 antibody dosage should be done simultaneously with the confirmatory testing to see if the patient is eligible for the gene therapy Zolgensma® (Figure 13) (143, 152).

As showed in the introduction, *SMN2* copy number is a phenotypic modifier in SMA and this test is used to decide if asymptomatic babies should start treatment right after the confirmation of the NBS positive result or if they should wait, with very close monitoring, until there are any signs of symptoms (91, 140, 151, 154, 155, 159). In a recent study, one baby was misdiagnosed with 4 *SMN2*

copies in the confirmatory testing and started to have symptoms at the age of 8 months (159). Since SMA therapies are more effective in the asymptomatic phase (51, 120, 129, 131, 132, 159), some authors advise that babies should receive treatment at the earliest possibility even if they have 4 *SMN2* copies or more. In fact, recently revised guidelines state that patients with 4 *SMN2* copies should start treatment promptly after the diagnosis confirmation (158). Also, if an abnormal confirmatory test arises, the baby should be referred to a specialist with the intent to undergo differential diagnostic and other clinical testing such as CMAP and/or electromyography to clarify the result (142, 155).

5. Conclusion

NBS is the best way to ensure that therapy is given in the asymptomatic phase. We have successfully implemented an *in house* qPCR assay that screens for the absence of *SMN1* exon 7 in DBS for NBS purposes at lower costs than the available commercial solutions. It allows clear identification of the *SMN1* homozygous deletion, without differentiating heterozygotes from normal samples, being adapted to high throughput. qPCR is, actually, the best molecular technique to use in the NBS for this purpose, since it is simple, cheap and reliable.

As a future perspective, it would be interesting to evaluate if, by adding probes, it could be possible to detect the second most common variant in the Portuguese population, therefore increasing SMA NBS sensitivity. This assay has also the potential to be multiplexed with other genetic conditions in the future (namely SCID screening) so costs can be kept down. NBS of SMA can and should be implemented in every country that have access to prompt treatment.

6. References

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