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Spinal Muscular Atrophy: Classification, Diagnosis, Background, Molecular Mechanism and Development of Therapeutics

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1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease and one of the most common genetic causes of infant death. The loss or mutation of the SMN1 gene results in reduced SMN protein level leading to motor neuron death and progressive muscle atrophy. Although recent progress has been made in our understanding of the molecular mechanisms underlying the pathogenesis of the disease, there is currently no cure for SMA. In this review, we summarize the clinical manifestations, molecular pathogenesis, diagnostic strategy and development of therapeutic regimes for the better understanding and treatment of SMA.

2. Epidemiology

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by the loss of motor neurons from the anterior horn of the spinal cord which leads to muscle weakness, hypotonia and ultimately muscle atrophy [1]. With a pan ethnic incidence of 1:11,000 live births and a carrier frequency of 1:50, SMA is one of the leading genetic causes of infant death globally [1-5].

3. Clinical classification

Due to the range of clinical severity, SMA is broadly classified into four major categories characterized by the age of onset as well as severity of the disease [6-9]. SMA type I, which was originally described by Werdnig and Hoffmann in the late 18th century is the most severe and prevalent form of the disease and accounts for more than 50% of the known diagnosed cases of SMA. Type I SMA presents within the first six months after birth and although historically patients succumbed within the first 2 years of life, with better ventilatory and nutritional support, the life expectancy of children with type I SMA can be increased beyond the 5th birthday. Infants with type I SMA experience a rapid loss of skeletal muscle mass with profound hypotonia and general muscle weakness characterized by poor head control, difficulty with suckling, swallowing and an inability to sit without support. These children develop problems with breathing over time due to impaired bulbar function and respiratory muscle weakness leading to respiratory insufficiency. Respiratory failure due to aspiration pneumonia is an important cause of SMA mortality [6, 10, 11]. The intermediate form of SMA, known as type II, has an onset between 6 and 18 months of age. Patients with type II SMA can sit unaided but still develop progressive muscle weakness and can never stand or walk on their own. Other symptoms and physical signs include respiratory insufficiency due to reduced bulbar function, poor weight gain, fine hand tremors and joint contractures [6]. SMA type III has an onset between 18 months to 30 years of age. Patients are able to stand and walk unaided, however they develop variable degree of muscle weakness which leads to a broad spectrum of physical signs and symptoms. While most walk independently, some lose ambulation during early adulthood and require wheelchair assistance. Others develop cramps and joint overuse problems; some develop scoliosis [6, 12, 13]. Type IV SMA is the mildest form of the condition and is characterized by adult onset with normal mobility. They have mild muscle weakness in adulthood with normal longevity [6] (Table 1).

SMA Type	Other Names	Age of Onset	Life Span	Highest Motor Activity
Type I years (Severe)	Werdnig- Hoffmann disease	0-6 months	2-5	Never sit
Type II (Intermediate)	SMA, Dubowitz type	7-18 months	>2 years	Sit, Never stand
Type III (Mild)	Kugelberg- Welander disease	>18 months	Adult	Stand and walk (may require assistance)
Type IV (Adult)	-------------	Adulthood	Normal	Walk during adulthood-unassisted (some muscle weakness)

Table 1. Classification of SMA disease

4. Diagnosis and treatment

The diagnosis of SMA is made by a thorough patient history and physical examination followed by genetic testing. The survival of motor neuron (SMN) -1 genotyping has to a large degree replaced electromyography (EMG) and muscle biopsies (Fig 1) [2, 14]. There is in 2012 no cure for SMA; current treatment is symptomatic and supportive. This includes clinical management through family education and counselling along with attention to pulmonary, gastrointestinal/nutrition and orthopedics/rehabilitation in an effort to managing symptoms of the patients [9].
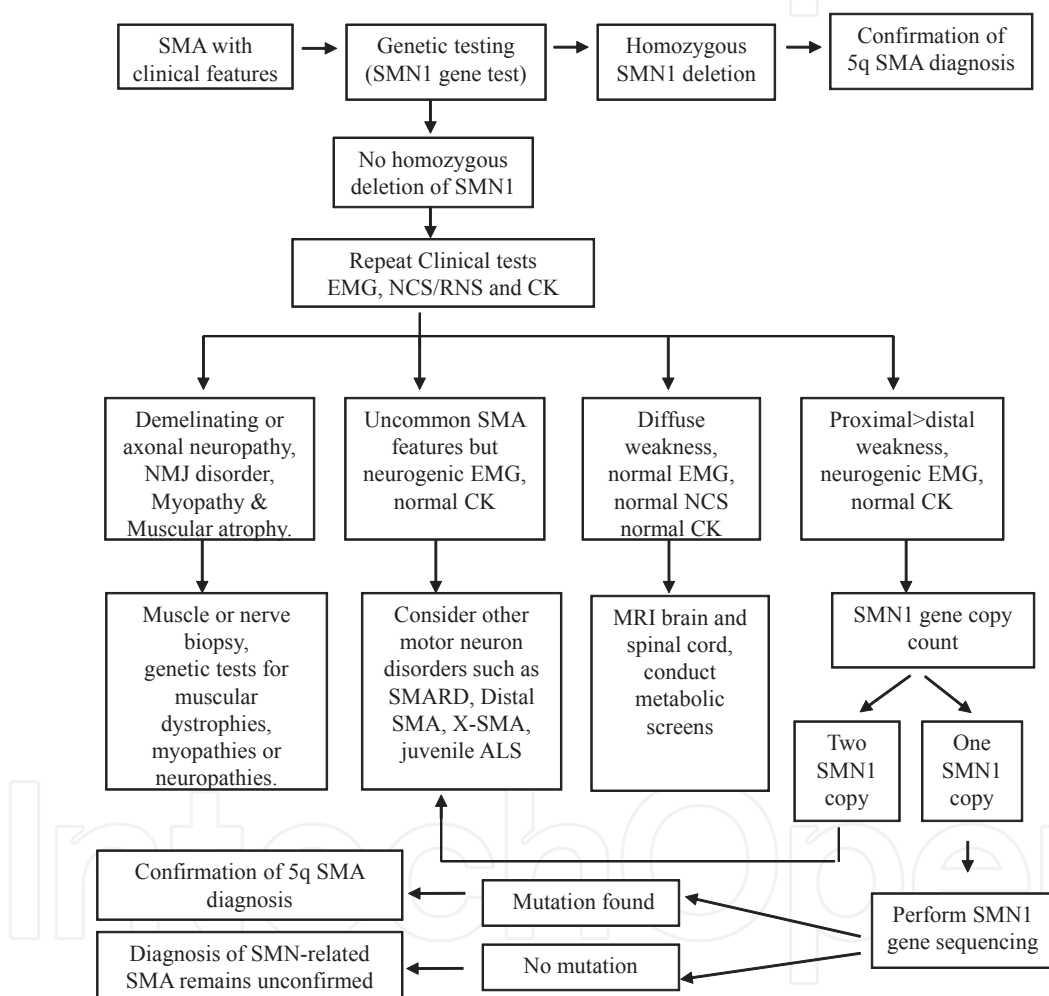

Figure 1. SMA diagnosis

5. Genetics of the disease

The SMA disease causing *SMN1* gene maps to a complex genomic region of chromosome 5q13.1. This region is characterized by an inverted duplication of the element with 4 genes

(*SMN*, neuronal apoptosis inhibitor protein *{NAIP}*, *SERF and GTFH2)* present in telomeric and centromeric copies (Fig 2a) [15, 16]. In 1995, it was reported that homozygous deletions of the *SMN1* gene were observed in and thus likely the cause of 95% of SMA patients [15]. All SMA patients have one or more copies of a nearly identical gene, *SMN2*. These two genes are distinguished by five nucleotide changes in exon 7 and 8. The critical nucleotide difference which makes *SMN2* only partially functional is a C to T transition at position 6 of exon 7. This change leads to the exclusion of exon 7 in the majority of transcripts. This mRNA is subsequently translated to form an unstable truncated non-oligomerizing isoform of SMN protein. However, *SMN2* gene still produces 5-10% functional full length SMN transcripts (Fig 1.2b) [15, 17, 18]. The *SMN2* gene is present in variable copy numbers in the population; all SMA patients have one or more copy of the *SMN2* gene which, due to its partial functionality, acts as a positive disease modifier. There is thus an inverse correlation between the number of *SMN2* gene (which can produce between 10-50% of SMN protein depending on copy number) and the severity of the disease [2]. Low levels of SMN protein allows embryonic development but is not enough, in the long term, to allow motor neurons to survive in the spinal cord [19, 20]. Type I patients usually have 2 copies whereas Type II have 3 copies of *SMN2*. Type III and IV have 3-4 copies of the *SMN2* gene. Individuals with 5 or more copies of the *SMN2* gene, despite having no functional *SMN1* gene are completely asymptomatic and are protected against the disease manifestation.
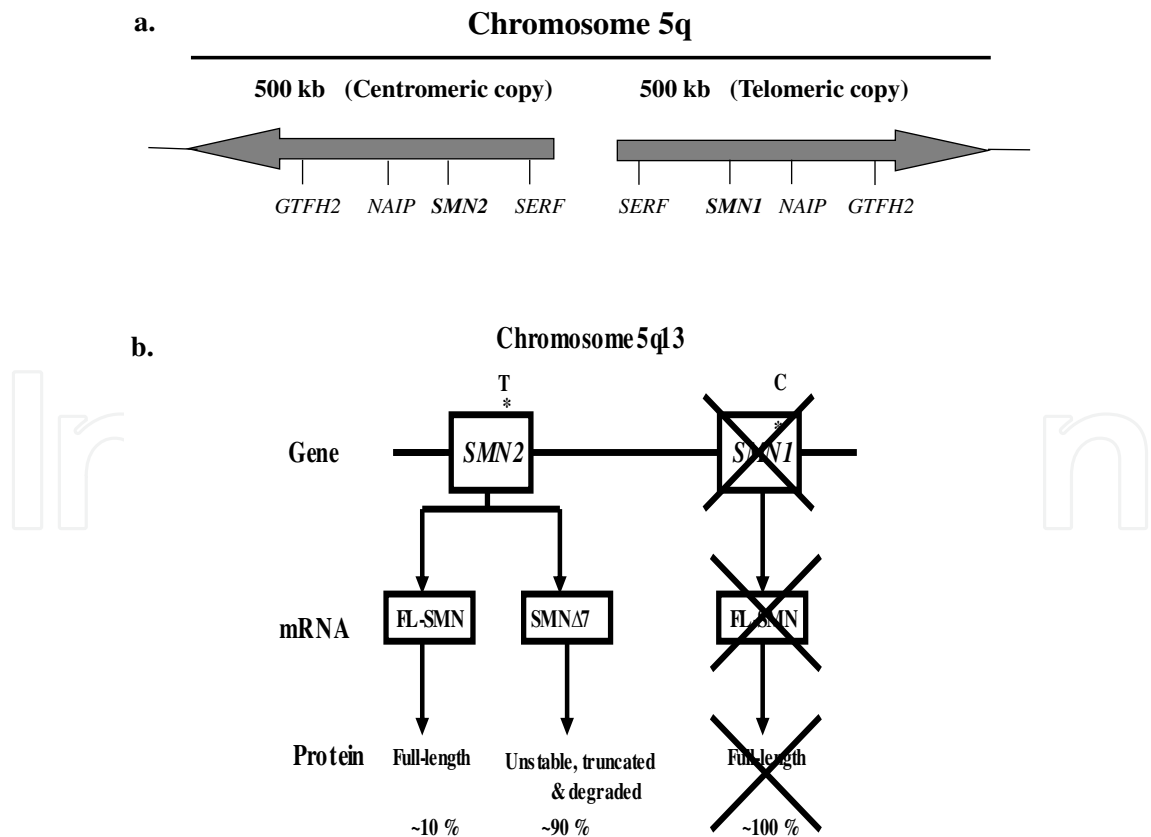

Figure 2. (a) Human SMN locus and (b) genetics of SMA patients

6. Pathology

The pathological hallmark of all forms of SMA is the loss of motor neurons from the lower brainstem and the anterior horn of the spinal cord [21]. Anterograde axonal degeneration results in denervation of the myocytes within the motor unit. This sometimes leads to reinnervation of muscle, where adjacent uninjured motor neurons sprout leads to fiber type grouping of myocytes. Histopathologic assessment of SMA muscle tissues reveals a large number of rounded atrophic fibers resulting from denervation. The widely held notion had been that SMA is primarily a neuronopathy (involving the cell body) with secondary degeneration of the axons. However, more recent observations in the field have shifted the focus of SMA pathology from the motor neuron cell body to the distal axon [22, 23] and the possibility of a synaptopathic defect [20, 24]. Specifically it has been suggested that the presynaptic transcriptome may be in some manner dysregulated; the direct inference is that SMN plays a role in the peripheral transport of critical mRNA, among which is that species encoding beta-actin. Regardless of the subcellular location of SMN mediated pathology, SMA is primarily considered as a motor neuron disease and consequently treatment strategies focus on drugs which can cross the blood brain barrier (BBB) to target the central nervous system (CNS). However, motor neuron autonomy of SMA pathogenesis has recently been called into question as multi-system involvement (including cardiovascular, peripheral necrosis and liver defects) have been reported recently in both SMA patients and SMA mice models [25-33]. In addition, one report has outlined the superiority of systemic SMN antisense oligonucleotide (ASO) therapy compared with intrathecal delivery in severe murine SMA calling into question the exclusive role of the motor neuron in disease causation [33].

7. Function of the SMN protein

SMN is a 294 amino acid long ubiquitously expressed protein with a molecular weight of 38 kilodaltons (kD). SMN is found in both the nucleus and cytoplasm. Within the nucleus, it is localized both throughout the nucleoplasm as well as in nuclear structures called Gems and Cajal bodies [34]. It is also found in abundance within the growth cones of the motor neurons [35]. SMN has been implicated in ribonucleoprotein biogenesis (e.g. assembly, metabolism and transport of various ribonucleoproteins), as well as playing a major role in the splicing machinery. It is part of a multiprotein complex comprised of Gemins [2-8], spliceosomal U-snRNPs, Sm proteins and profilins called the SMN complex. This complex is essential for the biogenesis of snRNPs [36-45]. Given the variety of roles that SMN has been implicated in, not surprisingly, the complete absence of SMN genes is embryonically lethal in virtually all metazoan life forms tested, indeed even cell cultures cannot survive without SMN [19, 20, 46].

8. Molecular mechanism: splicing defect in SMA

Splicing is mediated by a complex called the spliceosome, the activity of which depends on a number of factors. In particular, various cis- and trans-acting elements regulate the splicing of

both *SMN1* and *SMN2*. The C-T transition at position 6 of exon 7 in *SMN2* gene disrupts the function of an exonic splice enhancer (ESE; recognized by SF2/ASF to promote exon 7 inclusion) and/or creates an exonic splice suppressor (ESS; recognized by hnRNP A1/A2) which results in exon 7 skipping (Fig 3) [47-53].
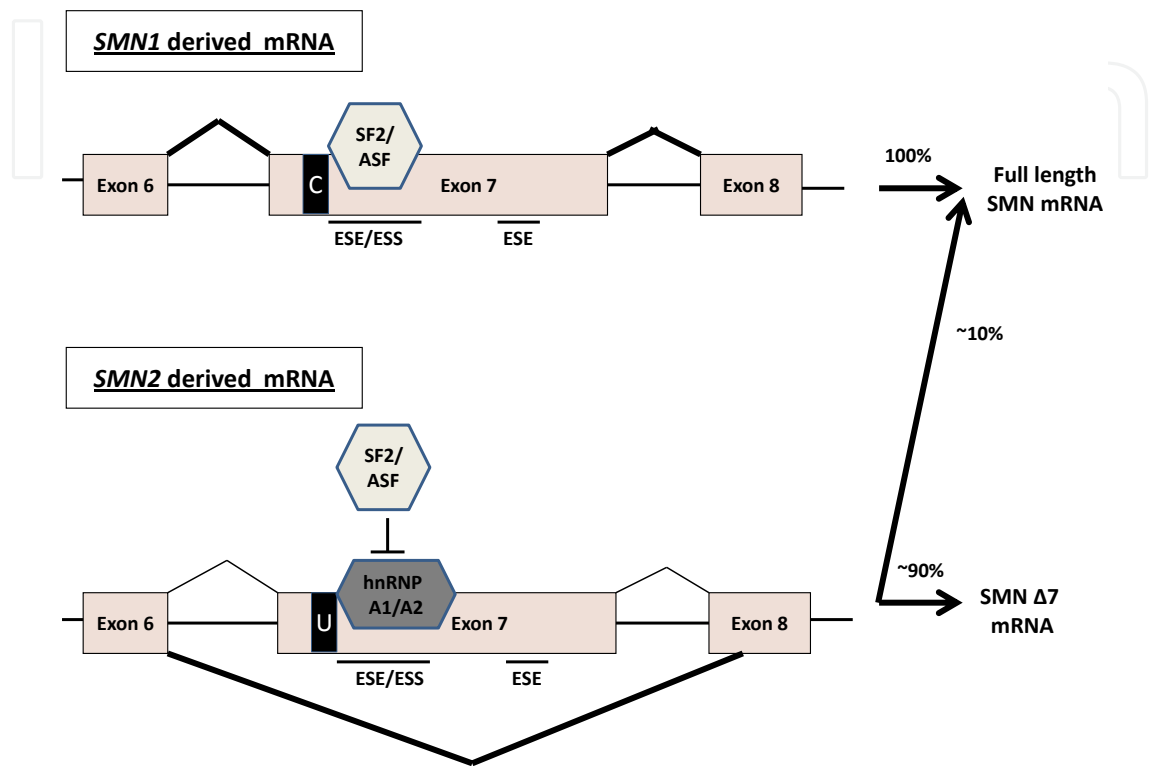

Figure 3. Splicing in SMA

9. Therapeutic strategies

Although there is no cure for SMA, the *SMN2* gene locus serves as a target for SMA treatment. The general treatment strategies for SMA are to compensate fully or in part for the absence of *SMN1* gene by increasing the levels of functional SMN protein levels though three distinct approaches: i) to induce the expression of *SMN2*, ii) to modulate splicing of *SMN2* transcript, and iii) to stabilize the full length SMN mRNA and/or protein. In addition, gene and stem cell therapies are also under development for the treatment of SMA. These and other strategies are discussed below.

1. **SMN dependent therapies**: As outlined above, there is an inverse correlation between the *SMN2* gene copy number and disease severity [54, 55] which implies that directly targeting the *SMN2* gene in SMA patients through different pathways could be one key for the development of a SMA drug treatment. Alternatively, SMN protein can also be produced through gene replacement therapy.

a. **Activation of *SMN2* promoter**: Histone deacetylases (HDACs) repress transcription of genes including *SMN2* by chromatin condensation. Thus, HDAC inhibitors can increase transcription of the *SMN2* gene and can produce more full length SMN transcripts and protein which may have a beneficial effect in patients. Various HDAC inhibitors have been analyzed in cell culture, mouse models and in clinical trials as potential therapeutic for SMA. Sodium butyrate, Valproic acid (VPA) and phenylbutyrate showed promise in cell culture and mouse models and were also well tolerated by the patients [56-63]. However, no clinical improvement was observed in SMA patients with HDAC inhibitors [61-63].

Recent studies with other HDAC inhibitors, LBH589, Trichostatin A (TSA) and Suberoylanilide hydroxamic acid (SAHA) showed *SMN2* gene induction in culture as well as in a number of animal models of neurodegeneration [62, 64-66]. In addition to these compounds, we have shown that the lactation hormone prolactin (PRL) which can both cross the blood brain barrier and, through binding to its receptor, activate the JAK2/STAT5 pathway also upregulates *SMN2* gene transcription [68]. Interestingly the degree of induction in SMN seen with the prolactin in the genetically engineered Δ7 SMA mouse model (where *SMN2* gene is the only source of SMN protein) is significantly greater than that seen in cell culture and wild type mice. We have determined that this is because of the difference between the promoter regions in *SMN1* and *SMN2* genes, the latter uniquely having STAT5a transcription binding motifs. This might prove beneficial as all SMA patients have *SMN2* as the only source of SMN protein. Since PRL has been successfully tested and proven safe in humans for the treatment of lactation deficient mothers [67], it may bypass other compounds which are yet to be tested for clinical safety and join the short list of drugs which may have immediate potential SMA therapeutic potential [68].

b. **Correction of splicing**: The suppression of exon 7 skipping to produce more full length transcript from the *SMN2* gene is another treatment strategy being explored for SMA. HDAC inhibitors such as VPA, TSA and sodium butyrate appear to have a dual effect on SMN mRNA expression; they not only open chromatin structure and therefore increase the rate of transcription but also appear to affect the splicing process [56-58, 64]. The antibiotic aclarubicin has been shown to increase full length SMN transcript by altering the splicing process *in vitro* [69]. The most promising compounds which correct splicing by preventing *SMN2* exon 7 skipping are antisense oligos (ASOs). An ASO complementary to *SMN2* exon 7 pre-mRNA sequences has been shown to inhibit binding of negative splicing factors and increase full length SMN transcript and protein production [30, 33, 70, 71]. The major hurdle in using ASOs for SMA therapeutics, however, is their inability to cross the blood brain barrier. However, Hua et al. 2011 documented a marked improvement in motor function along with an increase in survival in SMA mice with systemic delivery of ASO which results into increase in SMN levels mostly in peripheral tissues especially in liver. Interestingly, they documented only a slight increase in SMN levels in CNS tissues [33]. However, there are a number of issues which need to be addressed before clinical introduction of ASOs for SMA treatment (clinical safety, quantity of ASO, cost, immune response etc) [72].

c.   **Full length SMN transcript stabilization**: In this relatively new approach by by Singh *et al.*, decapping enzyme DcpS, an integral part of the RNA degradation machinery, was targeted by C5-substituted quinazolines which interact and open the enzyme into a catalytically inactivated conformation. Full length SMN mRNA decay is in this fashion blocked, ultimately increasing SMN protein in cell culture [73].

In a different approach, SMN mRNA has been shown to have a specific AU rich element (ARE) region in its 3' UTR which marks the mRNA for degradation. Our laboratory has shown that activation of the p38 pathway results in the accumulation of RNA binding protein HuR in the cytoplasm which then binds to the ARE in 3'UTR region of SMN mRNA and stabilizes the transcript. Importantly, transcript stabilization is not associated with any discernible inhibition of SMN protein translation. This study provided a novel mechanism through which SMN mRNA could be stabilized using p38 activating compounds which can cross the blood brain barrier to develop new therapeutics for SMA treatment [74].

d.   **Full length SMN protein stabilization**: Aminoglycosides are class of antibiotics which have been shown to mask premature stop codon mutations in some genes, allowing read through translation to occur. This moderates translation termination through an alteration in the conformation of the ribosomal reading site. Various aminoglycosides including tobramycin and amikacin have been used successfully in patient fibroblasts to increase SMN protein levels. However, their *in vivo* efficacy and safety has yet to be demonstrated [75-77].

An alternative potential therapeutic approach involves targeting the ubiquitin-proteasome pathway which mediates intracellular protein turnover. Proteins are marked with poly ubiquitin (Ub) molecules by the action of the enzymes E1 (Ub activating enzyme), E2 (Ub conjugating enzyme) and E3 (Ub ligase). The polyubiquitin modification marks the protein for destruction by the proteasome complex. SMN is one of the many proteins degraded by the ubiquitin proteasome pathway. It has been shown that FDA approved proteasome inhibitor bortezomib increases SMN both in vitro and in vivo by blocking proteolysis of SMN protein. However, it should be noted that bortezomib cannot cross the BBB; thus, it must be used in combination with other drugs which can cross the BBB for the treatment of SMA [78].

e.   **Gene therapy**: One of the most encouraging SMA therapeutic advances is the use of gene therapy which shows significant promise. In the last three years several groups have used self complementary adeno-associated virus (scAAV) 8 and 9 vectors carrying the *SMN1* cDNA to treat mice models of SMA, resulting in the most dramatic extension in the life span of mice yet observed combined with an overall amelioration of disease phenotype [79-82]. However, early pre-symptomatic intervention is necessary for the success of this therapy as is seen with other treatment strategies as well. Moreover, several challenges must be addressed for this mode of SMA treatment before bringing it to clinical application successfully. The most pressing issues are clinical safety, dealing with the cross-species barriers, the cost of virus production along with the possibility of an immune response to AAV which can neutralize its impact [83].

2. **SMN-independent strategies**: There have been some recent advances in SMN-independent strategies for the treatment of SMA. These include:

a. **Stem cell therapy**: Stem cell therapy has generated much attention as a treatment for motor neuron diseases, including SMA, through replacement of the lost motor neurons and, more realistically perhaps, supporting the existing neuron population. Primary murine neuronal stem cells as well as embryonic stem cell-derived neural stem cells injected into the spinal cord of animal models of SMA have been shown to ameliorate disease phenotype and increase survival [84, 85]. It is unclear whether this is through motor neuron and other cell replacement and/or through neuroprotection of host motor neurons by the numerous factors released from the donor cells. Although induced pluripotent stem (iPS) cells from an SMA patient have been differentiated into motor neurons [86, 87], there are several obstacles which hinder their use as a therapeutic for SMA treatment. These challenges include the production of the large number of stem cells and their successful transplantation into the patients, which could populate and cover the entire nervous system. Also, lentivirus vectors are used to deliver the cocktail of factors, required to produce iPS cells *in vitro*; these would be unsuitable for use in patients as they have the potential for insertional mutagenesis which could result into oncogenesis. Finally, even if motor neurons could develop *in situ*, the prospect that they would at a meaningful level connect with the host CNS must be viewed as highly unlikely at this time.

b. **Modifying neuromuscular junctions through actin dynamics**: The pharmacological Rho-kinase inhibitor (downstream effector of RhoA-GTP which plays role in actin dynamics) dramatically increases the life span of a mild SMA mouse model and improves disease phenotype. This improvement in the disease phenotype is independent of SMN increase, mainly through making neuromuscular junctions (NMJ) better, larger and more mature [88]. This suggests that there are novel SMN independent avenues for the development of therapeutics for SMA.

10. Future directions

Combination therapy: The impressive results seen so far with gene therapy and ASO's in the field of SMA will be difficult to equal with a monotherapy approach. However, unless and until gene therapy and ASO treatments are cleared for clinical safety as a therapeutic option for SMA treatment, combinatorial approaches for SMA shall likely be necessary to target not only CNS but also other tissues which are affected because of a lack of SMN. As outlined above, SMA can be targeted through different approaches, we can in a safe combination use compounds which are already FDA approved and can increase SMN levels through *SMN2* gene activation (such as PRL) along with *SMN2* transcript stabilizers (p38 pathway activators such as celecoxib) and/or SMN protein stabilizer (proteasome inhibitor bortezomib) [18] and/or neuroprotective compounds (Rho kinase inhibitor) [19], or a cocktail of the best suitable combination of these compounds (Fig 5.1). I believe that this approach will speed up the
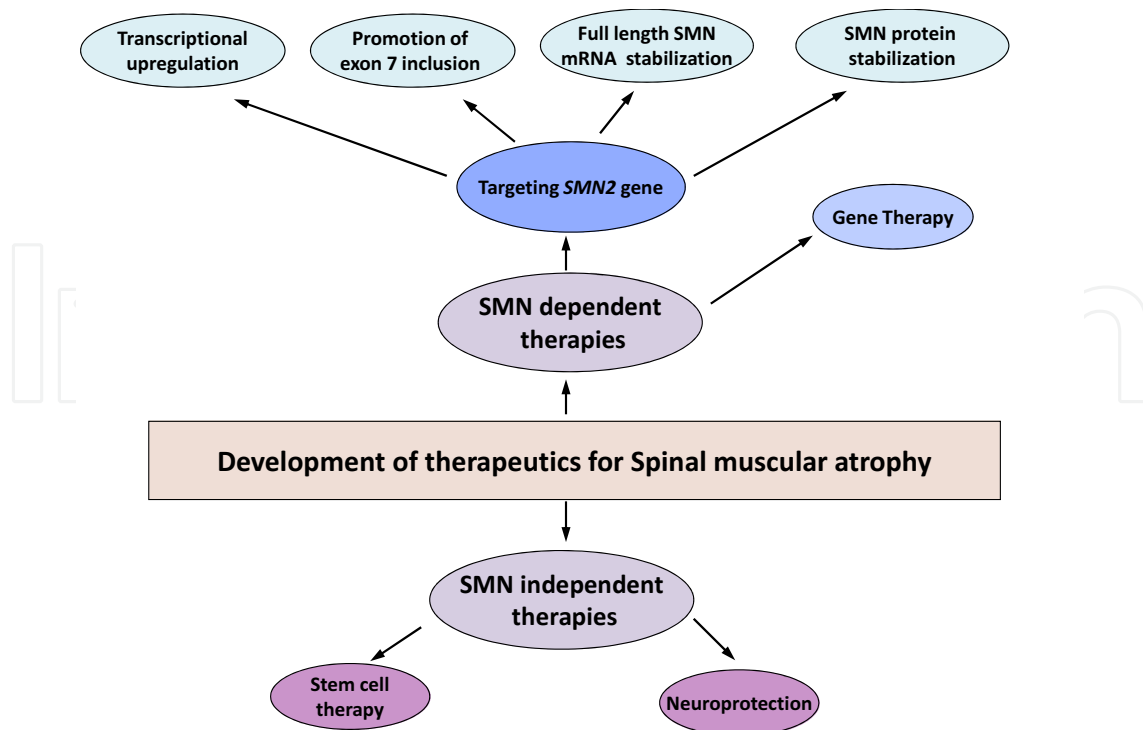
Figure 4. Development of therapeutics for SMA

process of finding the best possible and safest treatment of SMA. This approach is currently being assayed in our laboratory and others, showing some positive and promising results in the severe mouse model of the disease. More work is required to assess the potential drug interactions and their side effects in the animal models of the disease before pushing this approach for human clinical trials.

Designing clinical trials for SMA: In the last 5 years, a tremendous amount of promising translational work has been done using animal models of the SMA which is progressing rapidly towards the pre-clinical stage. However there are major challenges for designing a perfect clinical trial for SMA which includes 1) Variability of the disease phenotype, 2) lack of molecular biomarkers, 3) Accessibility of treatment centers and 4) lack of agreement for standard of care and disease management. However these issues are likely to be resolved as recently there has been a remarkable cooperation and collaboration between researchers, clinicians, industry, government and volunteer organizations which is bringing everyone on the same page to address these issues and reach a consensus for designing standard human clinical trials for SMA internationally.

Early intervention: New born screening: We and others have seen, irrespective of the modality, that early timing of the treatment is critical for maximum benefit in the mouse model of the disease. Presymptomatic identification of infants with SMA through new-born screening represents an important step in the effective treatment of SMA. In essence we shall need to intervene before the damage is done; to do so we need to rapidly identify infants with SMA, cases who will also serve as the best candidates to show the efficacy of promising therapeutic

treatments in the near future. Children in which the disease has already progressed may also benefit with the use of best combinational approach, however the aim will be more towards ameliorating the disease progress and preserving the function of remaining motor neurons and other tissues rather than a complete reversal of the disease phenotype.
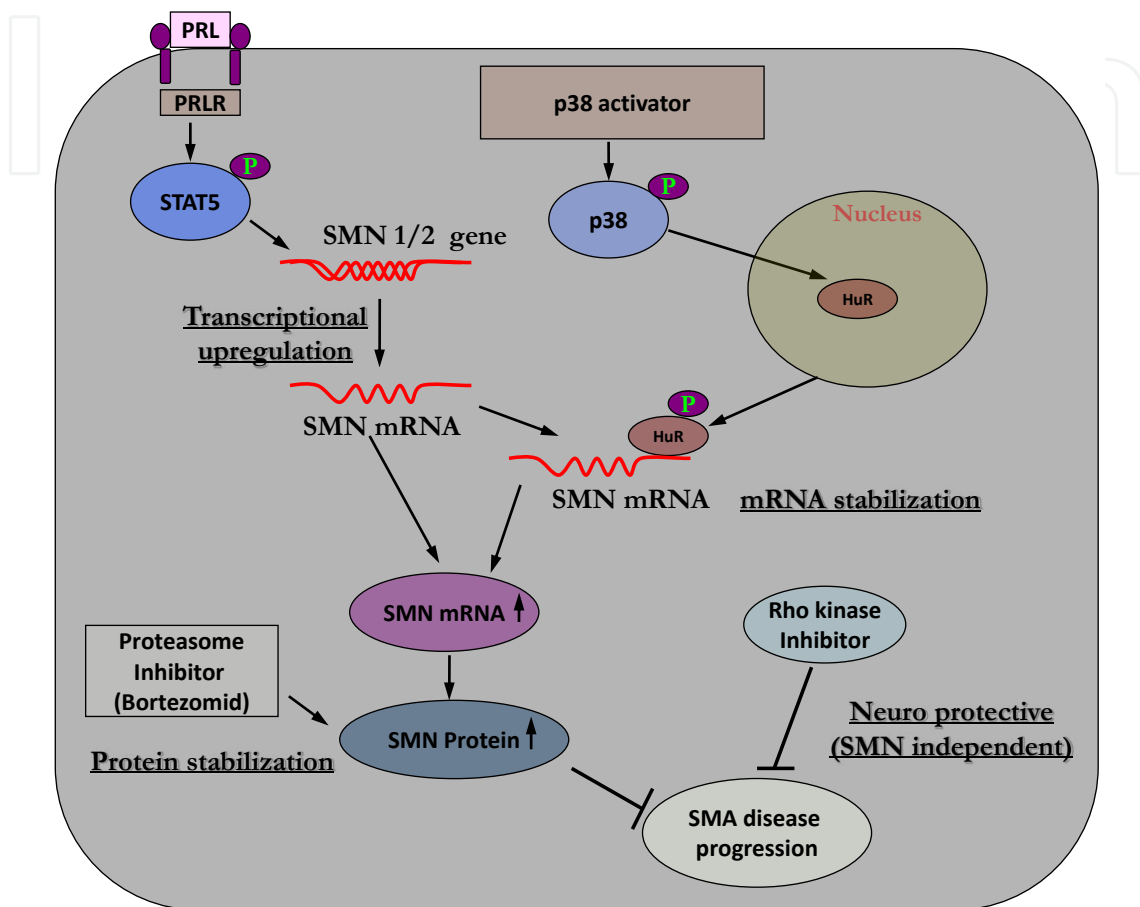

Figure 5. Proposed model of combination therapy for SMA treatment.

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