Identifying epigenetic regulators of survival motor neuron protein to treat spinal muscular atrophy

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Abstract

Spinal muscular atrophy (SMA) is a rare monogenic disease of mostly paediatric onset, affecting approximately 1:8,000 – 10,000 live births. If untreated, it leads to progressive muscle weakness and paralysis, due to degeneration of lower α-motor neurons in the spinal cord and brainstem. Three therapies are currently approved for SMA and while effective, they are extremely costly and not without limitations. It is becoming apparent that with SMA individuals living longer, new therapies, alone and/or in combination with the ones already on the market, are needed to improve quality of life and ensure sustained benefit for patients.

Epigenetic mechanisms have been implicated in several neuroprotection and neurodegeneration processes as well as SMA itself. The aim of this project is to identify novel epigenetic mechanisms involved in the regulation of SMN with the ultimate goal to identify new therapeutic targets. To this end, I have screened a library of epigenetic probes from the Structural Genomic Consortium, for their ability to modulate *SMN2* in SMA patient-derived fibroblasts. Lead molecules have been further validated in the SMA mouse model. Among these molecules, I have identified MS023, an inhibitor of type I protein arginine methyltransferases (PRMTs), and IOX2, an inhibitor of prolyl hydroxylase domain 2 (PHD2), and characterized MS023 in depth. MS023 is able to increase the levels of SMN protein *in vitro* and *in vivo, via* a splice switching mechanism and improve survival and weights in a severe mouse model of SMA. Furthermore, the combinatorial treatment of oral MS023 and the market-approved antisense oligonucleotide nusinersen, is able to prolong the lifespan of severe SMA mice to a greater extent than the oligonucleotide treatment alone. MS023 alters the methylation pattern of hnRNPA1, the main negative regulator of exon 7 inclusion, weakening its binding to *SMN2* pre-

mRNA. Transcriptomic analysis in spinal cords of SMA mice identified correction of immune-related pathways by the combinatorial treatment but not nusinersen alone. These results provide a new insight into epigenetic regulation of SMN and PRMT function in splicing and justify moving forward with MS023 preclinical development as an add-on therapy for SMA.

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List of abbreviations

3’UTR – 3’ untranslated region
4HQ – 4-Hydroxy-2-oxo-1,2-dihydroquinoline glycinamide
5’UTR – 5′ untranslated region
AD – Alzheimer’s disease
ADMA – asymmetric dimethylation
AdOx – adenosine dialdehyde
ALS – amyotrophic lateral sclerosis
AMLK – acute megakaryoblastic leukemia
AR – androgen receptor
Arc – activity regulated cytoskeleton associated protein
ARHGAP22 – Rho GTPase activating protein 22
Asns – asparagine synthetase
ASO – antisense oligonucleotide
BRSK2 – BR serine/threonine kinase 2
C9orf72 – chromosome 9 open reading frame 72
CACNA1H – calcium channel, voltage-dependent, T type, alpha 1H subunit
CARM1 – coactivator associated arginine methyltransferase 1
CBs – Cajal bodies
cDNA – complementary DNA
CHD2 – chromodomain helicase DNA binding protein 2
CHML – CHM Like Rab Escort Protein
CKD – chronic kidney disease
CLIP – crosslinking and immunoprecipitation (CLIP)
CNS – central nervous system
CPG15 – candidate plasticity-related gene 15
CSF – cerebrospinal fluid
CTD – C terminal domain
DDX – DEAD-box helicase 1
DLBCL – diffuse large B-cell Lymphoma
DMA – dimethylation
DMOG – dimethyloxalylglycine
DMSO – dimethyl sulfoxide
DNMT – DNA methyltransferases
DOT1L – DOT1-like histone H3K79 methyltransferase
DRG – the dorsal root ganglia
EGLN – egl-9 family hypoxia inducible factor
EMA – European Medicines Agency
EPO – erythropoietin
ESA – erythropoiesis-stimulating agent
ESS – exonic splicing silencer
FALS – familiar amyotrophic lateral sclerosis
FDA – Food and Drug Administration
FGFR3 – fibroblast growth factor receptor 3
FIH – factor-inhibiting HIF
FL SMN2 – full length survival motor neuron 2 transcript
FMRP – fragile X messenger ribonucleoprotein 1
FN3K – fructosamine 3 kinase

FOXM1 – forkhead box protein M1
FOXO3a – forkhead box O3
FUS – fused in sarcoma
Gadd45a – growth arrest and DNA-damage-inducible protein GADD45 alpha
GAP43 – growth-associated protein 43
GAR1 – H/ACA ribonucleoprotein complex subunit 1
GDNF – glial cell-derived neurotrophic factor
GFAP – glial fibrillary acidic protein
GLUT – glucose transporter
GSAP – gamma-secretase-activating protein
H2AX – H2A histone family member X
HD – Huntington's disease
HDAC – histone deacetylase
HDACi – histone deacetylase inhibitor
HFMSE – Hammersmith Functional Motor Score Expanded
HIF – hypoxia inducible factors
hnRNP – heterogeneous nuclear ribonucleoprotein
HRE – hypoxia-responsive elements
HSPA5 – heat shock protein 5
Htt – huntingtin
HuD – ELAV like RNA binding protein 4
HuR – Hu antigen R
IGF – insulin-like growth factor
IGFALS – IGF-binding protein acid labile subunit
IGFBP2 – insulin-like growth factor binding protein 2
IKKβ – nuclear factor kappa-B kinase subunit beta
IMP1 – insulin-like growth factor mRNA-binding protein 1
IPSC – Induced pluripotent stem cells
ISS–N1 – intronic splicing silencer N1
JMJD –Jumonji domain-containing 6
KD – knock–down
KDM – lysine demethylase
KSRP – KH-type splicing regulatory protein
LBH589 – panobinostat
LLPS – liquid–liquid phase separation
lncRNA – long noncoding RNA
M344 – benzamide
MAPK – mitogen-activated protein kinase
MDM4 (MDMX) – Mdm2-like p53-binding protein
MDS – myelodysplastic syndrome
MeCP2 – methyl CpG binding protein 2
miRNA – micro-RNA
MN – motor neurons
MND – motor neuron diseases
MRI – magnetic resonance imaging
MTS – (3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4- sulfophenyl]-2H-tetrazolium, inner salt)
MYC – Myc proto-oncogene protein
NCALD – neurocalcin delta
NF–κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NMD – nonsense mediated decay
NMDA – N-methyl-D-aspartate
NMJ – neuromuscular junction
NRCAM – neuronal cell adhesion molecule
NSMF – NMDA receptor synaptonuclear signalling and neuronal migration factor
PAD – peptidylarginine deiminase
PBA – phenylbutarate
PD – Parkinson's disease
PHD – plant homeodomain
PHD – prolyl hydroxylase domain
PHI – prolyl hydroxylase domain inhibitor
PRMT – protein arginine methyltransferase
PTM – posttranscriptional modification
RANK – receptor activator of nuclear factor κ B
RBM15 – RNA binding motif protein 15
RBP – RNA binding proteins
RNAP II – RNA polymerase II
RNP – ribonucleoprotein
ROS – reactive oxygen species
SAH – S–adenosylhomocysteine
SAHA – suberoylanilidehydroxamic acid
SALS – sporadic amyotrophic lateral sclerosis
SAM – S-adenosylmethionine
Sam68 – Src-associated in mitosis 68 KDa protein
SBMA – spinal and bulbar muscular atrophy
scAAV9 – self-complementary adeno associated virus serotype 9
SDMA – symmetric dimethylation
SFRS9 – Serine/arginine-rich splicing factor 9
SGC – Structural Genomics Consortium
SLC23A2 – solute carrier family 23 member 2
SMA – spinal muscular atrophy
SMN – survival motor neuron
SMN1 – survival motor neuron 1 gene
SMN2 – survival motor neuron 2 gene
snoRNP – small nucleolar ribonucleoproteins
snRNA – small nuclear RNA
snRNP – small nuclear ribonucleoproteins
SOD1 – superoxide dismutase 1
SPF30 – splicing factor 30
TARDBP – TAR DNA binding protein
TBI – traumatic brain injury
TCA – tricarboxylic acid
TCERG1 – transcription-elongation repressor CA150
TDRD – Tudor domain-containing proteins
TERC – telomerase RNA component
TERT – telomerase reverse transcriptase
TMA – thrombotic microangiopathy
TNF-α – tumour necrosis factor alpha
TRA2B – transformer 2 beta homolog
TSA – trichostatin A

TSS – transcriptional start site
U2AF1 – U2 small nuclear RNA auxiliary factor 1
UBA1 – ubiquitin-like modifier-activating enzyme 1
UPF1 – regulator of nonsense transcripts 1
VEGF – vascular endothelial growth factor
VHL – von Hippel-Lindau protein
WRAP53 – WD repeat containing antisense to TP53
XBP1 – X-box binding protein 1
ZPR1 – zinc finger protein ZPR1
Δ7 SMN2 – delta 7 survival motor neuron 2 transcript

Introduction

Introduction to spinal muscular atrophy

Spinal muscular atrophy (SMA) is a rare monogenic, recessive disorder of mostly paediatric onset, affecting 1:8,000 – 10,000 live births, with a carrier frequency of around 1:40 (Pearn 1978). To this date SMA remains the number one genetic cause of death of children below the age of two worldwide (Crawford and Pardo 1996). SMA is caused by a severe deficiency of survival motor neuron protein (SMN), which leads to degeneration of α-motor neurons in the anterior horn of the spinal cord and brainstem. Genetically, 95 % of SMA cases are caused by a homozygous deletion of *SMN1* gene on chromosome 5 (Lefebvre et al. 1995). The remaining 5 % are caused by a heterozygous deletion of *SMN1* and a point mutation on the other chromosome that renders that copy dysfunctional.

On the same chromosome, humans carry a nearly identical gene, *SMN2*, that differs from *SMN1* by five nucleotides (Lorson et al. 1999). A silent C to T substitution in position 6 of exon 7 of *SMN2* leads to preferential exclusion of exon 7 from the final transcript, resulting in a shorter SMN protein, so called Δ7 SMN, which fails to self-oligomerize and is quickly degraded (Cho and Dreyfuss 2010; Monani et al. 2000). Only around 10 to 15 % of *SMN2* transcripts retain exon 7 and generate a full length SMN protein. SMA patients rely only on the *SMN2* gene for SMN protein production and every patient has at least one copy of *SMN2*, as complete lack of SMN protein is embryonically lethal (Schrank et al. 1997).

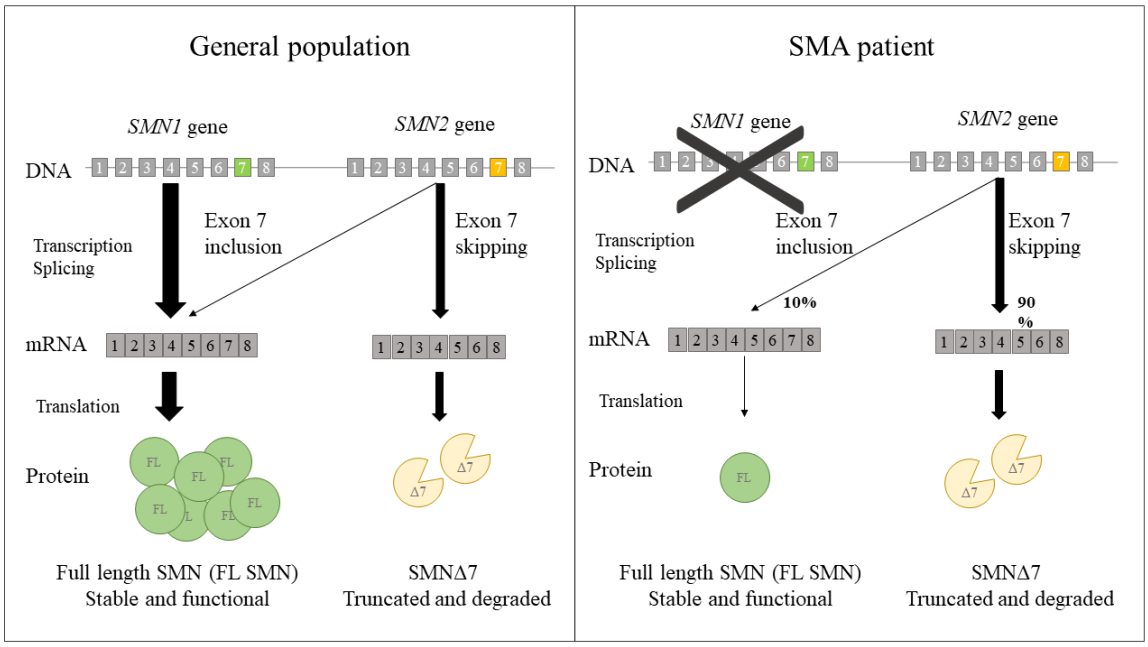


Figure *1*. A schematic showing genetics of general population and that of an SMA individual.

The number of *SMN2* copies vary between patients (and general population as well) and is a major disease modifier, with higher number of copies being generally linked to a milder phenotype (Feldkötter et al. 2002). Nevertheless, some SMA patients with high number of *SMN2* copies can display severe phenotype, indicating a presence of additional modifiers other than SMN protein (Wirth 2020). Proteins found to modulate disease severity include: Plastin3, Neurocalcin delta (NCALD), ZPR1 Zinc Finger (ZPR1) and Ubiquitin Like Modifier Activating Enzyme 1 (UBA1) (Kannan et al. 2019; Oprea et al. 2008; Powis et al. 2016; Riessland et al. 2017)

SMA has been first described in 1891 and is classified into four clinical types, depending on age at symptoms onset and the highest motor milestone achieved (Munsat and Davies 1992). The most severe and the most common type I, also referred to as Werdnig-Hoffmann disease, presents itself before 6 months of age, with infants never gaining the ability to sit unaided. Without treatment, by the age of two the patient is permanently

dependent on ventilation or has deceased (Russman 2007), however with advances in SMA care more SMA type I patients are surviving beyond infancy. Type II, also known as Dubowitz disease, presents itself between 6 and 18 months, with the infant being able to sit but not stand up. With time, without treatment, the sitting ability is lost, and type II individuals usually live 30-40 years. Type III patients (Kugelberg- Welander disease) are able to walk and start displaying symptoms after 18 months of age. Type IV is the mildest form of SMA, accounts for about 10 % of cases and can present itself in the middle age, when the patient starts experiencing muscle weakness and difficulties walking. Type III and IV patients usually have a normal lifespan. Sometimes a so called SMA type 0 is described separately from Type 1, and pertains to babies with respiratory insufficiency at birth in need of permanent ventilation (Macleod et al. 1999).

With the advent of disease modifying therapies (described in more detail below), the above classification is slowly becoming obsolete. The new classification is based on the current motor status of the patient: non-sitter, sitter or walker, as the motor function is more informative in planning care then the arbitral type of SMA (**Figure *2***) (Mercuri, Finkel, et al. 2018) .

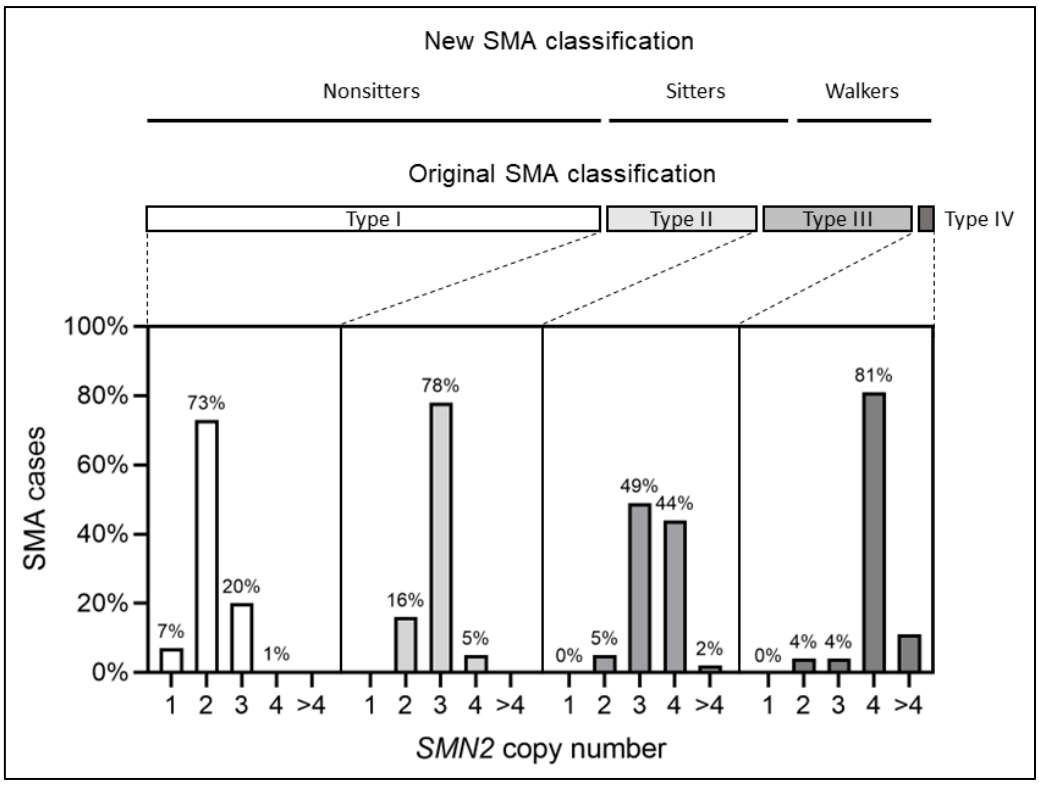


Figure *2*. Relationship between *SMN2* copy number and SMA type (old classification) and functional classification, adapted from Wirth 2020.

SMN1 and *SMN2* genes

The presence of *SMN1* and *SMN2* genes in humans is attributed to a ~500 kb intrachromosomal duplication on chromosome 5 (Lefebvre et al. 1995; Rochette, Gilbert, and Simard 2001). Both genes are ~ 34 kb long, including a ~6 kb long promoter sequence. Each *SMN* gene has 10 exons: 1, 2A, 2B, 3, 4, 5, 6, 6B, 7 and 8 (Singh and Singh 2018). About a third of exon 1 serves as a 5'UTR, and exon 8, the longest one, encodes 3'UTR (Singh et al. 2018). Nearly 40 % of *SMN* sequence are Alu elements located withing introns (Ottesen et al. 2019).

SMN1 and *SMN2* genes generate several transcripts. FL *SMN* contains exons 1, 2a, 2b, 3, 4, 5, 6, 7 and 8. As mentioned above, *SMNΔ7* transcript lacks exon 7. There are several

other *SMN* transcripts: *a-SMN*, *SMN6B*, *SMNΔ5* and *SMNΔ3*. *a-SMN* isoform is a result of intron 3 retention and plays and important developmental role in mammalian brain and promoting axon growth (Setola et al. 2007). In adults it is subjected to nonsense mediated decay (NMD) and not detected. *SMN6B* is formed by an exonization of and Alu-like sequence in intron 6 (Seo et al. 2016), and *SMNΔ5* and *SMNΔ3* lack exon 5 and 3 respectively. They too are subject to NMD and their specific functions are to be determined (Singh et al. 2017).

Exon 7 splicing regulation

Exon 7 splicing regulation is of most scientific interest in the context of SMA.
As mentioned above, a crucial C to T substitution in position 6 of exon 7 of *SMN2* leads to preferential exclusion of exon 7 from the final transcript, therefore the vast majority of protein encoded by *SMN2* is truncated (Δ7 SMN), fails to self-oligomerize and is quickly degraded (Kashima, Rao, and Manley 2007; Lorson et al. 1999). Over 40 proteins have been reported to be involved in regulating the splicing of exon 7 (Singh et al. 2018). An intronic splicing silencer (ISS-N1), a 15-nucleotide long sequence in intron 7, has been found to be an important negative regulator of exon 7 inclusion (Singh et al. 2006), and an antisense oligonucleotide targeting that sequence, nusinersen, has been approved for the treatment of SMA in 2016 (FDA 2016).
Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) is a splicing factor and a major negative regulator of *SMN2* (but not *SMN1*) exon 7 inclusion (Kashima and Manley 2003). The C to T substitution in exon 7 of *SMN2* creates a binding site for hnRNPA1 and an exonic splicing silencer (ESS), leading to skipping of exon 7 in most cases (Kashima and Manley 2003). hnRNPA1 knockdown results in significantly elevated levels of FL *SMN2* (Kashima et al. 2007).

SMN protein

Domain organization

Survival motor neuron (SMN) is a multifunctional protein expressed in all cell types of the animal kingdom. Within the cell it localizes both to cellular and nuclear compartments, and the relative abundance depends on the tissue type and development stage (Zhang et al. 2007). In the nucleus SMN is involved in the formation of nuclear gems and Cajal bodies (CBs) (Liu and Dreyfuss 1996), storage and maturation of ribonucleoproteins (RNPs), including small nuclear ribonucleoproteins (snRNPs) and small nucleolar ribonucleoproteins (snoRNPs) (Hebert et al. 2001). In the cytoplasm SMN localizes to several structures, including stress granules, the Golgi network, microtubules and sarcomeric Z-discs (Walker et al. 2008; Zhang et al. 2006).
SMN, a 38 kDA protein of 294 aa, is highly expressed during embryogenesis and its levels significantly drop in the first three months of life (Ramos et al. 2019). SMN harbours several domains and is highly conserved amongst higher vertebrates (Singh et al. 2017). The N terminus contains Gemin- and nucleic acid- binding domains encoded by exons 2A and 2B (Lorson and Androphy 1998). SMN-Gemin2 complex is critical to most SMN functions in vertebrates, including snRNPs assembly, translation regulation and DNA recombination (Piazzon et al. 2013; Sanchez et al. 2013; Takaku et al. 2011). The Tudor domain, responsible for binding to symmetrically dimethylated arginines within RGG/RG motifs, is located centrally in exon 3 (Tripsianes et al. 2011), and proline rich and YG domain are located C-terminally (Giesemann et al. 1999; Lorson and Androphy 1998). Proline rich domain binds profilins, small proteins involved in actin dynamics in the cell. The YG domain, encoded by exon 6 and partially by exon 7, are responsible for SMN self-oligomerization, a process critical for SMN stability and sub-cellular localization (Burnett et al. 2009; Giesemann et al. 1999). This function is impaired in Δ7 SMN.

Skipping of exon 7 also results in four amino acids addition encoded by exon 8 (EMLA), which serve as a degradation signal for Δ7 *SMN*, also contributing to its low stability (Cho and Dreyfuss 2010). Mutations in all domains have been found to cause SMA, indicating the importance of the overall protein structure for its function (Howell, Singh, and Singh 2014).

SMN protein function in RNA metabolism

SMN is a housekeeping protein involved in numerous essential processes. The most studied roles of SMN are connected to RNA biology, and include biogenesis of snRNPs, snoRNPs and telomerase, 3' processing of histones mRNAs, transcription, splicing, RNA trafficking and translation.

Spliceosomal snRNPs biogenesis

SMN involvement in the biogenesis of snRNPs is its most widely studied function (Friesen, Massenet, et al. 2001; Pellizzoni, Yong, and Dreyfuss 2002; Zhang et al. 2011). It is carried out by an SMN-Gemin complex consisting of SMN, Gemins 2-8 and unrip, a serine-threonine kinase receptor-associated protein, and is a multistep process occurring in the nucleus and cytosol (Fischer, Englbrecht, and Chari 2011). Spliceosomal snRNPs consist of snRNA and a heptameric ring of Sm proteins (B/B′, D1, D2, D3, E, F, and G). First, a snRNA is transcribed in the nucleus and undergoes co-transcriptional processing: the 5'end capping and cleavage of 3' end, generating pre-snRNA (Fischer, Englbrecht, and Chari 2011). Then, a multiprotein complex is formed, exporting pre-snRNA to the cytosol (Li et al. 2014). In the cytosol, the export complex is disassembled and SMN complex is responsible for the loading of a heptameric Sm protein ring onto to the correct pre-snRNA. Protein arginine methyltransferase 5 (PRMT5) symmetrically dimethylates three Sm proteins – SmB, SmD1 and SmD3, tightening their interaction with SMN *via* the

Tudor domain (Friesen, Paushkin, et al. 2001; Meister et al. 2001). Gemin5 also recognizes sites on pre-snRNA for Sm ring loading (Battle et al. 2006). After loading is completed, the pre-snRNA is hypermethylated at 5' end, and trimmed at 3' end (Mouaikel et al. 2002). The 5' cap and Sm core are nuclear import signals and the whole complex is transported back into the nucleus, when it undergoes final maturation process in the Cajal bodies.

Unsurprisingly, low levels of SMN (Bäumer et al. 2009; Zhenxi Zhang et al. 2008) and PRMT5 (Bezzi et al. 2013; Braun et al. 2017; Gerhart et al. 2018) cause wide spread splicing defects. It has been proposed that skipping of minor introns is particularly affected in SMA (Lotti et al. 2012). Deficiency of spliceosome assembly and function is one hypothesis behind preferential vulnerability of motor neurons in SMA (Burghes and Beattie 2009).

Histones 3' end pre-mRNA processing

Histone pre-mRNAs require different 3' end processing, as they are not polyadenylated, and U7 snRNP is responsible for carrying out this process (Marzluff, Wagner, and Duronio 2008). SMN role in U7 snRNP biogenesis is similar to its role in spliceosomal snRNPs generation, but the components of the heptameric ring differ and consist of Sm-like proteins (Lsm10 and Lsm11) instead of SmD1 and SmD2 (Pillai et al. 2001). Consequently, deficiency of SMN leads to defective processing of the 3' end of histones pre-mRNA and accumulation of U7 snRNA (Tisdale et al. 2013).

Transcription

SMN interacts with several splicing factors, like p53 and papillomavirus-encoded transcription factor E2, strengthening E2-dependent transcriptional activation (Strasswimmer et al. 1999). Binding to p53 and E2 indicates its role in transcription

initiation. SMN can be involved in transcription indirectly, *via* its regulation of U1 snRNPs biogenesis, the most abundant snRNP in the nucleus, which interacts with the nascent transcripts (Almada et al. 2013). SMN has been found to play a role in the control of transcription termination *via* the Tudor domain as well. It binds dimethylated arginines in the CTD of POLR2A unit of RNA polymerase II (RNAP II), recruiting senataxin, a RNA-DNA helicase. Together with senataxin, SMN is involved in resolution of R-loops. Consistently, deficiency of SMN leads to R-loop accumulation and genome instability, as assayed by the γ-H2A histone family member X (γ-H2AX) levels (Suraweera et al. 2009; Yanling Zhao et al. 2016).

Pre-mRNA splicing

In addition to being a core component of the spliceosome, SMN assists in the process of splicing as an auxiliary protein, independently from its involvement in snRNPs generation. It has been found that splicing of chicken δ-crystallin pre-mRNA is suppressed upon incubation with Δ27 SMN (a mutant that lacks the first amino-terminal 27 amino acids), but unaffected when incubated with full length SMN, suggesting a role of the N terminal of SMN in splicing (Pellizzoni et al. 1998). SMN is also a component of the E complex, or early commitment complex, bringing the 5’ and 3’ ends to close proximity, enabling correct splicing (Makarov et al. 2012). SMN also associates with RNA helicases - Gemin3, DDX1, DDX3 and DDX5, and influences splicing *via* unwinding pre-mRNA structures (Charroux et al. 1999; Shafey et al. 2010). Structural elements have been implicated in splicing of SMN and other genes, suggesting SMN can potentially regulate its own splicing.

RNA trafficking

In neurons, RNA binding proteins (RBPs) bind to mRNAs involved in axon growth, branching and synapse formation, and transport them away from cell body to dendrites, axons and synapses for local translation (Hörnberg and Holt 2013; Wong et al. 2017). The first evidence of SMN involvement in RNA trafficking came from a study showing β - actin transport along neuronal processed and growth cones (Rossoll et al. 2003). SMN is present in growth cones, axons and presynaptic terminals and has been found to be associated with several RBPs involved in neuronal mRNA transport, like, ELAV-like protein 4 (HuD), insulin-like growth factor mRNA-binding protein 1 (IMP1), KH-type splicing regulatory protein (KSRP) and heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) (Akten et al. 2011; Fallini et al. 2011, 2014; Hubers et al. 2011; Piazzon et al. 2008; Rage et al. 2013; Tadesse et al. 2008). Hud is only expressed in neuronal cells and one of its targets is *candidate plasticity-related gene 15 (Cpg15)*. SMN has been found to facilitate trafficking of *Cpg15* bound to Hud (Akten et al. 2011). Similarly, *growth-associated protein 43 (Gap43)* transcripts are transported in the motor neurons by the SMN/HuD/IMP1 complex (Fallini et al. 2016). In the same study the overexpression of HuD and IMP1 improved the phenotype of primary motor neurons derived from severe SMA mice. A transcriptome wide study has identified more than 200 mRNAs as potential targets of SMN, including *SMN* itself (Rage et al. 2013). Impaired neuronal mRNA trafficking is put forward as one of main causes of preferential motor neuron vulnerability to SMN depletion (Burghes and Beattie 2009).

Translation

SMN is emerging as a translational regulator, as it associates with polysomes and can influence translation *in vitro* and *in vivo* (Béchade et al. 1999; Fallini et al. 2016; Lauria et

al. 2020; Sanchez et al. 2013). Sanchez and colleagues showed that SMN co-represses translation of coactivator associated arginine methyltransferase 1 (PRMT4 or CARM1), and a mutation in the Tudor domain abrogates that repression. Interestingly, the mutation in the Tudor domain does not affect SMN association to polysomes. Consistently with CARM1 repression by SMN, its levels are elevated in tissues from SMA mice and cells from SMA type I patients, and are corrected upon SMN overexpression (Sanchez et al. 2013).

Global defects in mRNA recruitment onto polysomes have also observed in SMA models (Bernabò et al. 2017). Lauria and colleagues showed that SMN is a ribosome associated protein and a master regulator of translation of a subset of mRNAs relevant for SMA pathology (Lauria et al. 2020). SMN binds to a fraction of ribosomes ('SMN-primed ribosomes') in a tissue dependent manner. These ribosomes are preferentially positioned within the first five codons, and enriched in mRNAs encoding for proteins involved in neurogenesis, ubiquitination, chromatin regulation, translation and lipid metabolism. In conditions of SMN deficiency, the number of ribosomes at the beginning of coding sequences of SMN specific mRNAs is depleted, resulting in a dysregulation of proteins involved in motor neuron stability and function. The authors propose that impairment of translation is the underlying cause of preferential vulnerability of motor neurons to SMN depletion in SMA.

Telomerase biogenesis

Telomerase is a RNP complex catalysing the replication of chromosome ends, comprised of a reverse transcriptase (TERT), an RNA component (TERC) and protein component (Zhang, Kim, and Feigon 2011). SMN interacts with multiple components of telomerase, like Dyskerin, TERT, GAR1, and WRAP53 (Bachand et al. 2002; Pellizzoni et al. 2001; Poole and Hebert 2016), possibly transporting those to the Cajal bodies.

Other SMN protein roles in RNA metabolism and beyond

The above does not exhaust the roles of SMN in RNA biology. Other known functions involve biogenesis of snoRNPs, small snRNP complexes performing post-transcriptional modifications of non-coding RNAs (Jones et al. 2001), biogenesis of signal recognition particle (Piazzon et al. 2013), stress granule formation (Hua and Zhou 2004; Zou et al. 2011), and synthesis of selenoprotein (Wurth et al. 2014).

SMN protein roles extend beyond RNA biology, as the protein plays a role in DNA damage response (Takaku et al. 2011; Takizawa et al. 2010), signal transduction (Genabai et al. 2015; Nölle et al. 2011), autophagy (Garcera et al. 2013), endocytosis (Dimitriadi et al. 2016), and intracellular trafficking (Fallini et al. 2016; Rossoll et al. 2003). The most important roles of SMN identified so far are summarized in Error! Reference source not f ound..

Involved in so many housekeeping roles, it is not surprising that SMA is such debilitating disease. Except for SMA, SMN has been linked to amyotrophic later sclerosis (ALS), inclusion body myositis and osteoarthritis (Achsel et al. 2013; Broccolini et al. 2000; Cucchiarini, Madry, and Terwilliger 2014). SMN protein levels are elevated in osteoarthritis cartilage, but it remains to be seen if there are the cause or effect of the disease (Cucchiarini, Madry, and Terwilliger 2014). SMN is important to male fertility and testicular development, and protein levels in the testes are much higher compared to other organs and tissues (Chen et al. 2015; Ottesen et al. 2016).

Known SMN functions in:	Selected references
RNA biology	
Spliceosomal snRNPs biogenesis	(Friesen, Massenet, et al. 2001; Pellizzoni, Yong, and Dreyfuss 2002; Zhang et al. 2011)
Histones 3' end pre-mRNA processing	(Marzluff, Wagner, and Duronio 2008; Pillai et al. 2001; Tisdale et al. 2013)
Pre-mRNA splicing	(Charroux et al. 1999; Makarov et al. 2012; Pellizzoni et al. 1998; Shafey et al. 2010)
RNA trafficking	(Akten et al. 2011; Fallini et al. 2011, 2014; Hubers et al. 2011; Piazzon et al. 2008; Rage et al. 2013; Rossoll et al. 2003; Tadesse et al. 2008).
Translation	(Béchade et al. 1999; Bernabò et al. 2017; Fallini et al. 2016; Lauria et al. 2020; Sanchez et al. 2013).
Telomerase biogenesis	(Bachand et al. 2002; Pellizzoni et al. 2001; Poole and Hebert 2016)
Biogenesis of snoRNPs	(Lui and Lowe 2013; Reichow et al. 2007)
Biogenesis of signal recognition particle	(Piazzon et al. 2013)
Stress granule formation	(Fallini et al. 2011; Zou et al. 2011)
Synthesis of selenoprotein	(Rage et al. 2013; Wurth et al. 2014)
DNA recombination and repair	
DBS repair	(Morozumi et al. 2009; Takaku et al. 2011; Takizawa et al. 2010)
Centromeric Damage Response (iCDR)	(Sabra et al. 2013)
Signal transduction	
Signalling regulating the actin cytoskeleton	(Bowerman et al. 2009, 2010; Bowerman, Murray, et al. 2012; Bowerma, 2007;

Known SMN functions in:	Selected references
	Giesemann et al. 1999; Nölle et al. 2011)
Signalling pathways implicated in neurodegeneration	(Genabai et al. 2015; Sen et al. 2011; Wishart et al. 2014)
Endocytosis	(Dimitriadi et al. 2016; Hosseinibarkooie et al. 2016)
Autophagy	(Custer and Androphy 2014; Garcera et al. 2013; Periyakaruppiah et al. 2016; Wen et al. 2010)

Table *1*. A summary of known SMN protein functions

SMA as a multisystem disorder

SMN protein is ubiquitously expressed but its insufficiency predominantly leads to a severe neuromuscular phenotype due to degeneration of the lower α-motor neurons. However, the last two decades of research have shown involvement and pathology of multiple organs and systems, both in animal models and patients, and have changed our understanding of the disease, which is now widely recognized as multisystemic disorder. That is supported by studies suggesting that motor neuron specific restoration of SMN only provides a modest improvement in survival of SMA mice (Gogliotti et al. 2012; Martinez et al. 2012) and peripheral restoration is needed for long term rescue (Hua et al. 2011). As far as we know, except for motor neurons, SMA affects the neuromuscular junction, other neuronal populations, glia, skeletal muscle, heart and vasculature, bones, gastrointestinal system, spleen, liver and kidney. Characterizing changes that occur across tissues and cell types is crucial to developing new effective therapies.

Motor neurons

SMA is a disease with intrinsic deficits in motor neurons (Burghes and Beattie 2009; Monani 2005). Despite great research efforts it is still under debate, how a deficiency in a ubiquitous protein leads to their preferential degeneration. One major hypothesis is that disruption of snRNP assembly and splicing primarily affects motor neurons, as defects in snRNPs assembly correlate with disease severity in mice (Gabanella et al. 2007; Zhenxi Zhang et al. 2008). Deficiency in SMN leads to a more prominent defects in snRNPs in motor neurons compared to other neurons and non-neuronal cells (Ruggiu et al. 2012), which can lead to cell death. That can possibly be due to a low exon 7 inclusion in healthy motor neurons, that is further exacerbated by deficiency of snRNPs in disease, leading to a negative feedback loop and preferential vulnerability of motor neurons

(Jodelka et al. 2010; Ruggiu et al. 2012). Defects in snRNPs assembly can also lead to a disruption of key axon genes, resulting in cell death (Gabanella et al. 2007).

Splicing defects are observed in all SMA tissues and identification of motor neurons specific changes can inform their preferential vulnerability. Examples of splicing events that specifically affect motor neurons are *Stasimon* gene (Lotti et al. 2012), chondrolectin (Sleigh et al. 2014) and Agrin (Kim et al. 2017; Zhenxi Zhang et al. 2013).

Another major hypothesis for preferential MN vulnerability is that SMN in involved in mRNA axonal transport in the MNs, which is disrupted in SMA, and has been described in more detail above.

However, even within the motor neuron population the vulnerability to SMN deficiency is not equal. Motor neurons innervating proximal muscles are affected earlier than those innervating distal muscles (Mentis et al. 2011) and α-MNs are uniquely vulnerable while γ-MNs and other cholinergic neurons are largely spared (Powis and Gillingwater 2016).

Neuromuscular junction

Neuromuscular junction (NMJ) is a specialized synapse between the motor neurons and skeletal muscle fibres. Abnormalities in NMJ development, maturation and function are widely reported in SMA patients and animal models and they precede motor neuron loss (Courtney et al. 2019; Kong et al. 2009; Murray et al. 2008). SMA patients show abnormal aggregation of acetylcholine receptors at muscle endplate, neurofilament accumulation, denervation and electrophysiologic defects (Martínez-Hernández et al. 2013; Wadman et al. 2012). In mouse models, both pre-synaptic and post-synaptic pathology has been reported (Arnold et al. 2004; Kariya et al. 2008; Kong et al. 2009; Murray et al. 2008). Pre-synaptic includes abnormal accumulation of neurofilaments

at nerve terminal, as well as impaired density and distribution of synaptic vesicles. Post-synaptically, shrinkage of motor endplates is observed.

Other neuronal populations

Deficiency of SMN protein also affects neuronal populations other than motor neurons. Disruptions in sensory pathways have been reported both in patient and in mice, where the connectivity between the sensory and motor neurons is lost before the onset of motor neuron loss (Gogliotti et al. 2012; Jablonka et al. 2006; Martinez et al. 2012; Mentis et al. 2011; Sabine Rudnik-Schöneborn et al. 2003; Vukojicic et al. 2019). Some areas of the brain have been reported to be affected as well. In a severe mouse model, an abnormal development of the hippocampus was observed, together with impaired neurogenesis and decreased cellular proliferation (Wishart et al. 2010). Changes in the thalamus have been observed in several SMA patients with severe phenotype, suggesting that targeting the whole CNS can be beneficial for patients (Ito et al. 2004; Shishikura et al. 1983).

Glia

Glia cells are critical for maintaining neuronal homeostasis and survival and their contribution to SMA pathology has been reported by several groups. Motor neuron degeneration seem to be proceeded by activation of glial cells that can sustain and spread the neurodegenerative process (Wang and Bordey 2008). Oligodendrocytes development and CNS myelination seems to be intact in SMA mice (O'Meara et al. 2017; Ohuchi, Funato, Ando, et al. 2019). On the other hand, several pathological feature of astrocytes and microglia have been reported in SMA.

In SMA iPSC-derived astrocytes and SMNΔ7-tg-mice spinal cord sections astrocytes show signs of activation and up-regulation of GFAP and Nestin, as well as dysregulation

of basal calcium, reduced response to ATP stimulation and reduced GDNF production. (Mcgivern et al. 2013). Astrogliosis was prominent in end-stage SMA mice and in post-mortem patient spinal cords. Restoring SMN in astrocytes of SMA mice improved lifespan, gross motor function and defects in NMJs and proprioceptive synapses. (Rindt et al. 2015). Spinal cord astrocytes and motoneurons primary cultures and cocultures from WT and SMNΔ7-tg-mice show altered calcium concentrations, and disrupted motoneuron–astrocyte interactions in synapse formation and function in contact cocultures. (Zhou, Feng, and Ko 2016). In astrocytes and motoneurons cultures from spinal cord of WT and SMNΔ7-tg-mice and from control and SMA patients iPSCs, astrocyte conditioned medium from SMA mice provides decreased support of isolated MNs, and SMA astrocytes produce decreased amounts of neuroprotective monocyte chemoactive protein 1 (MCP1). Supplementation of MCP1 improves motoneuronal growth. (Martin et al. 2017). Finally, SMNΔ7-tg-mice spinal cord sections and SMA iPSCs-derived astrocytes show dysregulation of Notch signalling in the spinal cord in both early and late stages of SMA in mice. Motor function deficits of SMA mice were improved by pharmacological inhibition of Notch signalling (Ohuchi, Funato, Yoshino, et al. 2019).

Microglia have also been reported to be impaired in SMA. They show a proinflammatory phenotype, with increased levels of reactive oxygen species (ROS), release of TNF-α and increased NF-κB activity, which was rescued by increasing SMN expression (Ando, Osanai, et al. 2020). Phagocytic behaviour has been observed in SMA mice, and pharmacological inhibition of microglia rescued proprioceptive synapses and motor function in SMA mice (Vukojicic et al. 2019). More specifically, C1q and C3, proteins involved in the component system, locally produced by microglial cells, tag vulnerable synapses in SMA for removal by microglia, and that SMN deficiency drives C1q tagging

of proprioceptive synapses in the spinal cord. Block of C1q tagging resulted in synaptic and behavioural benefit in SMA mice while genetic deletion of C1q rescued synaptic deficit in SMN-deficient animals (Vukojicic et al. 2019). In SMA IPSC-derived model, microglia had increased cell migration, enhanced phagocytic activity and altered, reactive, transcriptome profile (Khayrullina et al. 2022). Incubating motor neurons with SMA microglia conditioned media resulted in alteration of cell morphology and electrophysiological properties of MNs (Khayrullina et al. 2022). In summary, it is very likely that neuroinflammation is contributing to neurodegeneration in SMA, like it is in ALS (Liu and Wang 2017).

Skeletal muscle

Motor neurons and skeletal muscles are intimately interconnected to form the motor unit and diseases specifically affecting one or the other will have functional consequences on the motor unit as a whole. For a long time, the question of intrinsic muscle pathologies in SMA have been debated. To date, several cell-autonomous events in skeletal muscles deficient in SMN have been described, independently of motor neuron pathology.
In SMA type I patients, myotubes are decreased in size and disorganized, with impaired maturation and altered expression of acetylcholine receptor (Arnold et al. 2004; Martínez-Hernández et al. 2009). The myoblasts do not fuse properly and form myotubes with smaller number of nuclei (Arnold et al. 2004). Myotubes from skeletal muscles of severely affected foetuses are also smaller, indicating a delay in muscle growth and differentiation (Martínez-Hernández et al. 2009). Mitochondrial impairment has been observed in several skeletal muscles from patient with SMA types I-III. Defective mitochondrial biogenesis, decreased levels of mitochondrial DNA, and defective activity of mitochondrial complexes 1, 2 and 4 has been observed in those individuals (Ripolone et al. 2015).

Mouse models of SMA recapitulate the skeletal muscle involvement in SMA.

In mice, muscle differentiation and growth impairment is present and proceeds neurodegeneration, confirming that SMN has cell-autonomous roles in muscle (Mutsaers et al. 2011). Muscle specific depletion of SMN in mature muscle fibres by HSA-Cre excision of SMN exon 7 in mice results in muscle degeneration and dystrophic phenotype (Cifuentes-Diaz et al. 2001).

Another mechanism that has been suggested to influence muscle function comes from SMN involvement in gene expression and transcription. A delay of transcription and expression of essential muscle contraction proteins such as ryanodine, sodium channel, and calcium channels has been observed in a presymptomatic mouse model, which likely contributes to disease pathology (Boyer et al. 2013). Deguise and colleagues on the other hand found a differential induction of muscle atrophy pathways in a severe and moderate mouse models. Proteasomal degradation was induced in skeletal muscle of both models, while autophagosomal degradation was only present in the moderate model (Deguise et al. 2016).

In vitro, myoblasts exhibit reduced proliferation and fusion defects, which correlate with SMN levels, confirming cell-autonomous defects in the skeletal muscle (Shafey, Côté, and Kothary 2005). Overall, it is accepted that there are intrinsic defects in skeletal muscle, independent of neurodegeneration in SMA, but the relative contribution to disease pathology is unclear. For example, no muscle phenotype was observed in a zebrafish model of SMA (McWhorter et al. 2003). Notably, SMN overexpression in skeletal muscle alone does not improve the SMA phenotype in mice, while selective overexpression in motor neurons does (Gavrilina et al. 2008).

Heart and vasculature

It is reported that around 15 % of SMA type I patients have bradycardia, a symptom also observed in a severe SMA mouse model, together with dilated cardiomyopathy (Bevan et al. 2010). Cardiac abnormalities and congenital heart defects are common in type 0 and 1 patients but until recently little evidence for those abnormalities in type 2 and 3 was available (Rudnik-Schöneborn et al. 2008). However, an elegant study of insurance claims of SMA patients with types 1 to 4 has found that overall SMA patients are more often diagnosed with cardiomyopathies, valve disorders, septal defect, premature beats, and more in general vascular issues then non-SMA individuals, before onset of neuromuscular symptoms (Lipnick et al. 2019). Vascular defects and capillary bed depletion are reported both in humans and mice, leading to hypoxia in the spinal cord in the animal models, and defect in blood-brain barrier (Araujo, Araujo, and Swoboda 2009; Somers et al. 2016). Distal necrosis is observed in some SMA mouse models, including the one used in this thesis, and occasionally in patients with severe phenotype as well (Sabine Rudnik-Schöneborn et al. 2010).

Together, these finding indicate an important role of SMN in development and function of the heart and vasculature and underlie the importance of therapies with a systemic distribution.

Bones and joints

Severe osteopenia has been observed in SMA patients types 1-3 and in mouse models, and type 2 and 3 patients have an increased risk of bone fractures and bone resorption (Vai et al. 2015; Vestergaard et al. 2001; Wasserman et al. 2017). Lack of ambulation contributes to osteopenia, as ambulatory patients have a better bone density compared to non-ambulant ones. However, intrinsic factors other than lack of ambulation contribute

to the osteopenia in SMA, as patients show a greater osteopenia than DMD patients in similar stages of the disease (Vestergaard et al. 2001). Children with SMA are at greater risk of joint pain, progressive foot collapse, joint hypermobility, abdominal hernia and poor wound healing, symptoms not observed in adults or mice models (Dhawan et al. 2019; Haaker and Fujak 2013). In mice, activation of osteoclasts *via* the RANK signalling pathway has been observed (Shanmugarajan et al. 2009). Interestingly, as mentioned above, the overexpression of SMN has been linked to osteoarthritis. SMN protein levels are elevated in osteoarthritis cartilage, but it remains to be seen if there are the cause or effect of the disease (Cucchiarini, Madry, and Terwilliger 2014).

Liver, pancreas and metabolism

In recent years, changes in the liver and pancreas have been observed in SMA. Autopsies from infants with SMA type I show fatty vacuolization in the liver, and around a third of children with types 1 to 3 have dyslipidaemia (Crawford et al. 1999; Deguise et al. 2019). Mice with liver specific *Smn* deletion show impaired embryonic liver development, iron overload, elevated triglycerides and dyslipidaemia, features characteristic of a non-alcoholic fatty liver disease (Deguise et al. 2019; Hua et al. 2011; Szunyogova et al. 2016; Vitte et al. 2004). Levels of insulin-like growth factor 1 (IGF-1), a hormone and a plasma protein produced by the liver, are reduced in SMA patients and mice. That is due to a downregulation of another hepatic protein, a IGF-binding protein acid labile subunit (IGFALS), responsible for stabilizing IGF-1 (Hua et al. 2011; Yesbek Kaymaz et al. 2016). Reduction in IGFALS levels precedes neuromuscular defect and can be reversed upon SMN protein elevation. An infant biomarker study, NeuroNEXT, revealed that levels of several serum proteins produced in the liver decrease with age (Kolb et al. 2016).

In the pancreas, a disorganized cellular function has been observed in severe patients (Melissa Bowerman, Swoboda, et al. 2012). Infants with type I and severe mice exhibit pancreatic defects and glucose intolerance. In pancreatic islets, an increased number of glucagon producing cells and a decreased number of insulin producing cells has been observed, leading to fasting hyperglycaemia and glucose resistance (Melissa Bowerman, Swoboda, et al. 2012). Also, hyperleptinemia has been reported in patients with types 1 to 3 (Kölbel et al. 2017). Overall, metabolic pathways are affected in SMA.

Gastrointestinal system

SMA patients experience gastroesophageal reflux, constipation and delayed gastric emptying, a phenomenon observed also in mice (Davis et al. 2014; Gombash et al. 2015). Moreover, severe SMA mice show abnormal number of small intestine villi (Sintusek et al. 2016).

Kidney, spleen and immune system

Kidney structure and function is impaired in type I patients (Nery et al. 2019).
On a histological level, nephrocalcinosis and medullary fibrosis has been reported. Crystals in urine and granular casts have been observed consistent with renal tubular dysfunction (Nery et al. 2019). In the spleen, higher number of megakaryocytes and lower number and smaller size of lymphocytes have been reported (Deguise and Kothary 2017). Spleen dysfunction might be responsible for the systemic inflammation observed in a severe mouse model, with upregulation of proinflammatory cytokines in all tissues and an activation of acute phase response in the liver (Wan et al. 2018).

Summary

While motor neurons are undoubtedly the primary cellular target in SMA, cumulative evidence highlights the role of other cells and tissues, and their clinical relevance remains to be elucidated. So far, most of the studies have investigated these tissues or cells in isolation, and therefore relative contribution of each of these to SMA pathology across the disease spectrum needs further investigation.

Disease modifying therapies

Mutations in *SMN1* genes have been identified as causing SMA in 1995 and shortly after *SMN2* has been suggested as potential drug target, which prompted vast efforts to develop therapies (Lefebvre et al. 1995, 1998). A monogenic cause of the disease and accurate and robust animal models made the therapeutic development quicker and more successful than in case of other motor neuron diseases, for examples ALS. Right now, less than thirty years after the identification of SMA causing gene, three therapies are approved for SMA: nusinersen (Spinraza, Biogen), onasemnogene abeparvovec (Zolgensma, Novartis Gene Therapies) and risdiplam (Evrysdi, Roche), two of which (nusinersen and risdiplam) target *SMN2* splicing.

Nusinersen (Spinraza, Ionis / Biogen)

Nusinersen is an antisense oligonucleotide (18-mer) targeting an intronic splicing silencer (ISS-N1) in the intron 7 of *SMN2*, facilitating inclusion of exon 7 into the final *SMN2* transcript, and elevating levels of functional SMN protein. Discovery of ISS-N1 in 2006 was followed by preclinical development of ASOs targeting the sequence with positive results (Hua et al. 2008; Porensky et al. 2012; Singh et al. 2006). That led to several clinical trials in type I, II and III patients, including a double-blind placebo controlled trial in 126 type I children and subsequently also presymptomatic newborns (Chiriboga et al. 2016; Finkel et al. 2016, 2017; Mercuri, Darras, et al. 2018; De Vivo et al. 2019). Nusinersen increases SMN protein level in the CSF by around 2- fold (Chiriboga et al. 2016). It has been approved by the FDA in 2016 and by EMA in 2017. It can be used in all SMA patients regardless of age, type and functional status and is currently given at 12 mg dose regardless of the patients' weight. Nusinersen does not cross the blood brain barrier and is administered intrathecally by a lumbar puncture, thereby only targeting the central

nervous system (Geary, Yu, and Levin 2001). Initial four loading doses every two weeks are followed by a four-monthly dose indefinitely. Nusinersen is in general safe and well tolerated, side effects are often caused by the delivery route and encompass a post puncture syndrome with headaches, dizziness, and vomiting. Scoliosis and other spinal deformities can make the intrathecal injection impossible. Repeated intrathecal injections are a major disadvantage of nusinersen, as they become more difficult and burdensome with time. Nusinersen is extremely costly and the US price is $ 750,000 in the first year and $ 375,000 in subsequent years.

Onasemnogene abeparvovec (Zolgensma, Novartis Gene Therapies)

Zolgensma is a self-complementary adeno associated virus serotype 9 (scAAV9)-based vector containing *SMN1* cDNA, administered as a one-time intravenous infusion, potentially reaching all or nearly all tissue types, including the central nervous system. Zolgensma is thought to elevate SMN levels quickly, within the matter of hours from administration in comparison to weeks for nusinersen, and risdiplam, which is especially important in the severe form of the disease where patients loose motor neurons and motor function very rapidly (Al-Zaidy and Mendell 2019). *SMN1* cDNA does not integrate into the host genome and exists in the nucleus ectopically. As a consequence, with every cell division, the dose of *SMN1* cDNA in the dividing tissues is halved. That is not the case for the crucial non-dividing motor neurons, which means that long term, the peripheral effects of Zolgensma are going to fade away. It can only be given once due to mounting immunity against the AAV9 capsid. Following successful preclinical development, several clinical trials were carried out in type I patients (Day et al. 2021; Mendell et al. 2017) (SPR1NT -NCT03505099, STR1VE -NCT03306277, START -NCT02122952). Approved by the FDA in 2019 for children under 2 years old (FDA 2019) and by the EMA in 2020 for

SMA patients with 2 or 3 *SMN2* copies under 21 kg, it is the second *in vivo* gene therapy after Luxturna, and is regarded as the most expensive drug in the world with the price of $ 2.1 million per dose. The main barrier, expect for the price, is the AAV9 dose, which is proportional to body weight, as serious adverse events, especially liver toxicity including liver failure and thrombotic microangiopathy (TMA), thrombocytopenia and cardiac toxicity are more common in heavier patients (Chand et al. 2021) (and as per Zolgensma drug label). An intrathecal delivery was being studied to decrease the dose of AAV but the trial was put on hold due to pathology of the dorsal root ganglia (DRG) sensory neurons in non-human primates (Hinderer et al. 2018). However, even if successful, that route of delivery is going to only target the CNS.

Risdiplam (Evrysdi, Roche)

Risdiplam is a small molecule, which like nusinersen targets *SMN2* splicing (Naryshkin et al. 2014; Ratni et al. 2018). Risdiplam promotes the inclusion of exon 7, binding selectively to two sites in *SMN2* pre-mRNA: the exonic splicing enhancer 2 in exon 7 and the 5′ splice site in intron 7 (Campagne et al. 2019). It is given orally on a daily basis and is a first ‘home-based’ SMA therapy. Risdiplam was originally identified in a screen of small molecule modulators of *SMN2* splicing and does not seem to affect splicing globally (Campagne et al. 2019; Naryshkin et al. 2014; Ratni et al. 2018). Like Zolgensma, it crosses the blood brain barrier and can reach nearly all tissue types, including the CNS, blood, and muscles (Poirier et al. 2018). Good distribution profile, combined with daily dosing, gives risdiplam the potential for a stable and sustained SMN elevation across tissues over time. It was approved in FDA in 2020 (FDA 2020) and in EMA in 2021 following a clinical programme encompassing the most diverse SMA population. Several clinical trials, including presymptomatic newborns are ongoing. The clinical trials involve:

FIREFISH (NCT02913482) (Baranello et al. 2021; Darras et al. 2021), SUNFISH (NCT02908685) (Eugenio Mercuri et al. 2022), JEWELFISH (NCT03032172), and RAINBOWFISH (NCT03779334). As any other drug, risdiplam is not without its limitations. It is contraindicated in patients with liver conditions, and as mentioned earlier liver is affected in SMA, and those abnormalities are likely to worsen with age, making it unavailable for some patients (Kakazu et al. 2021). Concerns about teratogenicity and male infertility have been raised but so far only observed in animal models (Ottesen et al. 2016). Risdiplam targets splicing and has shown specificity towards *SMN2*, but in large doses it also targets *Forkhead box M1* (*FOXM1)*, a regulator of cell cycle, posing a risk of oncogenesis (Ratni et al. 2018). Price tag of risdiplam is $ 340,000 for a year of treatment.

Limitation of currently approved therapies and a necessity of combinatorial and complex approach

The recently approved therapies have undoubtedly changed the life expectancy and quality of life of SMA patients and their families, especially those treated presymptomatically or shortly after the onset of symptoms. They all elevate SMN protein levels and are most efficacious when administered at earlier stages of the disease. Thanks to the available therapies, new phenotypes are becoming apparent (Mercuri et al. 2020; Wirth 2020). Right now, individuals with SMA are more likely to live a normal or close to normal lifespan, making comprehensive and life-long approach to treatment and care extremely important. All available therapies have their limitations and are far from cure, particularly for individuals in more advanced stages of the diseases and adults. While newborn screening programmes are underway in several countries, the majority of the world population is not covered by them. Some patients do not respond to treatment, and those who do respond after being treated postsymptomatically, remain disabled, have complex multisystemic need and require multidisciplinary care (Lavie et al. 2021; Matesanz et al. 2021).

Despite the large amount of clinical data generated in the last decade, many gaps remain, with the main and obvious one being the lack of long-term follow-up. Unless the SMN enhancing therapies are given presymptomatically, and elevate SMN very efficiently, the neurodegeneration is unlikely to be completely halted throughout the lifespan of the individual. For babies born with two copies of *SMN2*, even treatment in the first weeks of life might not be sufficient to provide full rescue of phenotype (Schwartz et al. 2022). In recent years it has become accepted that SMA is a systemic disorder and systemic elevation of SMN is needed to ensure the best long-term benefit. Suboptimal SMN elevation, combined with the lack of or insufficient targeting of peripheral organs poses

a risk of developing other, CNS related and non-CNS related comorbidities later in life, stressing a need for more therapies, including SMN independent ones.

At the moment, there are no clinical guidelines to help clinicals and affected families choose one treatment over another. A direct comparison of the three therapies is very difficult, due to different inclusion criteria, assessments and outcome measures used in the clinical trials and varying amounts of follow up time.

Adult individuals with SMA remain the most understudied population. The clinical development of SMN enhancing drugs focused mainly on the paediatric population and the vast majority of clinical data on adults comes from the real word. The natural history is lacking and no specific tools to evaluate the adult population have been developed. They are more likely to present with contractures, scoliosis and other concomitant diseases than children. The guidelines on therapy and management that do exists are based on the paediatric population, but are meant to apply to adults as well. Gene therapy is not suitable for adult population and nusinersen delivery is impossible in that group more often than in children. Therefore adults, more than other groups, are going to rely on orally available therapies.

And finally, while none of the treatments provide cure, they are all extremely expensive and all have been deemed not-cost effective (Dangouloff et al. 2021).

In summary, many medical needs remain unmet for SMA patients, who are going to live lives longer than ever before. A comprehensive, multidisciplinary approach to SMA therapies is required as well as new SMN-dependent and in SMN-independent therapies.

Efforts to address limitations of current SMA therapies

Vast research efforts are underway to optimize SMA treatment and care. They include optimizing and combining current therapies, and development of both new SMN-dependent and SMN-independent therapies that can potentially be also efficacious in other neuromuscular diseases.

Optimizing and combining current therapies

Several clinical trials are underway to address optimizing and combining current therapies. Currently nusinersen is administered at 12 mg regardless of patients age and weight.
A DEVOTE study (NCT04089566) is a 2/3 phase clinical trial examining the safety and efficacy of nusinersen at 28 mg and 50 mg with a long-term follow up (ONWARD study - NCT04729907).
Alternative approach is combining currently approved therapies. Right now there is very little data on any additional benefit in motor function progression or ventilation and data available from the real world shows no added benefit (Harada et al. 2020; Mirea et al. 2021).
Nevertheless, several clinical trials are underway. JEWELFISH is an ongoing study assessing safety of risdiplam in patients previously treated with nusinersen, onasemnogene abeparvovec, olesoxime, or R06885247 (RG7800) (Chiriboga et al. 2021). RESPOND (NCT04488133) is an open label phase 4 trial assessing clinical outcomes of 12 mg nusinersen in infants and children with sub-optimal response to gene therapy. Similarly, ASCEND (NCT05067790) is a 3b open label study assessing higher doses of nusinersen in individuals previously treated with risdiplam. No readily available data is currently available for those studies.

Other potential therapies targeting *SMN2* splicing

Methods and compounds targeting *SMN2* splicing continue to be researched. HDAC inhibitors where one of the first studied molecules aimed at increasing SMN protein expression *via SMN2* splicing and are still studied, mostly in combination with nusinersen (Marasco et al. 2022; Pagliarini et al. 2020).

A new antisense oligonucleotide targeting *SMN2* splicing, BIIB115 has been acquired by Biogen from Ionis in early 2022 based on its potential to extend dosing intervals (Biogen 2022). Clinical trials are planned to assess its safety, tolerability and efficacy. Research efforts are also underway to improve ASO chemistry and delivery, including cell-penetrating peptides (Hammond et al. 2016; Osman et al. 2016).

Branaplam, like risdiplam, is a small molecule compound leading to improved exon 7 inclusion (Palacino et al. 2015). Despite promising clinical trial results in infants (NCT02268552), its development has been abandoned by Novartis, due to business reasons and competition of risdiplam, rather than lack of efficiency.

Recently, TEC-1, a new small molecule targeting *SMN2* splicing with better selectivity and less target effects that risdiplam has been reported (Ando, Suzuki, et al. 2020). TEC-1 improves the phenotype of SMA mice while not inducing micronuclear formation and not strongly affecting *FOXM1* splicing, contrary to risdiplam. Another molecule, flunarizine, a calcium channel blocker, has been identified *via* screening to alter splicing events *in vitro*, including intron retention of *SMN* genes (Younis et al. 2010). Further study showed increased SMN localization to Cajal bodies and improved survival of murine SMA motor neurons upon treatment (Sapaly et al. 2018).

SMN-independent therapies

Current therapies have changed phenotypes and lifespan of SMA affected individuals but currently available data shows the need for adjunctive ('SMN plus') therapies to improve the quality of life long term. Due to systemic nature of the disease, SMN independent therapies, for example enhancing muscle function or improving NMJ function, can provide additional benefit and address widely reported muscle fatigue and weakness. Since those therapies do not target the genetic cause of the disease but have a potential to ameliorate disease phenotype, there are also attractive for other neuromuscular disorders beyond SMA.

Muscle targeting therapies

Myostatin is a growth factor, negatively regulating muscle growth. Several compounds to inhibit myostatin are being developed for muscle wasting conditions including SMA, with the most advanced being Apitegromab (SRK-015, Scholar rock) and RO7204239 (GYM329, Roche). Apitegromab is a monoclonal antibody targeting pro-myostatin, which improves muscle mass and strength in animal models (Long et al. 2018). A Phase 2 clinical trial TOPAZ (NCT03921528) assessing its safety and efficacy in SMA type II and type III patients is underway. Interim results presented at the MDA conference in 2021 have shown that intravenous injection of 20 mg/kg of Apitegromab resulted in 60 % of patients achieving a minimum 3 point increase in motor function tests, compared to 27 % of patient in the nusinersen only group (Crawford et al. 2021).
RO7204239 (GYM329) is an antibody binding to a latent form of myostatin and inhibiting its activation which results in muscle mass improvement in three disease models (Muramatsu et al. 2021). MANATEE clinical trial assessing its efficacy and safety

in combination with risdiplam, compared to risdiplam alone, is currently recruiting (NCT05115110).

Another muscle targeting molecule studied in the context of SMA is reldesemtiv (CK-2127107, Cytokinetics), a fast skeletal troponin activator. It prolongs calcium binding to the troponin complex of the skeletal muscle, resulting in reduced energetic cost of contraction and increased muscle contractility (Cheng et al. 2019). Its efficiency has been recently assessed in a phase 2 clinical trial (NCT02644668), where unfortunately only one of 10 primary outcomes, an improvement in maximum expiratory pressure, was met (Rudnicki et al. 2021).

Ameliorating neuromuscular junction pathology

Neuromuscular junction (NMJ) is a specialized synapse between the motor neurons and skeletal muscle fibres. Abnormalities in NMJ development, maturation and function are widely reported in SMA patients and animal models and they can be a cause of muscle weakness and fatiguability, especially in SMA types 2, 3 and 4 (Wadman et al. 2012). Therefore improving NMJ function seems like a viable target. Pyridostigmine is an acetylcholinesterase inhibitor used in the treatment of myasthenia gravis. It slows down degradation of acetylcholine in the synaptic cleft leading to improved cholinergic transmission. It is currently being investigated in a SPACE trial (NCT02941328), a phase 2 double-blind trial in SMA patients and initial results are positive, showing a decrease in fatiguability (Stam et al. 2018).

Another compound targeting NMJ function is 4-aminopyridine (4-AP), used in multiple sclerosis. It is a potassium channel blocker, increasing neurotransmitter release and duration of action potential at the NMJ (Imlach et al. 2012). It has shown improved motor function in preclinical models like flies and *C. elegans* (Sleigh et al. 2011), however it has recently failed to improve ambulation in individuals with SMA in a clinical trial

(NCT01645787) (Chiriboga et al. 2020). Another aminopyridine, amifampridine phosphate (3, 4 DAP) was investigated in phase 2 clinical trial and met the primary outcome of achieving at least a 3-point improvement in Hammersmith Functional Motor Score Expanded (HFMSE) but not the secondary outcomes (NCT03781479) (Bonanno et al. 2022).

Other strategies

The aforementioned therapies and strategies are in the most advanced stages of development but do not exhaust the vast body of research aimed at new improving the lives of patients with SMA. Numerous other strategies are being investigated, including, but not limited to stabilizing SMN protein (Foran et al. 2016), improving neuroprotection (Bertini et al. 2017), targeting endocytosis (Riessland et al. 2017), cytoskeleton (Villalón et al. 2019), and autophagy (Piras et al. 2017). These efforts, combined with currently approved therapies, can contribute to the quickly changing landscape of SMA management and give SMA patients a hope for a better quality of live.

Epigenetics of motor neuron diseases

Motor neuron diseases (MNDs) are rare neurological conditions primarily leading to degeneration of motor neurons. They can affect upper motor neurons originating from the primary motor cortex, lower motor neurons originating from the brainstem and spinal cord, or both. SMA affects lower motor neurons, while another debilitating MND, amyotrophic lateral sclerosis (ALS) affects both the upper and the lower MNs. In recent decades epigenetic mechanisms have been found to be dysregulated in MNDs. The major epigenetic mechanisms investigated in this context are DNA methylation, modifications of histone tails and regulation of gene expression *via* non coding RNAs (Bennett et al. 2018; Cerrato et al. 2020; Kariyawasam et al. 2019).

DNA methylation is an addition of a methyl group to a 5 carbon of cytosine, resulting a formation of a 5-methylcytosine (5-mC), a reaction catalysed by DNA methyltransferases (DNMTs) (Goll and Bestor 2005; Jones 2012). DNA methylation occurs at CpG sites – cytosines followed by guanine. When CpG sites (islands) are methylated near the promoter regions, it leads to gene silencing, while the same methylation in the body of the genes is found in actively transcribed genes (Coppedè 2020).

Histones are the most abundant proteins associated with DNA, forming an octamer. They are critical in the regulation of gene expression and chromatin state (Bannister and Kouzarides 2011). Posttranscriptional modifications (PTMs) of N-terminus of histones determine the open or closed structure of the chromatin and therefore gene expression, and include methylation, acetylation, phosphorylation and ubiquitinoylation, forming a so-called histone code (Berger 2007).

Non coding RNAs can be short (microRNAs - miRNAs) or long (lncRNAs) RNAs, also falling under the umbrella of epigenetic mechanisms, as they can regulate gene expression

posttranscriptionally (miRNAs) or prevent transcription (lncRNAs) (Wilczynska and Bushell 2015).

Epigenetics of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a rare, adult onset motor neuron disease, affecting both upper and lower motor neurons, leading to progressive muscle weakness and paralysis (Grad et al. 2017). In European population it has an incidence of around 2.6 in 100,000 and a mean life expectancy of 2.5 years, due to respiratory failure (Chiò et al. 2009; Longinetti and Fang 2019). Currently approved therapies provide minimal clinical benefit and do not address the cause of the disease. Around 10 % of cases are familiar (FALS) and 90 % are sporadic ALS cases (SALS) (Grad et al. 2017). Four main genes related to ALS are *SOD1, FUS, TARBD* and *C9orf72* (Bigio 2011; Ji et al. 2017; Renton et al. 2011; Van Rheenen et al. 2016). *SOD1* gene encodes for a superoxide dismutase 1, *TARDBP (TAR DNA binding protein)* and *FUS (Fused in sarcoma)* are both coding for RNA-interacting proteins, and *C9orf72* encodes the C9orf72 protein, which is abundant in motor neurons and involved in RNA processing. Hundreds of other genes have been identified to contribute to ALS, together with environmental factors including early-live events, metal exposure and organophosphate exposure (Coppedè 2020; Van Rheenen et al. 2016). It is becoming increasingly clear that epigenetic mechanisms play an important role in ALS formation and progression.

An increase in global DNA methylation levels is observed in DNA from post mortem spinal cords of ALS patients in comparison to unaffected controls, and in the peripheral blood (Figueroa-Romero et al. 2012; Hamzeiy et al. 2018; Tremolizzo et al. 2014). Global methylation levels in a ALS patients with *SOD1* mutations not only show increased global DNA methylation in the blood, but also an increase of methylation levels over time

and disease progression (Coppedè et al. 2018). Overall, levels of methylation are increased in ALS both in motor neurons and in peripheral tissues like blood. Consistently, levels of DNA methyltransferases (DNMTs) are upregulated in the brain and spinal cords of ALS patients, specifically DNMT3A and DNMT1 (Chestnut et al. 2011). On the other hand, major ALS causing genes - *SOD1, FUS* and *TARDBP* are demethylated in ALS tissues (Coppedè et al. 2018; Morahan et al. 2009; Tarr et al. 2019).

Histone tail modifications in ALS has been mostly investigated in the context of histone deacetylases (HDAC) inhibitors and their effect on disease progression. It has been found that on disease onset in ALS mice, histone acetylation is decreased (Rouaux et al. 2003). Several HDAC inhibitors have been studied in ALS models. Pan-HDAC inhibitors like valproate, sodium butyrate and trichostatin A slowed down ALS progression and improved survival in *SOD1* mice (Ryu et al. 2005; Sugai et al. 2004; Yoo and Ko 2011). Class I and II HDACi showed neuroprotective results in FUS models of the disease (Kuta et al. 2020; Rossaert et al. 2019). That is despite the fact that in post mortem ALS tissue no changes to class I and II HDACi have been observed (Dios et al. 2019). C9orf72 mice show global changes in histone modification and chromatin structure (Zhang et al. 2019).

Dysregulation of miRNAs in ALS is a well-established and widely studied phenomenon. A variety of studies have shown a dysregulation in spinal cords, cerebrospinal fluid (CSF), skeletal muscle, and blood of ALS patients (Foggin et al. 2019; Joilin et al. 2019; Lin Wang and Zhang 2020). Four miRNAs overlap between tissues and represent potential biomarkers, namely miR-124-3p, miR-127-3p, let-7a-5p and miR-9-5p (Foggin et al. 2019). Other examples include hsa-miR-451 and has-3935 which are downregulated in SALS patients, and are both involved in the regulation of the MAPK signalling pathway (Chen et al. 2016). In SOD1 mice, a constitutive activation of MAPK pathways in motor neurons and glial cells was connected to MNs degeneration (Tortarolo et al. 2003).

The miRNAs landscape in ALS is extremely complex and further studies are necessary to disentangle their contribution to ALS pathology.

Epigenetics of SMA

The initial link between SMA and epigenetics comes from studies of histone deacetylase inhibitors (HDACi). Histone acetylation is a prevalent posttranslational modification regulating gene expression. Acetylation of N-terminus of core histones results in a more relaxed chromatin structure and leads to increased transcription. That is because the acetylation of lysines imparts a negative charge, relaxing the chromatin. Histone deacetylases are a group of enzymes repressing transcription by the deacetylation of lysine and forming a chromatin structure less accessible to the transcriptional machinery. There two main groups of HDAC, classic HDACs and Sir-2 related HDACs (or Class III HDACs). The classic HDACs are further subdivided into HDAC I, HDAC II and HDAC IV classes. Each of these smaller classes contains enzymes (HDAC1 – HDAC 11), which are targeted by different histone deacetylases inhibitors. More than ten HDAC inhibitors have been studied in the context of SMA, including valproic acid (VPA), phenylbutarate (PBA), benzamide (M344), LBH589 (Panobinostat), suberoylanilidehydroxamic acid (SAHA), trichostatin A (TSA), entinostat, romidepsin, resveratrol and curcumin (Mohseni, Zabidi-Hussin, and Sasongko 2013). The fold increase of FL *SMN2* varied considerably between all of the above compounds, from 0.4 to 10. Several of those inhibitors are thought to act by two mechanisms: by increasing the overall *SMN2* expression, or by facilitating the inclusion of exon 7 into the final transcript of *SMN2*. Valproic acid and phenylbutarate had been the most widely studied for SMA and entered the clinical stage, with results which were generally negative or inconclusive (Kissel et al. 2014; Krosschell et al. 2018; Mercuri et al. 2007). Their lack of specificity, combined with lack

of understanding of their specific mechanism in SMA, limited their translational applicability. Failure in the clinic, combined with the discovery of precise *SMN2* targeting therapies led to a substantial decline in interest and research addressing HDAC inhibitors and SMN upregulation. However research is still ongoing, making use of novel HDACi, like dacinostat, a less toxic hydroxamate-based HDACi (Mohseni et al. 2016), or using a more relevant disease models like neuronal cells derived from induced pluripotent stem cells of SMA patients (Lai et al. 2017). It seems like HDAC inhibitors cannot provide clinically relevant SMN elevation on its own but might be attractive in combination with other SMN-targeting drugs. An added benefit of low dose nusinersen and LBH589 (Panobinostat) has been recently shown in SMA cells (Pagliarini et al. 2020). A recent study from Marasco and colleagues has shown a synergistic effect of nusinersen and valproic acid, revealing a new mechanism of action where nusinersen increases exon 7 inclusion, while at the same time promoting the silencing H3K9me2 mark. Addition of valproic acid results in a change of PTM on that histone lysine from H3K9me2 to H3K9ac2, and enhances exon 7 inclusion by nusinersen (Marasco et al. 2022).
Several other mechanisms of epigenetic regulation of SMN have been uncovered.
A transcriptomic analysis of murine SMA motor neurons has identified chromatin regulator and chromatin methylation gene clusters as altered in the disease (Murray et al. 2015). SMN is a chromatin binding protein, which binds to mono- and dimethylated lysine 79 on histone 3 (K79H3me1/2) a gene expression- and splicing-associated histone modification., an interaction mediated by the Tudor domain. That interaction is abrogated upon knockdown of disruptor of telomeric silencing 1-like (DOT1L), the only known methyltransferase with a specificity to H3K79, an epigenetic mark of active transcription and DNA damage response (Sabra et al. 2013).

It has been reported that *SMN2* promoter has 4 CpG islands, three of which are heavily methylated (Hauke et al. 2009). The level of methylation at specific sites, surrounding transcriptional start site (TSS), correlated inversely with disease type, where samples from SMA type III patients exhibited lower levels of methylation than from type I patients. Transcriptional repressor methyl CpG binding protein 2 (MeCP2) binds to the *SMN2* promoter region in a methylation-dependent manner (Hauke et al. 2009). Different levels of promoter methylation can partially explain why individuals with same *SMN2* copy number exhibit a range of phenotypes. Interestingly, in that study, several non-specific HDACi were able to increase *SMN2* transcription without affecting the methylation levels. A later study of 35 children confirms that *SMN2* methylation levels can modulate transcription and affect SMA phenotype (Cao et al. 2016). In 2013, a genome-wide methylation study was performed on SMA patients and healthy control samples, where the authors identified 40 CpG islands with significantly different levels of methylation. Of those, *Rho GTPase Activating Protein 22 (ARHGAP22), CHM Like Rab Escort Protein (CHML)* and *Solute Carrier Family 23 Member 2 (SLC23A2)* genes are implicated in neuronal development, axon genesis and cytoskeleton dynamics (Zheleznyakova et al. 2013). It has been reported that histones 3 and 4 around the TSS of *SMN2* are heavily acetylated, while histone 3 and 4 upstream of TSS are hypoacetylated. Upon HDACi treatment, upstream histones show a significant increase in the acetylation levels (Kernochan et al. 2005).

Non coding RNAs are also investigated in the context of SMA, as biomarkers of the disease progression and response to treatment and contributors to neurodegeneration. Interestingly miR-431, which has been shown to regulate genes involved in motor axon outgrowth, can potentially contribute to preferential motor neuron vulnerability (Wertz et al. 2016).

Recently two long non coding RNAs (lncRNAs) - *SMN-AS1* and *SMN-AS1**– originating form *SMN* loci have been described (d'Ydewalle et al. 2017; Woo et al. 2017). Both recruit polycomb repressive complex 2 (PRC2) to SMN promoter and ASOs against respective lncRNAs improves SMA mice survival in combination with currently approved ASO (targeting ISS-N1 sequence in intron 7).

Interestingly, one group found that *SMN-A1* knockdown increased SMN pre-mRNA and FL *SMN2* mRNA, but had no effect on Δ7 *SMN2* transcript levels. That discrepancy between FL and Δ7 transcripts may mean, that *SMN-A1* can regulate not only the transcription rate of *SMN* genes but also splicing. Indeed, PRC2 has been shown to regulate splicing of *fibroblast growth factor receptor 3 (FGFR3)* (d'Ydewalle et al. 2017).

Circular RNAs are another class of non-coding RNAs implicated in, amongst others, regulation of transcription and sponging of mi-RNAs. Recently it has been reported that *SMN* loci generate a vast repertoire of circular RNAs, but their specific roles remain to be determined (Ottesen et al. 2019).

Overall, there is an increasing body of evidence that epigenetic mechanisms including DNA methylation and non-coding RNAs are dysregulated in SMA and can potentially contribute to the disease pathology.

Physiology of protein arginine methylation

Protein arginine methylation is a prevalent posttranscriptional modification of proteins, influencing their stability, function and interactions with other macromolecules. It plays important roles in splicing regulation, RNA metabolism, gene transcription, and DNA damage repair (Blanc and Richard 2017). It is estimated that around 4000 of proteins have methylated arginines (Hornbeck et al. 2015). In that group, there is an overrepresentation of RNA-binding proteins (RBPs) and proteins involved in RNA biology, including transcription, splicing and translation.

Methylation occurs at the nitrogen of guanidinium group, which has a positive charge in physiological conditions. S-adenosyl-L-methionine (SAM) is a methyl group donor, converted into S-adenosyl-L-homocysteine (SAH) in the process of arginine methylation. Addition of methyl group does not change the charge, but distributes it across more atoms, therefore affecting hydrophobicity (Evich et al. 2016). It also increases the bulkiness of the amino acid, and decreases the number of hydrogen bonds arginine can form (Fuhrmann, Clancy, and Thompson 2015). Therefore, methylated arginine can regulate the binding of proteins that read that epigenetic mark, like Tudor domain containing proteins and plant homeodomain (PHD) zinc fingers (Gayatri and Bedford 2014), while methylation of histone arginines has an important effect on gene expression (Guccione and Richard 2019).

In mammals, arginine methylation of histone and non-histone proteins is performed by nine enzymes called protein arginine methyltransferases (PRMTs), divided into three families (Bedford and Clarke 2009; Blanc and Richard 2017; Jarrold and Davies 2019). Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8) perform monomethylation followed by an asymmetric dimethylation (ADMA) of arginines, with both methyl groups on one of the guanidino nitrogen atoms. PRMT1 is the primary enzyme, responsible for around 85 % of ADMA in the cell. Type II PRMTs (PRMT5

and PRMT9) catalyse monomethylation, followed by a symmetric dimethylation (SDMA), where one methyl group is transferred to each of the nitrogen atoms, with PRMT5 being the primary one. PRMT7 is the only described member of type III PRMTs and only performs a monomethylation (MMA). It has been recently found that PRMT1 and PRMT5 can also monomethylate their substrates, but this observation needs more in depth studies (Fong et al. 2019; Musiani et al. 2019). PRMTs have an overlapping substrate specificity and therefore only a small number of arginine methylated proteins have been assigned to an individual PRMT (Hornbeck et al. 2015). PRMT1 and PRMT5 are also known to scavenge each other substrates, which means that in the absence of one enzyme, the substrate is methylated by another, complicating matters even further (Dhar et al. 2013). The common PRMTs targets sites are glycine rich RG/RGG motifs (Thandapani et al. 2013).

Methylarginines are recognized by Tudor domains, PHD (plant homeodomains) and WD40 domains. There are more than 35 Tudor domain containing proteins, but only a subset has currently validated interactors. That includes SMN (survival of motor neuron), SPF30 (splicing factor 30), and Tudor domain-containing proteins 1/2/3/6/9/11 (TDRDs) (Chen et al. 2011).

The existence of arginine demethylases is being debated. Several enzymes have been reported to perform arginine demethylation. Jumonji domain-containing 6 (JMJD6) had been reported to decrease ADMA in cells, but later it was proposed it catalyses hydroxylation rather than demethylation (Poulard et al. 2014; Thompson and Fast 2006). KDM4E and KDM5C have been shown to demethylase methyl-arginines *in vitro*, but there is no *in vivo* evidence (Walport et al. 2016). Peptidylarginine deiminases (PADIs) were proposed as demethylases as well, however they convert arginine into citrulline,

and therefore might prevent arginine methylation, rather than directly remove it (Cuthbert et al. 2004).

In the recent decade protein arginine methylation has been a topic of great scientific interest, as PRMTs are thought to play a prominent role in several types of cancer and cardiovascular diseases (Jarrold and Davies 2019; Wu et al. 2021). Their involvement in neuromuscular conditions is emerging as well (Lee et al. 2022).

Role of arginine methylation in pre-mRNA splicing

One of the best recognized roles of PRMTs is regulation of constitutive and alternative splicing, reported for PRMT1 (Zhang et al. 2015), PRMT4 (Cheng et al. 2007), PRMT5 (Bezzi et al. 2013; Braun et al. 2017; Gerhart et al. 2018; Koh et al. 2015), PRMT7 (Gonsalvez et al. 2007) and PRMT9 (Yang et al. 2015). Profiling of arginine methylome has identified several RBPs, including splicing factors (Boisvert et al. 2003; Larsen et al. 2016; Musiani et al. 2019).

PRMT1 has been found to regulate the localization and function of several RBPs. PRMT1 is overexpressed in acute megakaryoblastic leukaemia (AMKL) and correlates with poor patient survival (Zhang et al. 2015). PRMT1 methylates RNA binding motif protein 15 (RBM15), a RBP regulating splicing and RNA export, scheduling it for degradation. Reduced levels of RBM15 result in aberrant splicing and a block of differentiation, a hallmark of AMKL, and therefore targeting PRMT1 can be a promising therapeutic target.

PRMT4 methylates several RBPs including two splicing factors, SAP49 and U1 snRNP C as well as the transcription - elongation repressor CA150 (also known as TCERG1) (Cheng et al. 2007). Interestingly, PRMT4 can also asymmetrically dimethylate Sm proteins which are PRMT5 targets (Cheng et al. 2007).

The most characterized PRMT in the context of splicing is PRMT5, a type II PRMT. As mentioned before, it plays a key role in maturation of snRNPs and ensuring splicing fidelity. Deletion of PRMT5 leads to decreased spliceosome assembly, and alternative splicing: exon skipping and retention of introns with weak 5'-splice acceptor sites (Bezzi et al. 2013; Braun et al. 2017; Gerhart et al. 2018). In neuronal stem cells, depletion of PRMTs leads to mis-splicing of hundreds of genes regulating cell proliferation and signalling (Bezzi et al. 2013). Another example is MDM4 (MDMX), a p53 inhibitor, which is abnormally spliced upon *Prmt5* deletion in murine neuronal cells, leading to a production of a shorter isoform, that cannot inhibit p53 and is degraded *via* NMD (Bezzi et al. 2013). This has been reported in several cancers, including melanoma and haematological malignancies (Dewaele et al. 2016; Gerhart et al. 2018; Koh et al. 2015).

PRMT5 has also been linked to splicing induced by the Myc proto-oncogene during lymphomagenesis. Lymphomas with high MYC expression show high PRMT5 levels, which correlates with poor prognosis. A deletion of a single *Prmt5* allele in a mouse model of lymphoma significantly slowed down tumorigenesis (Koh et al. 2015).

Overall, there is strong evidence indicating PRMTs involvement in splicing regulation.

PRMTs and SMN

As SMN protein contains a Tudor domain, it has unsurprisingly been linked to PRMTs function. PRMT5 is the PRMT that has been most closely linked to SMN. As described previously, one of the main functions of SMN is spliceosome assembly, namely snRNPs biogenesis (Matera and Wang 2014). The SMN complex, consisting of SMN and GEMINs 2-8 is thought to control the cytoplasmic phase of the snRNP assembly. During that process a heptameric ring of Sm proteins undergoes loading onto the correct snRNAs, that have been exported from nucleus. To ensure fidelity of loading, three of seven Sm proteins (SmB, SmD1 and SmD3) undergo symmetric methylation by PRMT5, and that modification is recognized by SMN *via* its Tudor domain (Friesen, Paushkin, et al. 2001; Meister et al. 2001). The loading of Sm proteins onto the snRNAs protects them from nucleases and is necessary for further maturation and nuclear import (Matera and Wang 2014). As mentioned before, mutations in SMN or PRMT5 cause widespread splicing defects.

PRMT5 and SMN have also been found to control termination of Pol II transcription. Symmetric dimethylation of RNA polymerase II (RNAP II) at position R1810 (R1810mes) is recognized by SMN protein, and SMN interacts with senataxin, a DNA-RNA helicase, to resolve R-loops and control termination of RNAP II. Interaction between SMN and senataxin is reduced upon PRMT5 knockdown, suggesting the interaction depends on the dimethylated arginine (Yanling Zhao et al. 2016).

Several studies have looked into the link between PRMT4 (or CARM1) and SMN. Sanchez and colleagues have shown that SMN represses PRMT4 on a translational level (Sanchez et al. 2013). Consequently, due to insufficiency of SMN, PRMT4 levels are upregulated in SMA mice spinal cord and patients' cells. SMA causing mutation in the Tudor domain of SMN abrogates that repression. A more recent study from the same

group demonstrated upregulated PRMT4 levels in skeletal muscle (Gastrocnemius) of an intermediate SMA mouse mode (Ravel-Chapuis et al. 2022). PRMT4 has been found to promote nonsense mediated decay (NMD), as it interacts with regulator of nonsense transcripts 1 (UPF1), a major effector of NMD. Consistent with upregulation of PRMT4 in SMA tissues, PRMT4 NMD targets, like *growth arrest and DNA-damage-inducible protein GADD45 alpha (Gadd45a), activity regulated cytoskeleton associated protein (Arc) and asparagine synthetase (Asns)* are misregulated in the spinal cords of SMA mice (Sanchez et al. 2015). Therefore strategies targeting PRMT4, but not PRMT5 can have a potential to be beneficial in SMA.

PRMTs in other neuromuscular disorders

PRMTs have been found to be involved in muscle strength, function, and development, namely myogenesis, and in neuronal function. Their involvement in neuromuscular and neurodegenerative diseases such as ALS and spinal and bulbar muscular atrophy (SBMA) is emerging.

Amyotrophic lateral sclerosis

Due to the complexity of the disease, epigenetics is widely studied in the context of ALS, not excluding PRMTs. PRMT1 immunoreactivity in post mortem brain tissues is more prominent in ALS patients compared to controls. Cytoplasmic aggregates of fused in sarcoma (FUS) protein or TAR DNA-binding-43KDa (TDP-43)-positive inclusions are a hallmark of the disease and present in 90 % of post mortem brain samples. It has been shown that FUS has over twenty dimethylated arginine residues and is a target of PRMT1 and PRMT8 (Rappsilber et al. 2003; Scaramuzzino et al. 2013; Tradewell et al. 2012). Adenosine dialdehyde (AdOx), a general inhibitor of methylation can restore nuclear transport of ALS-FUS mutants, and so can PRMT1 knockdown (Dormann et al. 2012; Suárez-Calvet et al. 2016), clearing out cytoplasmic aggregates. Studies have shown that unmethylated or monomethylated FUS has a higher affinity to transportin, necessary for nuclear import, than asymmetrically dimethylated FUS (Dormann et al. 2012; Suárez-Calvet et al. 2016). In post mortem tissue, unmethylated and monomethylated FUS is not found, further suggesting that asymmetric dimethylation of FUS is prevalent in ALS and contributes to FUS aggregates in the cytoplasm (Suárez-Calvet et al. 2016).
The cytoplasmic accumulation of protein aggregates increases local protein concentration and can lead to liquid-liquid phase separation (LLPS) of the RNA binding proteins, forming reversible membrane-less structures. When persistent, those structure can have

fibrotic and ultimately cytotoxic consequences (Holt and Schuman 2013). How and why LLPS form is under investigation but it has been found that RGG motives in FUS are necessary for its formation, and methylation of arginines suppresses the LLPS of FUS, making it more complex to disentangle PRMTs role in ALS (Hofweber et al. 2018; Qamar et al. 2018). On one hand, PRMTs prevent nuclear transport of FUS and contribute to cytoplasmic aggregates, but on the other, they prevent the formation of toxic LLPS. Therefore, consequences of PRMT inhibition in ALS can depend on timing and context of inhibition. It has been shown that AdOx treatment or PRMT1 inhibition decreases the cytosolic aggregates if performed 48 h before the transfection of ALS-FUS (Tradewell et al. 2012). The same group showed that if the PRMT1 KD is performed at the same time as ALS-FUS transfection, it has an opposite effect and increases cytoplasmic aggregates, a result corroborated by another group in cortical neurons (Jäckel et al. 2015). It is possible that early PRMT1 KD prevents cytoplasmic aggregates formation but fails to do so if performed simultaneously with ALS-FUS transduction, while exacerbating LLPS of already present ALS-FUS aggregates. That dual effect means employing PRMT1 KD as a therapeutic approach for ALS requires further studies.

Spinal and bulbar muscular atrophy (SBMA)

Spinal and bulbar muscular atrophy is and X-linked motor neuron disease characterized by degeneration of lower motor neurons (Kennedy, Alter, and Sung 1968). It is caused by expansion of CAG trinucleotide repeat in the androgen receptor (*AR*) leading to a toxic gain of function. It has been found that PRMT6 is a coactivator of AR, and methylates two arginines at Akt consensus sites, exacerbating the diseases phenotype in a cell model of SBMA (Scaramuzzino et al. 2015). On the other hand, PRMT6 orthologue inhibition

improved the phenotype in a *Drosophila* model, suggesting PRMT6 has a role in facilitating mutant AR-mediated toxicity.

Other neurodegenerative disorders

New evidence regarding PRMT involvement in neurodegeneration is rapidly emerging. It has been found that β-amyloid leads to PRMT5 repression in neurons, causing apoptosis and PRMT5 provides protection against β-amyloid in a *C. elegans* model of Alzheimer's disease (Quan et al. 2015). In a model of Huntington's diseases (HD), PRMT5 co-localizes and interacts with mutant huntingtin (Htt) and SDMA of PRMT5 target histone residues was reduced (Ratovitski et al. 2015). While involvement of PRMTs in neuromuscular and broader neurodegenerative disorders is still in its infancy, they are emerging as important contributors to the disease pathology. If their clinical development for cancer is successful, it is going to pave the way for other indications.

Clinical value of targeting epigenetic proteins

Epigenetic mechanisms influence fundamental processes in the body, like differentiation and development, and are responsive to changes in the environment. As such, not surprisingly they are dysregulated in numerous diseases like cancer, inflammatory diseases and neuromuscular disorders. They represent promising biomarkers of disease progression and response to treatment (Arrowsmith et al. 2012). In recent years epigenetic protein families like histone deacetylases, protein methyltransferases or lysine demethylases have emerged as druggable enzymes (Scheer et al. 2019; Q. Wu et al. 2019). Epigenetic mechanisms are an attractive target, as they are dynamic, reversible, and can be targeted with orally available, cost-effective small molecules.

Aim of the study

The primary aims of the project are to identify novel small molecule(s) targeting epigenetic mechanisms involved in SMA pathology, characterize their mechanism of action and evaluate therapeutic potential in SMA animal models alone and in combination with nusinersen.

Results chapter 1 - Epigenetic screening of *SMN2* modulators

In order to identify small molecules that selectively modulate *SMN2* pre-mRNA splicing to include exon 7, I have performed a cell-based screen in SMA type II-patient derived fibroblasts carrying three copies of the *SMN2* gene, using a collection of 54 chemical probes from the Structural Genomics Consortium (SGC) collection (**Table *2***) (https://www.thesgc.org/chemical-probes) (Scheer et al. 2019; Q. Wu et al. 2019). This unique library includes compounds targeting key epigenetic regulatory proteins with high degree of potency and selectivity, and a favourable therapeutic index (Ackloo, Brown, and Müller 2017). Each probe is at least 30-fold more selective towards the target over proteins in the same family, the IC_{50} or K_d is below 100 nM, and the probes show a significant cellular activity at 1 µM concentration.

Determination of the highest non-toxic concentration of epigenetic regulators in SMA patient fibroblast cell line

First, the maximum non-toxic concentrations were identified *via* an MTS assay, which informed the concentration used in the screen (**Figure *3***). Briefly, cells were plated in a 96 well plate and after 6 hours treated with increasing concentrations of each small molecule, from 1 µM to 10 µM. After 48 h MTS reagent was added and 4 h later the absorbance was read at 490 nm. The concentration was deemed non-toxic if the absorbance was insignificantly different from the absorbance of untreated cells, the cells looked viable after visual inspection and the confluency of treated cells was comparable to untreated via visual inspection. The vast majority of the compounds were tolerated up to 10 µM, with a few exceptions (**Table *2***). Unless otherwise stated, the concentrations listed in **Table *2*** were used for each respective compound in subsequent experiments.

Effect of epigenetic regulators on SMN mRNA and protein levels in SMA patient fibroblasts

To test the effect of the library of small molecules on SMN transcript and protein levels, patient-derived fibroblasts were incubated with the epigenetic probes for 48 h (RNA) or 72 h (protein), followed by a qPCR or a Western Blot, respectively. The transcripts amplified were: FL *SMN2* (amplicon spanning exons 6, 7 and 8), Δ7 *SMN2* (amplicon spanning exons 6 and 8), Tot *SMN2* (amplicon spanning exons 1 and 2a), and *GAPDH*. In the screen, only one molecule, selective type I PRMT inhibitor MS023, was able to promote exon 7 inclusion in *SMN2* pre-mRNA, as demonstrated by the increase in full length (FL) and not total *SMN2* mRNA levels and concomitant reduction in mRNA transcripts lacking exon 7 (Δ7) (**Figure *4***). MS023 treatment in these fibroblasts also increased SMN protein levels up to 1.6 fold, as determined by Western blot analysis (**Figure *5***). Notably, other compounds, including bromodomain inhibitors (BAY-299, BI-9564, JQ1), lysine demethylase inhibitors (GSK-J1, GSK-LSD), and prolyl hydroxylase domain 2 (PHD2) inhibitor, IOX2, also determined a ≥1.5 fold increase in SMN protein without affecting mRNA levels, suggesting a direct or indirect effect on SMN protein regulation (**Figure *5***). PRMT5 inhibitors GSK591 and LLY-283, and bromodomain inhibitor I-BRD9 slightly reduced FL *SMN2* without affecting Δ7 *SMN2* levels, with GSK591 treatment also resulting in reduction of SMN protein, supporting a mechanism of transcriptional repression (**Figure *4***). No compound changed levels of total *SMN2* transcripts. Overall these results suggest that protein arginine asymmetric and symmetric dimethylation by different classes of PRMTs can exert opposite effects on SMN regulation.

PRMTs are involved in several critical biological functions (Blanc and Richard 2017) and represent a promising therapeutic target for many human diseases from cancer

to neurodegeneration, with eight PRMT inhibitors attaining clinical trial testing in human cancers (Guccione and Richard 2019; Hwang et al. 2021; Yang and Bedford 2013). Altogether the direct effect of PRMT type I inhibition on *SMN2* exon 7 inclusion and the potential for clinical impact of this class of molecules, was a reason to further investigate MS023 as a therapeutic agent for SMA. Another molecule characterized further but to a lesser extend is PHD2 inhibitor, IOX2, due to its effect on SMN protein levels (Results chapter 4).

Small molecule	Target protein family	Specific Targets	Concentration used
GSK8814	Bromodomains	ATAD2, ATAD2B	10 μM
BAZ2ICR	Bromodomains	BAZ2A, BAZ2B	10 μM
GSK2801	Bromodomains	BAZ2A, BAZ2B	10 μM
BAY299	Bromodomains	BRD1, TAF1	10 μM
JQ1(+)	Bromodomains	BRD2, BRD3, BRD4, BRDT (BET)	10 μM
PFI1	Bromodomains	BRD2, BRD3, BRD4, BRDT (BET)	10 μM
IBRD9	Bromodomains	BRD9	10 μM
BI9564	Bromodomains	BRD9, BRD7	10 μM
LP99	Bromodomains	BRD9, BRD7	10 μM
TP472	Bromodomains	BRD9, BRD7	10 μM
GSK6853	Bromodomains	BRPF1	10 μM
NI57	Bromodomains	BRPF1, BRPF2, BRPF3	10 μM
OF1	Bromodomains	BRPF1, BRPF2, BRPF3	10 μM
PFI4	Bromodomains	BRPF1B	10 μM
NVSCER21	Bromodomains	CECR2	1 μM
ICBP112	Bromodomains	CREBBP, EP300	10 μM
SGCCBP30	Bromodomains	CREBBP, EP300	10 μM
SGC0946	Methyltransferase	DOT1L	10 μM
A395	Methyl Lysine Binder	EED	10 μM
UNC0642	Methyltransferase	EHMT2 (G9a), EHTM1 (GLP)	2 μM
GSK343	Methyltransferase	EZH2	10 μM
UNC1999	Methyltransferase	EZH2	5 μM
A366	Methyltransferase	G9a, GLP	10 μM
UNC0638	Methyltransferase	G9a, GLP	2 μM
BAY876	Glucose transporter	GLUT1	10 μM
GSKJ1	Lysine Demethylase	JMJD3, UTX, JARID1B	10 μM
UNC1215	Methyl Lysine Binder	L3MBTL3	10 μM
GSKLSD1	Lysine Demethylase	LSD1	10 μM
T26c	Matrix metalloproteinase	MMP-13	10 μM
GSK864	Dehydrogenase	Mutant isocitrate dehydrogenase 1	10 μM
BAY678	Serine Proteases	Neutrophil Elastase	10 μM
A485	Acetyltransferase	p300, CBP	10 μM
GSK484	Arginine deiminases	PAD-4	10 μM

Small molecule	Target protein family	Specific Targets	Concentration used
NVSPAK11	Serine/threonine-protein kinase	PAK1	10 µM
IOX1	Lysine Demethylase	pan-2-OG	10 µM
BSP	Bromodomains	pan-Bromodomain	10 µM
LMOSES	Bromodomains	PCAF bromodomain	10 µM
GSK4027	Bromodomains	PCAF, GCN5	10 µM
IOX2	2OG	PHD2	10 µM
SGC707	Methyltransferase	PRMT3	10 µM
TP064	Methyltransferase	PRMT4	10 µM
MS049	Methyltransferase	PRMT4,6	10 µM
GSK591	Methyltransferase	PRMT5	10 µM
LLY283	Methyltransferase	PRMT5	10 µM
SGC3027	Methyltransferase	PRMT7	5 µM
MS023	Methyltransferase	PRMTs type I	10 µM
PFI2	Methyltransferase	SETD7	10 µM
PFI3	Bromodomains	SMARCA,PB1	10 µM
PFI5	Methyltransferase	SMDY2	5 µM
BAY598	Methyltransferase	SMYD2	10 µM
BAY6035	Methyltransferase	SMYD3	10 µM
A196	Methyltransferase	SUV420H1/H2	10 µM
OICR9429	WD40	WDR5	10 µM

Table *2*. Library of small molecule epigenetic inhibitors obtained from the Structural Genomic Consortium used in the screen, their specific targets and the concentration established in the viability assay and used in the initial screen.

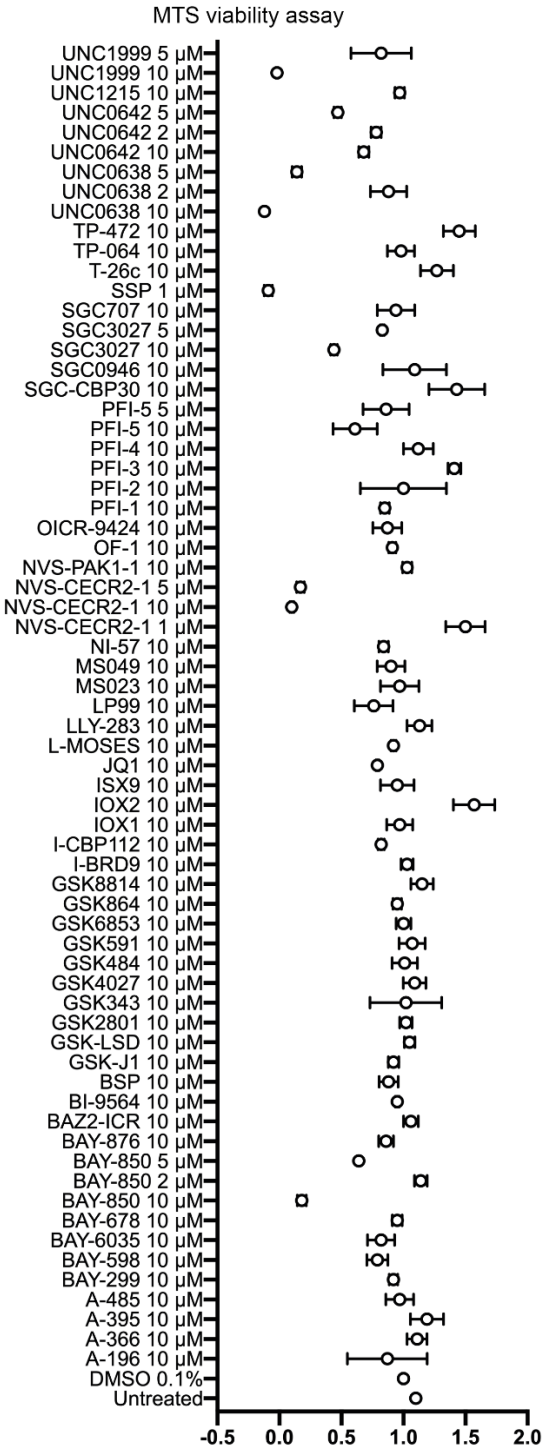


Figure *3*. Effect of the epigenetic small molecules on SMA type II patient fibroblasts. Fraction of metabolically active cells treated with epigenetic small molecules, relative to vehicle-treated cells (0.1 % DMSO), normalized to one, assayed by an MTS assay

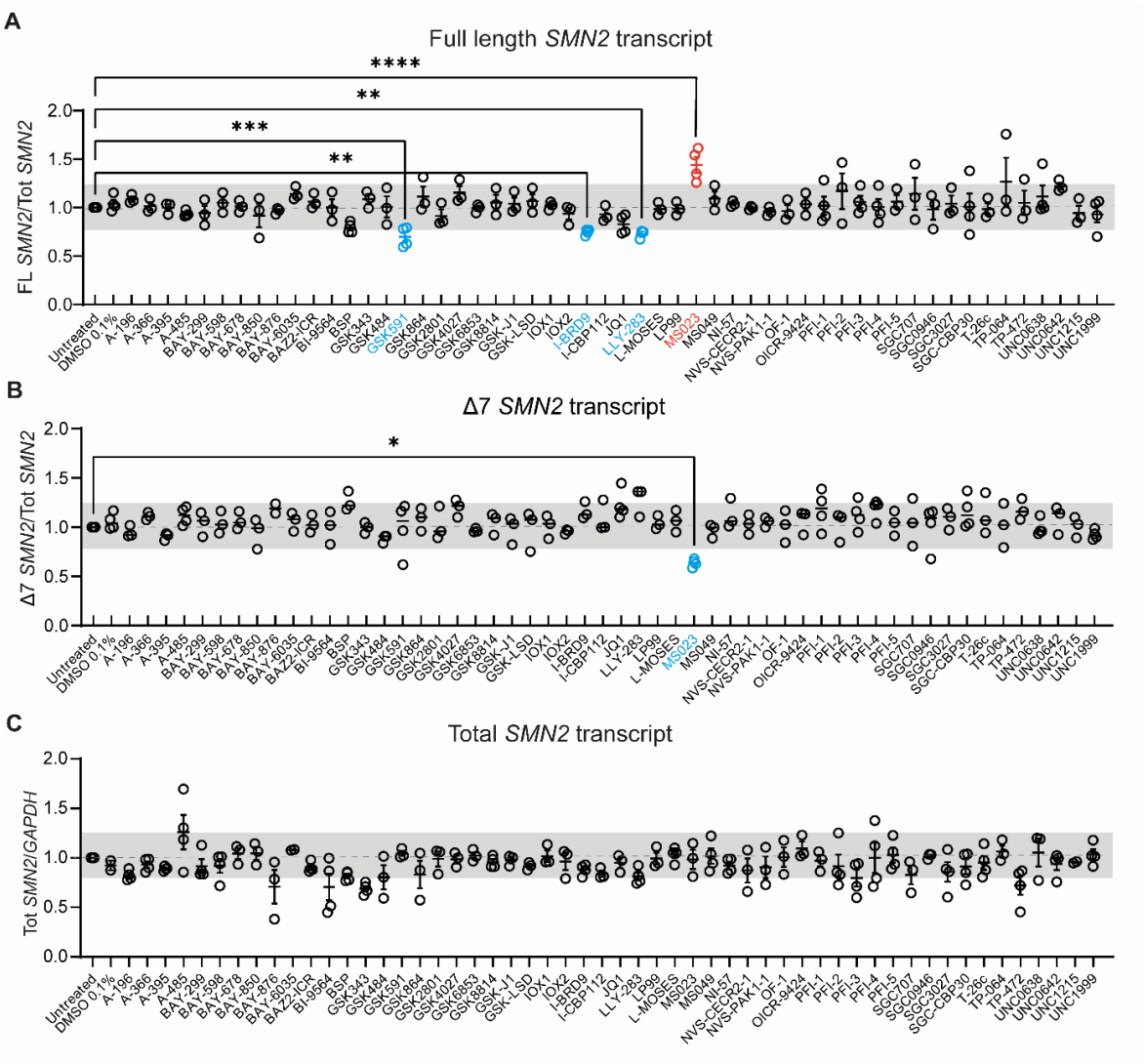


Figure *4*. Screening of epigenetic small molecules in SMA fibroblasts – *SMN2* transcript levels.

SMA type II patient-derived fibroblasts were treated with the indicated small molecule at the appropriate maximum tolerated dose (range: 1-10 µM) (n = 3-4). Cells were harvested for RNA (**A-C**) and protein (**D**) quantification, respectively 48 and 72 hours post treatment. **A**, FL *SMN2* transcript levels relative to Tot *SMN2* are expressed as fold change compared to untreated SMA fibroblasts, normalized to one (dashed line). Each dot represents a biological replicate (n = 3-4). FL *SMN2* / Tot *SMN2* ratio is significantly increased by MS023 and decreased by IBRD9, GSK591 and LLY-283. **B**, Δ7 *SMN2* transcript levels relative to Tot *SMN2* are expressed as fold change compared to untreated SMA fibroblasts, normalized to one (dashed line). Each dot represents a biological

replicate (n = 3-4). MS023 significantly decreases the Δ7 *SMN2* / Tot *SMN2* ratio. **C**, Tot *SMN2* transcript levels relative to *GAPDH* are expressed as fold change compared to untreated SMA fibroblasts, normalized to one (dashed line). Each dot represents a biological replicate (n = 3-4). **A-C**, Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

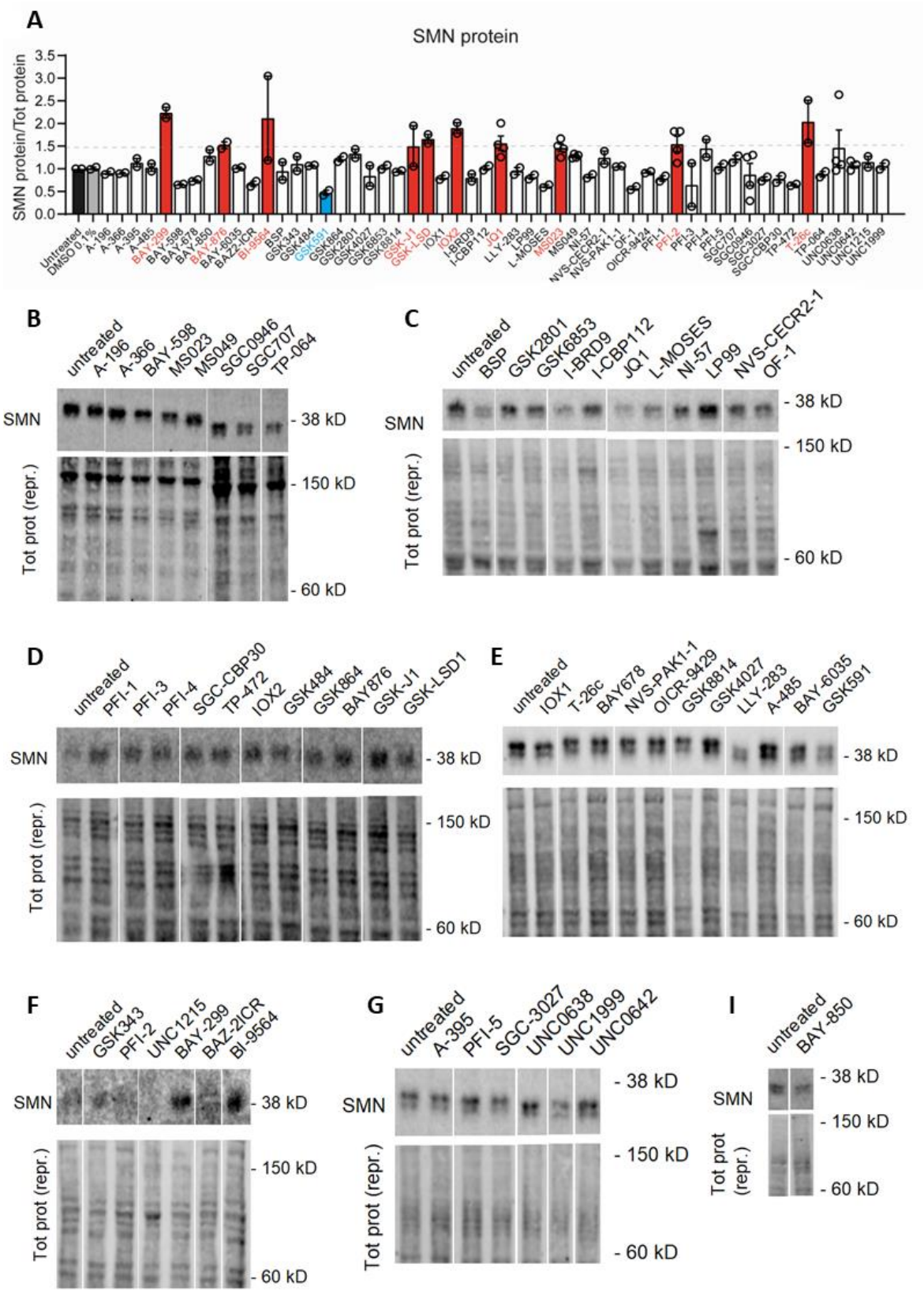


Figure 5. Screening of epigenetic small molecules in SMA fibroblasts – SMN protein

A, SMN protein levels relative to total protein are expressed as fold change compared

to untreated SMA fibroblasts, normalized to one (dashed line). Each dot represents a biological replicate (n = 2-4). Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons. **B-I** Representative western blots show SMN protein levels upon treatment with epigenetic probes (top). A representative section of total protein stain, used for protein normalization, is shown (bottom).

Results chapter 2 - Type I PRMT inhibition promotes exon 7 inclusion in *SMN2* pre-mRNA by decreasing hnRNPA1 binding

After selecting MS023 as a primary molecule of interest, I have endeavoured to further characterize its effect on *SMN2* and its mechanism of action. MS023 is a recently identified potent and selective inhibitor of type I PRMTs harbouring an ethylenediamino group, which is a critical moiety for its activity (**Figure *7*A**) (Eram et al. 2016).

Treatment with MS023 shows a dose response *in vitro*

Treatment of SMA fibroblasts with MS023 led to a dose-responsive increase in both *SMN2* exon 7 inclusion and protein levels and a concomitant decrease of Δ7 *SMN2* transcripts (**Figure *7*B-E)**. This effect was not observed upon treatment with MS094, an inactive MS023 analogue where the terminal primary amino group in the ethylenediamino group is switched to a hydroxyl group (Eram et al. 2016), further confirming its dependency on PRMT activity (**Figure *8*A-B**).

Consequences of PRMT knockdown on SMN expression

To determine if MS023 effect is mediated by a single or a pair of PRMTs, I have checked the relative expression of PRMTs in patients fibroblasts and later on performed a knock-down of each and any combination of two of the type I PRMTs (PRMT 1, 3, 4, 6, and 8) (**Figure *6***). It resulted in no change in SMN transcript or protein levels, suggesting a high degree of functional overlap between these proteins.

MS023 decreases hnRNPA1 binding to *SMN2* pre-mRNA

Subsequently, in order to identify the PRMT substrate mediating the effect of MS023 on *SMN2*, I have interrogated a recently published database of the arginine methyl

proteome in human NB4 cells upon treatment with MS023 (Fong et al. 2019). Out of 72 responsive targets, MS023 affected the methylation of 9 splicing factors reported to affect exon 7 inclusion, including heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) (Fong et al. 2019), which directly binds *SMN2* pre-mRNA across multiple sites and is a well-established negative regulator of exon 7 splicing (Bose et al. 2008; Cartegni et al. 2006; Chen et al. 2008; Hua et al. 2008; Kashima et al. 2007; Kashima and Manley 2003; Kashima, Rao, and Manley 2007; Singh et al. 2013; Xiao et al. 2012). Specifically, treatment with MS023 increased levels of MMA (around four-fold), and decreased levels of ADMA (around-two fold). I therefore hypothesised that MS023-induced alteration in hnRNPA1 methylation pattern affects its binding to *SMN*2 pre-mRNA, resulting in exon 7 inclusion. In order to test this hypothesis, SMA fibroblasts were treated with increasing concentration of MS023 and I observed a dose-responsive reduction of FL *SMN2* transcripts bound to hnRNPA1 by a crosslinking and immunoprecipitation (CLIP) assay (**Figure *7*F**), which validated the working model (**Figure *7*G**). Notably, treatment with MS023 did not change hnRNPA1 levels nor its subcellular localization, further suggesting that the treatment-induced alteration of arginine methylation pattern specifically affects hnRNPA1 binding affinity with its RNA target (**Figure *8*C-D**). Taken together, these findings indicate that MS023 promotes *SMN2* exon 7 inclusion *via* decreasing the binding of hnRNPA1 to *SMN2* pre-mRNA.

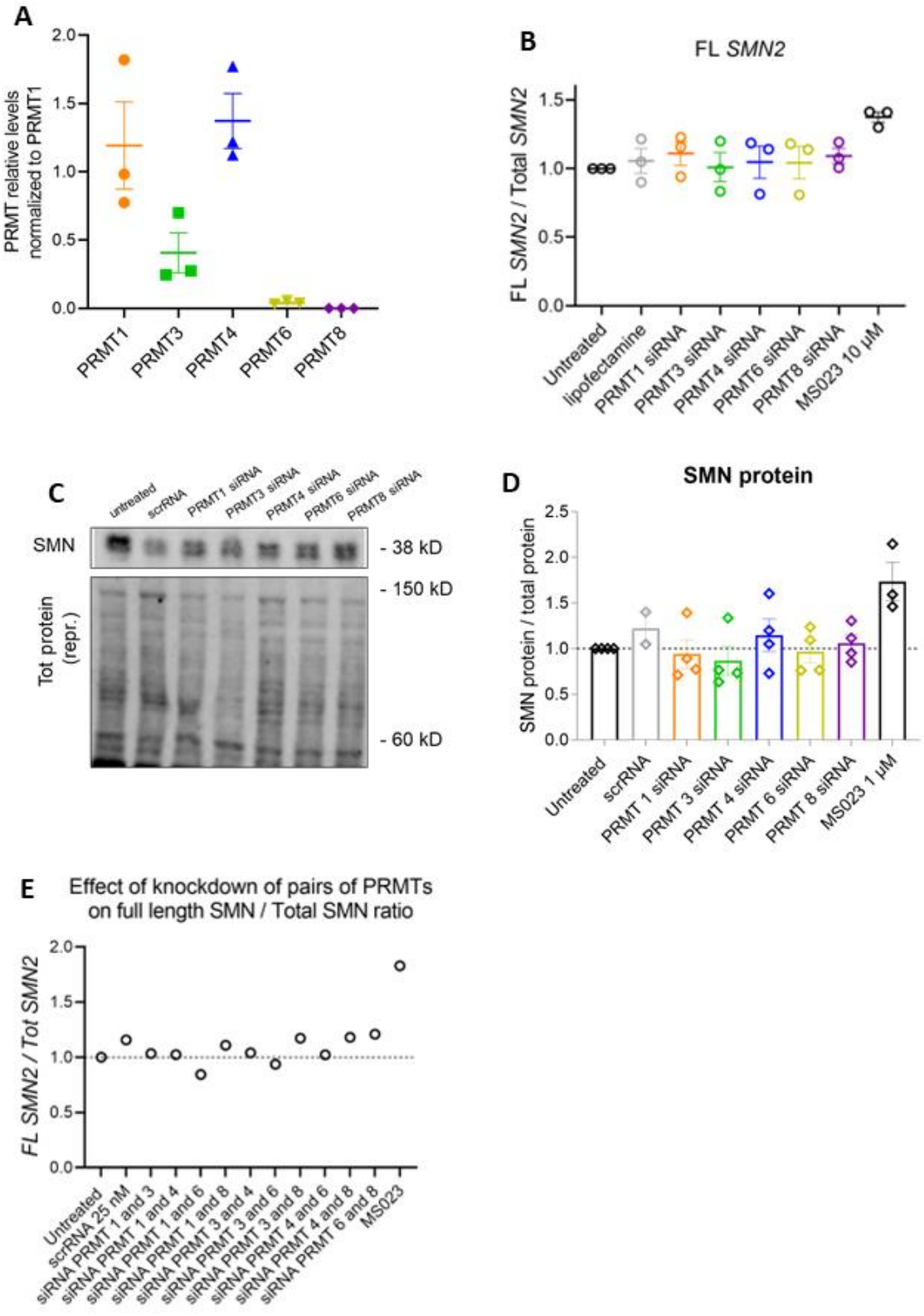


Figure *6*. Effect of type I PRMTs knockdown on SMN transcript and protein.

A Relative type I PRMTs levels in SMA type II patient fibroblasts, each dot represents a biological replicate (n = 3). **B**, FL *SMN2* transcript levels relative to Tot *SMN2* upon knockdown of individual PRMTs are expressed as fold change compared to untreated

SMA fibroblasts, normalized to one. Each dot represents a biological replicate (n = 3). **C**, Western blot shows SMN protein levels upon knockdown of individual type I PRMTs (top). A representative section of total protein stain, used for protein normalization, is shown (bottom). Size in kilodalton is indicated on the right. **D**, Quantification of SMN protein levels related to total protein is shown. Each dot represents a biological replicate (n = 3). **E**, FL *SMN2* transcript levels relative to Tot *SMN2* upon knockdown of pairs of PRMTs are expressed as fold change compared to untreated SMA fibroblasts, normalized to one. Each dot represents a biological replicate (n = 1). **B, D**, Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons.

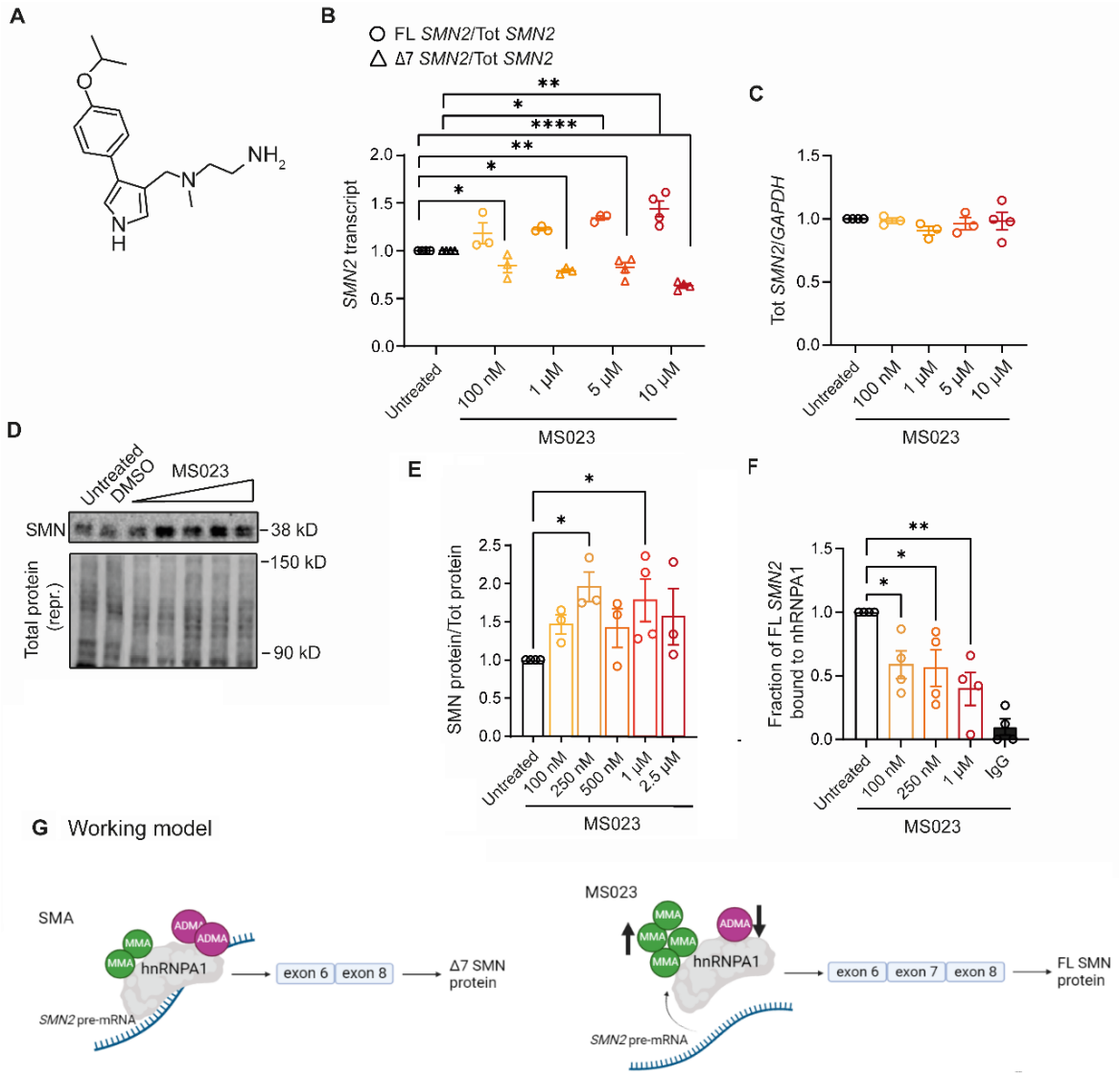


Figure *7*. MS023 increases exon 7 inclusion in *SMN2* transcript *via* hnRNPA1 binding inhibition.

A, Chemical structure depiction of MS023 (PubChem CID: 92136227). **B**, FL *SMN2* (circles) and Δ7 *SMN2* (triangles) transcript levels relative to Tot *SMN2* are expressed as fold change compared to untreated SMA fibroblasts, normalized to one upon treatment with increasing concentrations of MS023. MS023 treatment results in increased exon 7 inclusion into the *SMN2* transcript. Each dot represents a biological replicate (n = 3-4). **C**, Tot *SMN2* transcript levels relative to *GAPDH* are expressed as fold change compared to untreated SMA fibroblasts, normalized to one. Each dot represents a biological replicate (n = 3-4). **D**, Western blot shows SMN protein levels upon treatment with increasing MS023 concentrations (top). A representative section of total protein stain, used for protein normalization, is shown (bottom). Size in kilodalton is indicated on the right.

E, Quantification of SMN protein levels related to total protein is shown. Each dot represents a biological replicate (n = 3). **F**, Ratio of FL *SMN2* transcripts bound to hnRNPA1 protein and relative to *GAPDH* was assayed using crosslinking and immunoprecipitation (CLIP) in SMA patient fibroblasts treated with increasing concentration of MS023. IgG was used a control. Each dot represents a biological replicate (n = 4). **B, C, E, F** Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).
G, Cartoon represents the mechanism of exon 7 inclusion into the *SMN2* transcript by MS023, ADMA – asymmetric dimethylation, MMA – monomethylation.

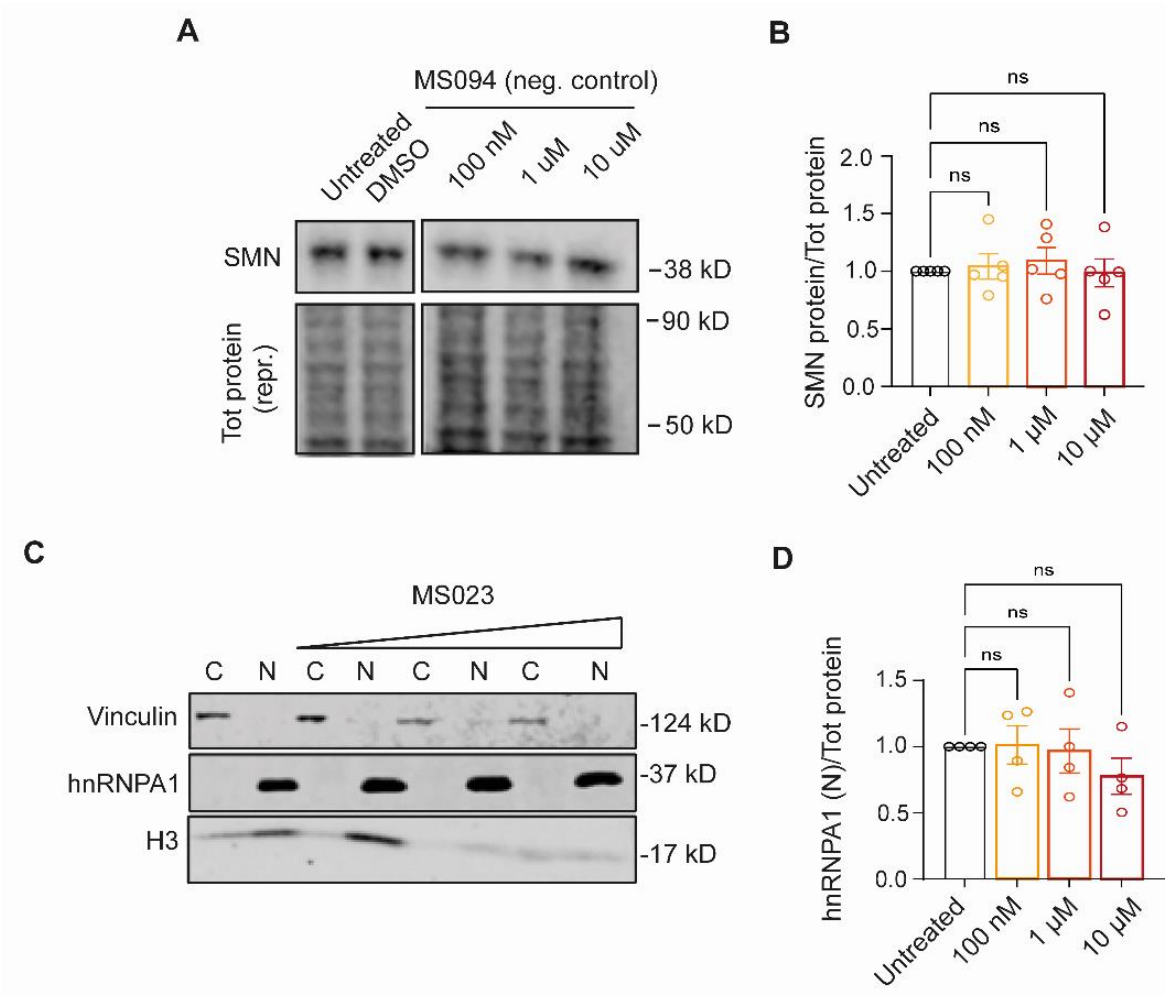


Figure *8*. MS023 has no effect on hnRNPA1 localization or expression levels, MS094, a MS023 negative control has no effect on SMN protein levels.

A, Western blot shows SMN protein levels upon treatment with increasing MS094 (MS023 negative control) concentrations (top). A representative section of total protein stain, used for protein normalization, is shown (bottom). Size in kilodalton is indicated on the right. **B**, Quantification of SMN protein levels related to total protein is shown. Each dot represents a biological replicate (n = 5). **C**, Western blot shows vinculin protein (top), hnRNPA1 (middle) and histone 3 (bottom) levels upon treatment with increasing MS023 concentrations and in cytoplasm (C) and nucleus (N). Size in kilodalton is indicated on the right. **D**, Quantification of hnRNPA1 protein levels related to total protein is shown. Each dot represents a biological replicate (n = 4). **B**, and **D**, Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

Oral administration of MS023 improves the phenotype of SMA mice alone and in synergy with nusinersen.

Next, I evaluated the efficacy and tolerability of MS023 treatment *in vivo* in a severe, Taiwanese, preclinical model of SMA (Hsieh-Li et al. 2000). These mice, which lack the *Smn* gene and only carry a single copy of the *hSMN2* gene, display a phenotype with weight loss and reduced motor activity starting at postnatal day 5 (P5) and typically reach human end point by P8-9.

Oral administration of MS023 alone

Daily oral administration of MS023 or vehicle (0.5 % DMSO in saline solution) was performed in SMA mice from P0 to P11 (**Figure *9*A**). I tested a range of doses (1, 2, 5, and 40 mg/kg) and found that treatment with both 2 mg/kg and 5 mg/kg resulted in significant increase in survival time, with the 2mg/kg dose achieving the best effect (median: 10 days), compared to both untreated (median: 7) and vehicle-treated (median: 6 days) mice ($P < 0.0001$) (**Figure *9*B**). Mice treated with this regimen also showed an improvement in the disease-associated weight loss (**Figure *9*C**). Notably, no further amelioration was observed with 40 mg/kg MS023, hinting that with this dose the therapeutic window has been surpassed (**Figure *9*B, D**). In accordance to MS023 proposed mechanism of action, I observed an increase in FL *SMN2* transcript and SMN protein levels in spinal cords and skeletal muscles, and not brain, kidney and liver of SMA mice treated with 2 mg/kg MS023, possibly reflecting the different levels of expression of hnRNPA1 in these tissues (**Figure *10*)** (Gagné et al. 2021; Liu et al. 2017) .

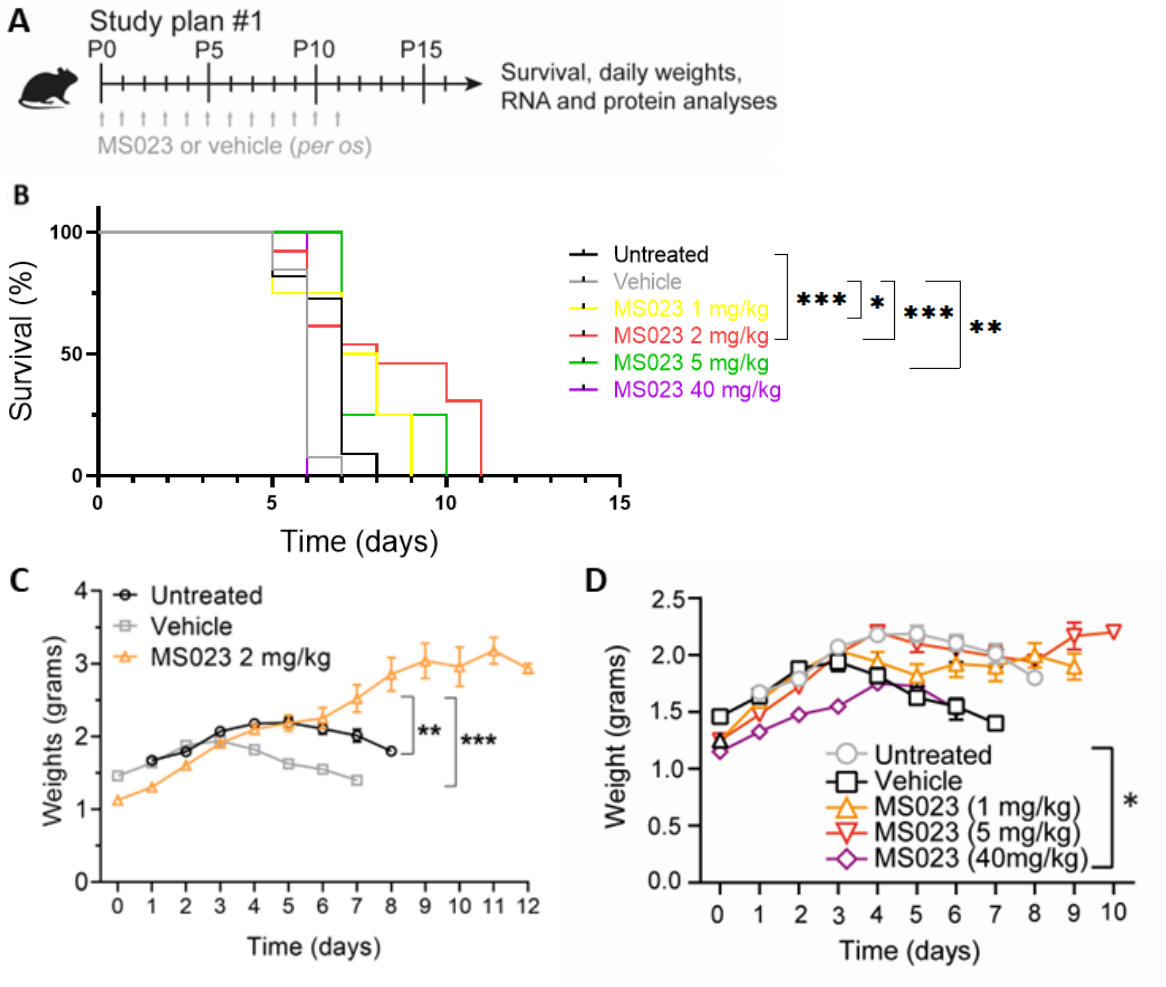


Figure 9. Oral administration of MS023 improves the phenotype of SMA mice.

A, Diagram of study design; Mice were treated daily with oral administrations of MS023 (2m/kg) or vehicle (0.5 % DMSO in saline) from postnatal day 0 (P0) using a Hamilton syringe **B**, Kaplan-Meier survival estimation of untreated (n = 15), treated with vehicle (n = 14), and MS023 (n = 26) SMA mice. **C, D**, Body weights of untreated, MS023- and vehicle-treated SMA mice from postnatal day 0 are shown. **B-D,** Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

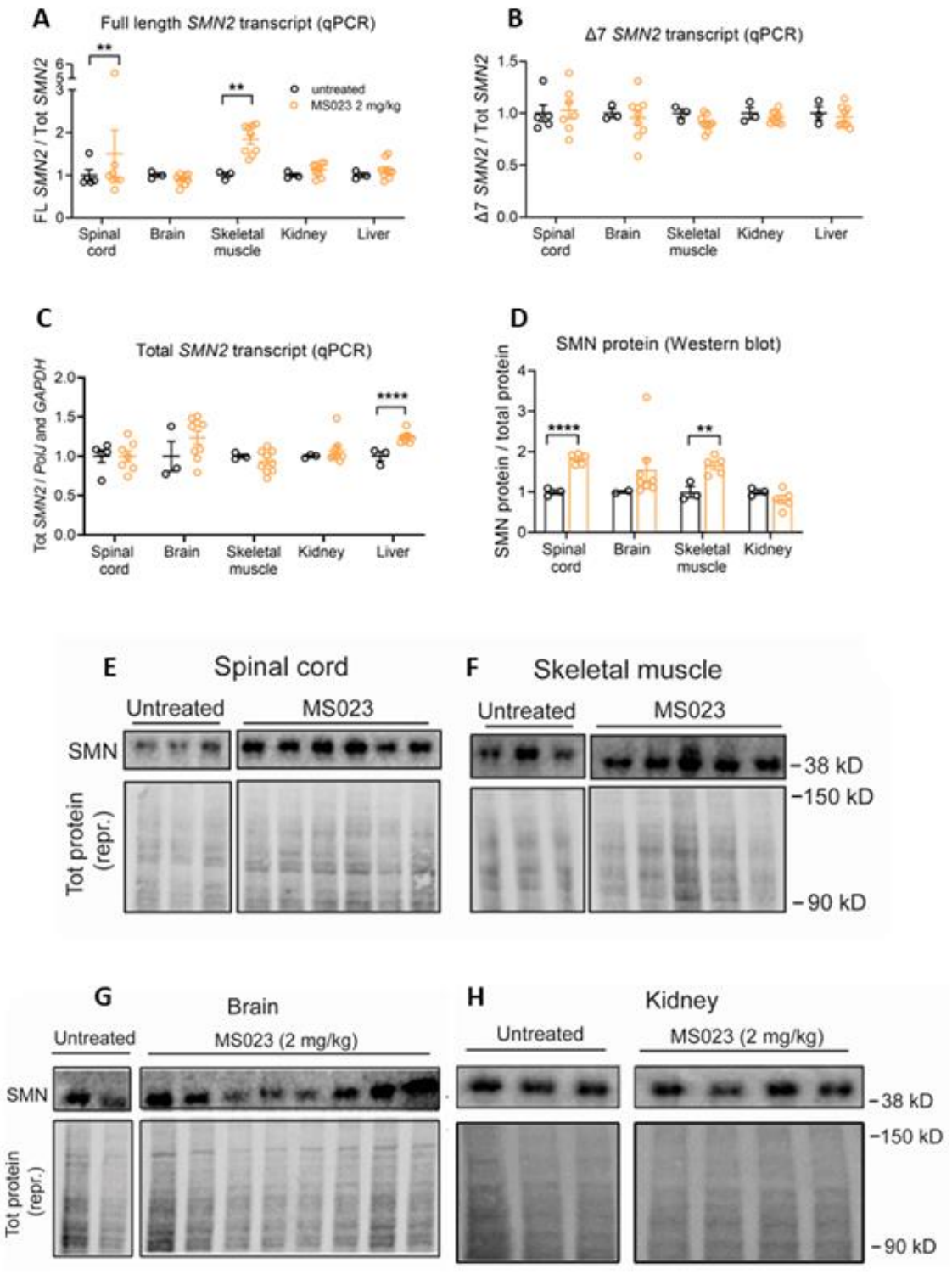


Figure *10*. Effect of oral administration of MS023 on SMN transcript and protein level in severe SMA mouse model.

A, FL *SMN2* and **B**, Δ7 *SMN2* transcript levels relative to Tot *SMN2* in spinal cord, brain, skeletal muscle, kidney and liver of treated mice compared to vehicle-treated mice, normalized to one. Each dot represents a biological replicate (n = 3-9). **C**, Tot *SMN2* transcript levels relative to *PolJ* and *Gapdh* in spinal cord, brain, skeletal muscle, kidney

and liver of treated mice compared to vehicle-treated mice, normalized to one. Each dot represents a biological replicate (n = 3-9). **D**, Quantification of SMN protein levels related to total protein is shown. Each dot represents a biological replicate (n = 3-9). **E**-**H**, Western blots show SMN protein levels upon MS023 treatment in **E,** spinal cords, **F,** skeletal muscles, **G**, brain and **H** kidney of treated mice (top). A representative section of total protein stain, used for protein normalization, is also shown (bottom). Size in kilodalton is indicated on the right. **A**-**D,** Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ****P ≤ 0.0001).

Combinatorial therapy of MS023 and currently approved nusinersen

Since the mechanism of action of the 2′-O-methoxyethyl phosphorothioate-modified drug nusinersen, a currently approved antisense oligonucleotide therapy for SMA patients, consists of promoting exon 7 inclusion in *SMN2* pre-mRNA (Chiriboga et al. 2016; Finkel et al. 2016, 2017; Haché et al. 2016; Mercuri, Darras, et al. 2018), I postulated that a combinatorial treatment with MS023 would synergistically lead to improved therapeutic benefit. In order to test this hypothesis SMA mice were treated at P0 with a single subcutaneous administration of 30 mg/kg nusinersen, a minimum effective dose previously shown to extend the SMA mice life span (Hammond et al. 2016), alone or in combination with oral 2 mg/kg MS023 from P1 to P6 (**Figure *11*A***). Spinal cord, brain, skeletal muscle, kidney and liver were harvested at P7. RT-PCR analysis has shown, that combining nusinersen and MS023 results in increased FL *SMN2* levels in comparison to nusinersen alone in the brain and skeletal muscle of treated animals and decreased Δ7 *SMN2* levels in comparison to nusinersen alone in the kidney (**Figure *11*B-D**). Administration of MS023 and saline significantly increases SMN protein expression in the spinal cord of severe SMA mice in comparison to the vehicle treated mice, but combining nusinersen and MS023 does not increase SMN protein levels to a greater extent than MS023 or nusinersen alone (**Figure *11*E** and **Figure *12***).

A further follow-up study designed to assess the effect on survival function (**Figure *13*A**) revealed that the combinatorial treatment of nusinersen and MS023 dramatically prolonged the lifespan of treated mice (median: 97.5 days) compared to nusinersen alone (median: 19.5; $P = 0.02$) (**Figure *13*B**), and improved body weight of SMA mice (**Figure *13*C**). Overall, these results suggest that oral administration of MS023 synergises with nusinersen to provide a therapeutic benefit in SMA.

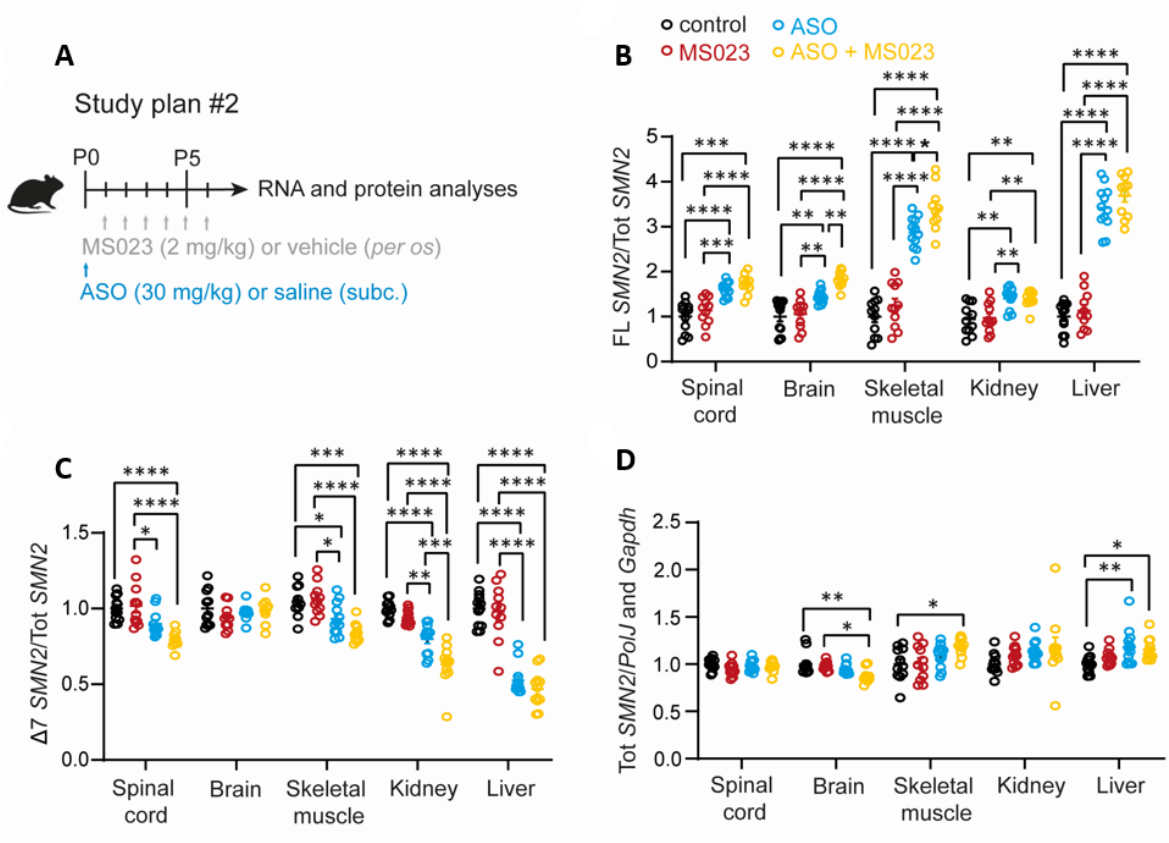


Figure *11*. Combinatorial treatment with MS023 and ASO exerts synergistic effects in SMA mice on RNA levels.

A, Diagram of study design: At P0 SMA mice were injected subcutaneously with *SMN2*- targeting ASO (30 mg/kg) or saline; from P1 mice were treated daily with oral administrations of MS023 (2 m/kg) or vehicle (0.5 % DMSO in saline) using a Hamilton syringe until P6. At P7, mice were sacrificed and tissues collected for analysis.

B, FL *SMN2* and **C**, Δ7 *SMN2* transcript levels relative to Tot *SMN2* in spinal cord, brain, skeletal muscle, kidney and liver of treated SMA mice compared to vehicle-treated SMA mice, normalized to one. Each dot represents a biological replicate (n = 10-12).

D, Tot *SMN2* transcript levels relative to *PolJ* and *Gapdh* in spinal cord, brain, skeletal muscle, kidney and liver of treated SMA mice compared to vehicle-treated SMA mice, normalized to one. Each dot represents a biological replicate (n = 10-12) **B-D**, Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001, ****P ≤ 0.0001).

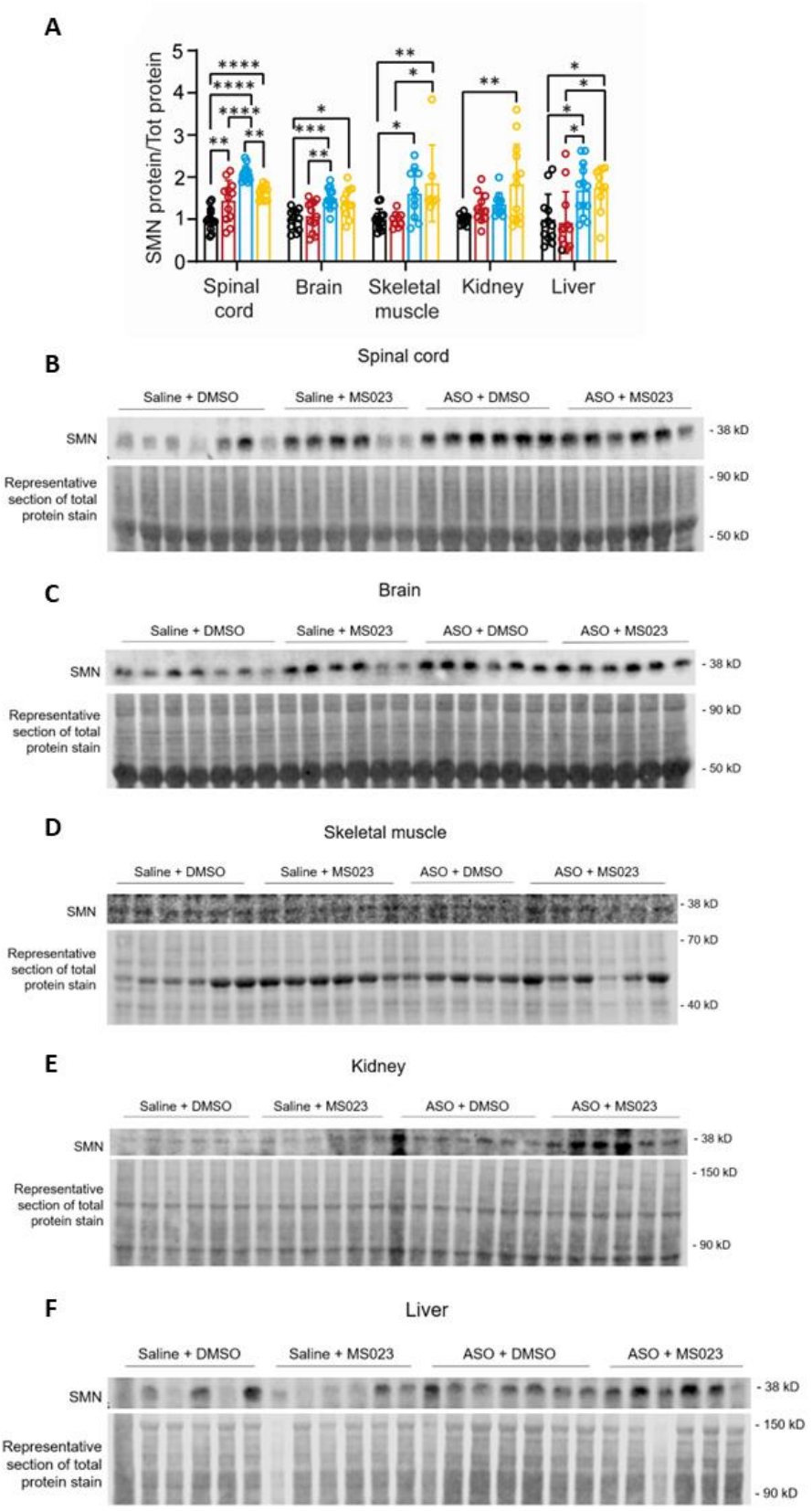


Figure *12* . Effect of combinatorial treatment with MS023 and ASO in SMA mice on SMN protein levels.

A, Quantification of SMN protein levels related to total protein is shown. Each dot represents a biological replicate (n = 7-13). **B-F**, Western blot shows SMN protein levels upon the indicated treatments in **B**, spinal cords, **C**, brain, **D**, skeletal muscle, **E**, kidney, **F**, liver of SMA mice (top). A representative section of total protein stain, used for protein normalization, is also shown (bottom). Size in kilodalton is indicated on the right **A**, Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001, ****P ≤ 0.0001).

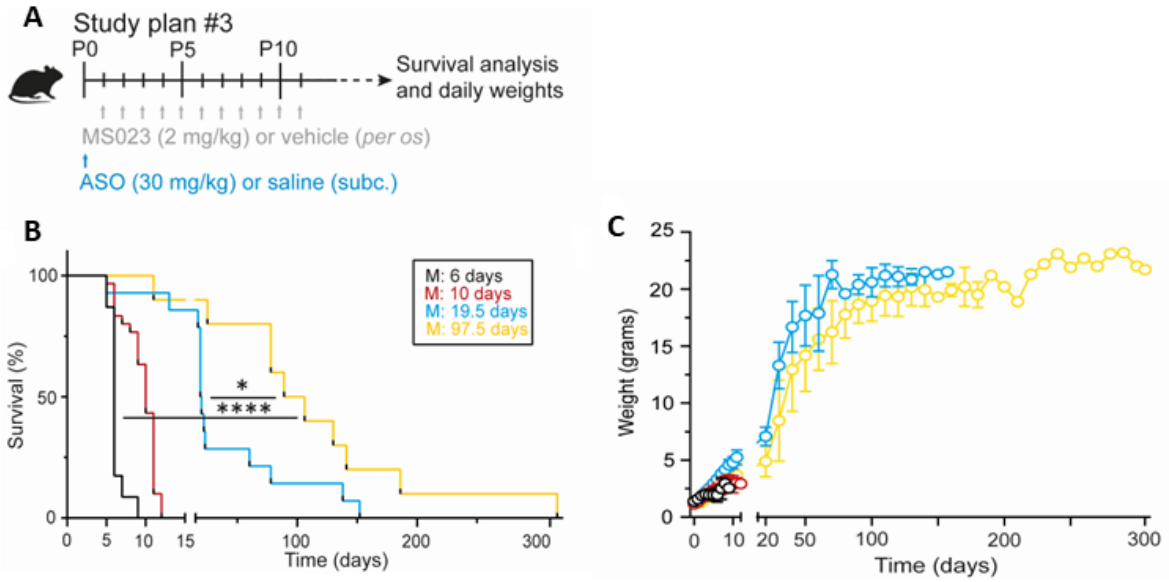


Figure *13*. Combinatorial treatment with MS023 and ASO exerts synergistic effects on survival in SMA mice.

A, Diagram of study design: at P0 mice were injected subcutaneously with *SMN2*-targeting ASO (30 mg/kg) or saline. From P1 mice were treated daily with oral administrations of MS023 (2 m/kg) or vehicle (0.5 % DMSO in saline) using a Hamilton syringe until P11. Mice were weighed daily until they reached their humane end point. **B**, Kaplan-Meier survival estimation of SMA mice treated with *SMN2*-targeting ASO (n = 15), MS023 (n = 14), *SMN2*-targeting ASO and MS023 (n = 10), and vehicles (n = 23). **C**, Body weights of SMA mice from postnatal day 0 are shown. **B,C,** Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; ****P ≤ 0.0001).

Results chapter 3 – Transcriptomic analysis of SMA mouse spinal cords treated with single and combinatorial therapies reveals molecular signature of combinatorial treatment

In order to understand the molecular underpinnings of the added benefit provided by the combinatorial treatment, a bulk transcriptomic analysis was performed in P7 spinal cords of SMA mice treated with MS023 only, nusinersen only, and MS023 in combination with nusinersen, compared to wild type and SMA littermates, following the treatment paradigm described above (**Figure *11*A**). The samples passed all the quality checks required by the company carrying out the sequencing, Novogene (described in materials and methods section). The Pearson coefficient between samples was above 0.95 in all cases (**Figure *14*A**). Principal component analysis reveals clear clustering of untreated sample and control animals along PC1, explaining 16.34 % of the variance. Samples treated with nusinersen, MS023 or both, as expected, all cluster in between the untreated and control animals, but no clear clustering between the three treatment regimens can be observed along PC1 or PC2 (12.20 % of the variance) (**Figure *14*B**).

Molecular signature of the combinatorial treatment

Differentially expressed genes

Between untreated and control animals, I have identified 5509 significantly differentially expressed genes, of which 2874 were upregulated, and 2635 were downregulated (**Figure *15*A**). Out of the 2874 upregulated genes, 2482 (86.4 %), were corrected by both nusinersen treatment and nusinersen and MS023 alone, 75 (2.6 %) genes were only corrected by nusinersen, 192 (6.7 %) were only corrected by the combination and 125 (4.3 %) remained uncorrected. Out of the 2635 downregulated genes, 2060 (78.2 %), were

corrected by both nusinersen treatment and nusinersen and MS023 alone, 130 (4.9 %) genes were only corrected by nusinersen, 167 (6.3 %) were only corrected by the combination and 278 (10.6 %) remained uncorrected.

Genes only corrected by combinatorial approach, but not either therapy alone

Overall, the combinatorial treatment was able to rescue 359 (6.5 % out of 5509) additional genes that were not corrected upon treatment with nusinersen alone, and their correction may be responsible for increase in lifespan (**Figure *14*** and **Appendix *1***, where the full list of 359 genes can be found). I generated a heatmap of the top significant genes ($P < 0.01$) of this category (**Figure *15*B**) and performed a hallmark analysis, to depict and identify the pathways that could explain the added beneficial effects of the combinatorial treatment (**Figure *15***,**C** and **D**). The TNF-α signalling, together with several other immune-related pathways, including the interferon response and complement activation, were highly enriched, overall suggesting that targeting neuroinflammation is key to achieving a beneficial effect in SMA. Interestingly, astrocyte dysfunction and chronic microglia activation have been observed early in SMA and other neurological conditions (Eikelenboom et al. 2002; Heneka, Kummer, and Latz 2014; Sargsyan, Monk, and Shaw 2005; Vukojicic et al. 2019) and their contribution to neuronal dysfunction and death is abundantly supported (Martin et al. 2017; Mcgivern et al. 2013; Rindt et al. 2015; Zhou, Feng, and Ko 2016).

Alternative splicing events

Given the role of SMN in RNA biogenesis and spliceosomal proteins assembly, and the observation of increased mis-splicing events upon SMN depletion (Bäumer et al.

2009; Custer et al. 2016; Doktor et al. 2017; Huo et al. 2014; Zhenxi Zhang et al. 2008, 2013), I wondered whether the beneficial effect of the combinatorial treatment was due to restoration in the splicing profile. 446 mis-splicing events in the spinal cord of SMA mice were identified (**Figure *16***) of which 353 events were skipped exons, 46 were retained exons, 29 were alternative 5' splice sites, 14 were mutually exclusive exons and 4 events were alternative 3' splice sites. The vast majority (nearly 80 %) were skipped exons, in accordance with previous reports (Bäumer et al. 2009; Custer et al. 2016; Doktor et al. 2017; Huo et al. 2014; Zhenxi Zhang et al. 2008, 2013). Both treatments with nusinersen and MS023 alone were able to restore the splicing profile almost fully (347/446 and 368/446, respectively), with a minimal additive effect upon combinatorial treatment (370/446) and only 14 unique splicing events corrected (**Figure *16*E** and **Table *3***) suggesting that mis-splicing has a low threshold for normalization in SMA and is a poor predictor of treatment response.

Of those fourteen genes, several are implicated in neuronal function. *BR Serine/Threonine Kinase 2 (Brsk2)* encodes a serine/threonine-protein kinase, which is involved in axonogenesis and the polarization of cortical neurons. (Kishi et al. 2005) and has been associated with a neurodevelopmental disorder (Hiatt et al. 2019). *Gamma-secretase-activating protein (Gsap*) is a regulator of gamma-secretase activity, which specifically activates the production of β-amyloid protein, a hallmark of Alzheimer's disease (He et al. 2010). *Neuronal cell adhesion molecule (Nrcam)* is required for normal responses to cell-cell contacts in brain and in the peripheral nervous system, and plays a role in neurite outgrowth (Sakurai et al. 2001). It is involved in mediating contact between axons and Schwann cells (Feinberg et al. 2010) and formation and maintenance of nodes of Ranvier (Custer et al. 2003). *NMDA receptor synaptonuclear signalling and neuronal migration factor (Nsmf)* couples NMDA-sensitive glutamate receptor signalling to the

nucleus and triggers long-lasting changes in the cytoarchitecture of dendrites and spine synapse processes (Xu et al. 2010).

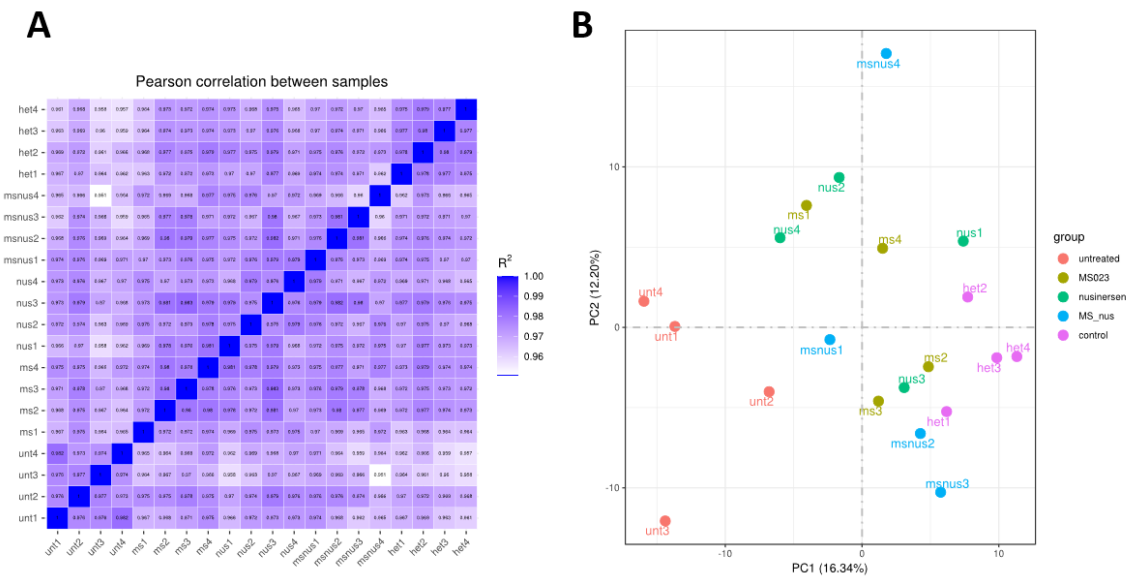


Figure *14*. Transcriptomics quality control analysis.

A, Pearson correlation between samples in transcriptomic dataset. All samples showed a minumum correlation of 0.95 or more. **B,** Principal component analysis of spinal cord samples sent for transcriptomics. PC1 explains 16.34 % of the variance, while PC2 12.20 %. Unt: untreated SMA mice, nus: nusinersen treated SMA mice, ms: MS023 treated SMA mice, msnus: SMA mice treated with MS023 and nusinersen, het:unaffected littermates.

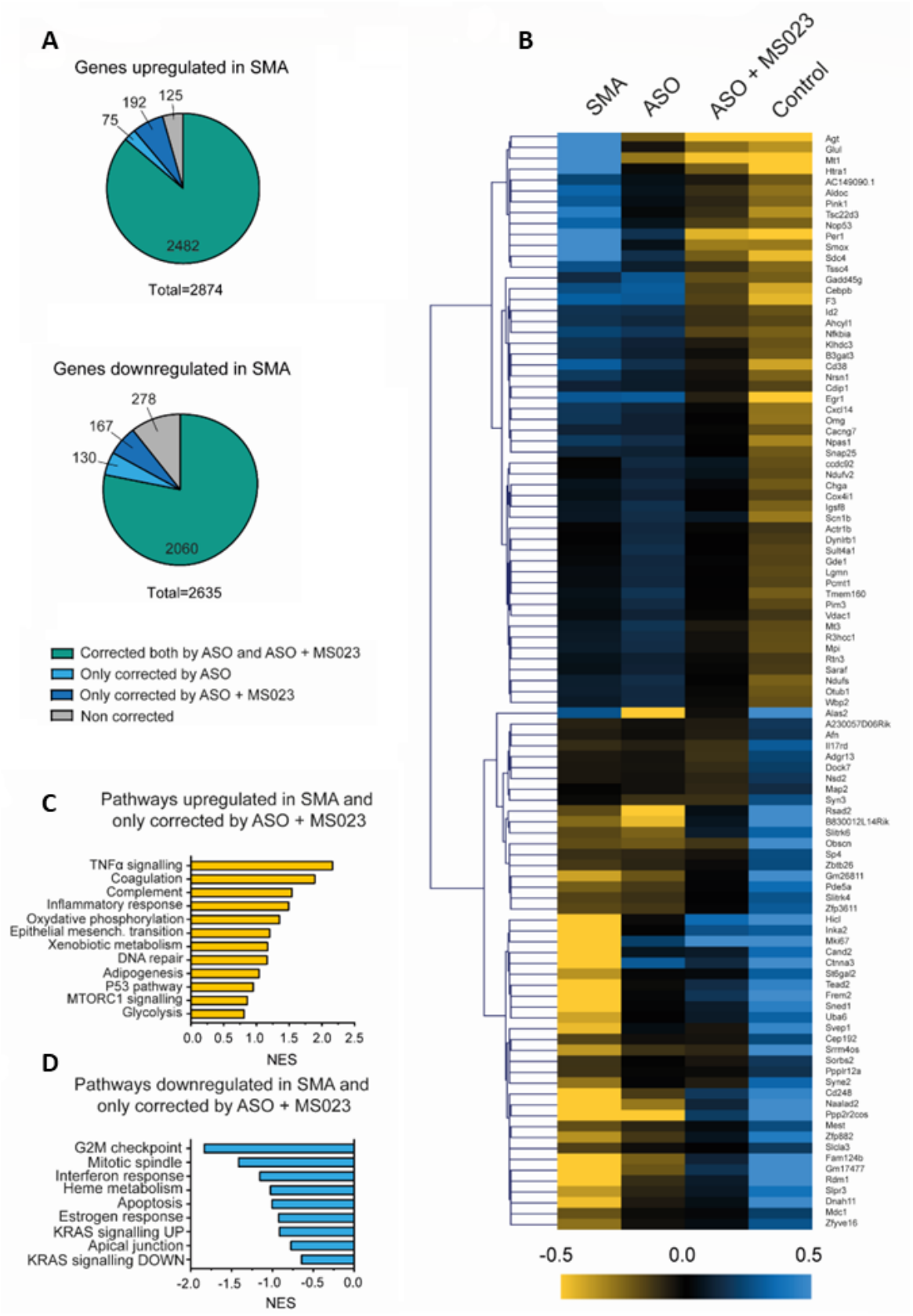


Figure *15*. Combinatorial treatment with MS023 and ASO results in improved correction of the SMA transcriptomic signature compared to ASO alone.

A, Pie charts showing the proportion of transcripts normalised upon the treatments, among the genes that are upregulated (top) and downregulated (bottom) in SMA mice.
B, Normalised counts were used to generate the hierarchical clustering heatmap. Upregulated and downregulated genes are displayed in yellow and blue, respectively.
C, Hallmark pathway analysis of genes upregulated and **D**, downregulated in SMA corrected by the combinatorial treatment only.

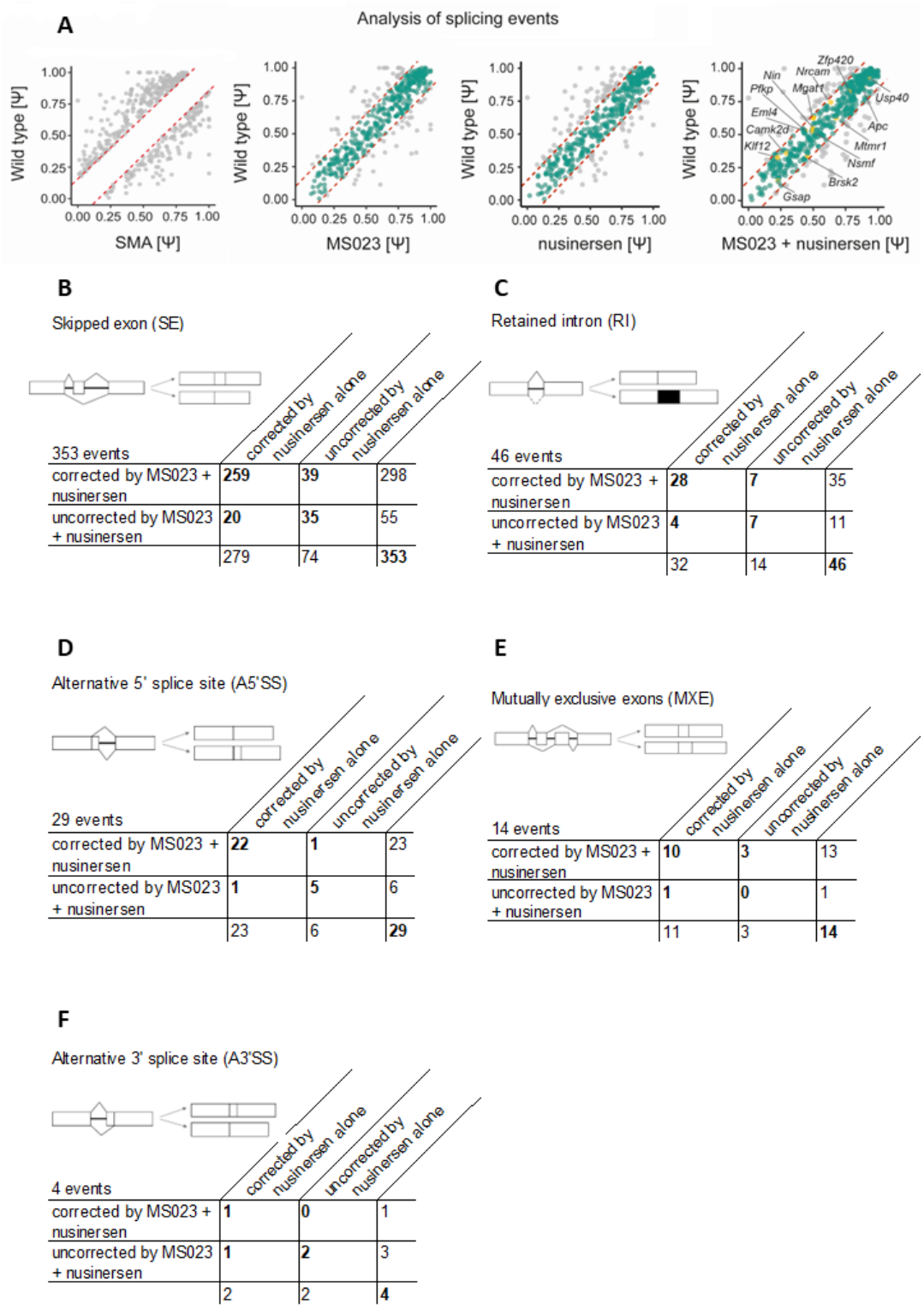


Figure *16*. Analysis of alternative splicing events

A, Plot charts show the distribution of splicing events (Ψ) in SMA mice upon treatment relative to wild type littermates. The red dotted lines mark the ± 15 % normalization range, corrected values are indicated in green. **B-F,** detailed description of individual splicing

events and their correction status upon nusinersen or MS023 and nusinersen treatment. **B**, skipped exon, **C**, retained intron, **D**, alternative 5' splice site, **E**, mutually exclusive exons, **F**, alternative 3' splice site.

Transcript name	Alternative splicing event
Apc	Skipped exon
Brsk2	Skipped exon
Camk2d	Intron retention
Eml4	Skipped exon
Gsap	Skipped exon
Klf12	Mutually exclusive exon
Mgat1	Skipped exon
Mtmr1	Skipped exon
Nin	Skipped exon
Nrcam	Skipped exon
Nsmf	Skipped exon
Pfkp	Skipped exon
Usp40	Skipped exon
Zfp420	Skipped exon

Table *3*. Fourteen transcripts whose splicing was corrected by the combinatorial treatment but not nusinersen alone, and specific alternative splicing event they undergo.

Results chapter 4 - Inhibitor of prolyl hydroxylase domains (PHDs) IOX2 improves the lifespan and weights in a severe mouse model of SMA

Introduction

Hypoxia inducible factors and their regulation

Animals utilize molecular oxygen (O_2) and have developed mechanisms to cope with hypoxia, a state when oxygen demand exceeds supply. Hypoxia response is orchestrated mainly by the upregulation of hypoxia inducible factors (HIFs), transcription factors regulating gene expression in response to hypoxic conditions first described by Semenza and Wang in 1992 (Semenza and Wang 1992; Semenza 2011). HIFs consists of two subunits, HIF-α and HIF-β, and there are three HIF-α homologues (1, 2, and 3, with HIF-1 and HIF2 being the major transcription factors), which form a heterodimer with a constitutively expressed HIF-β (Keith, Johnson, and Simon 2011; P. Lee, Chandel, and Simon 2020). HIF-α is transcribed at a high basal level and its regulation and activity vary depending on the presence of oxygen: in normoxic conditions, two proline residues of the α subunit (HIF-1α: Pro402 and 564; HIF-2α: Pro405 and Pro531) are hydroxylated by 2-oxo-glutarate dependent prolyl hydroxylase domain enzymes (PHD1-3, also known as EGLN enzymes), scheduling HIF-α for degradation (Markolovic, Wilkins, and Schofield 2015) (**Figure *17***). Hydroxylation of HIF-α facilitates its binding to von Hippel-Lindau (VHL) protein, and leads to ubiquitination and proteolysis (Yuan et al. 2003). In hypoxic conditions, due to oxygen deficiency and lack of substrate, PHD enzymes are not able to hydroxylate HIF-α and mark them for degradation, therefore HIF-α forms

a heterodimer with HIF-1β, translocates to the nucleus, binds to hypoxia-responsive elements (HRE) and induces the transcription of several hundred of target genes, exerting cellular response to hypoxia (Schödel et al. 2011) (**Figure *17***). Among the target genes are those involved in angiogenesis, cell survival, glycolysis and invasion/metastasis, the main ones being erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1) (Kim et al. 2006; Lee, Chandel, and Simon 2020; Mole et al. 2009; Semenza et al. 1996).

Another level of regulation is provided by a factor-inhibiting HIF (FIH), which under normoxic conditions hydroxylates HIF asparagine residue (Asn803 in human HIF-1α; Asn851 in human HIF-2α) (Lando et al. 2002). Hydroxylation prevents the binding of HIF to a transcriptional co-activators (CBP/p300), decreasing its transcriptional activity (Lee, Chandel, and Simon 2020). As with prolyl hydroxylation, asparagine hydroxylation is reduced under hypoxia. Together, PHD and FIH are oxygen/hypoxia sensors and key regulators of HIF stability.

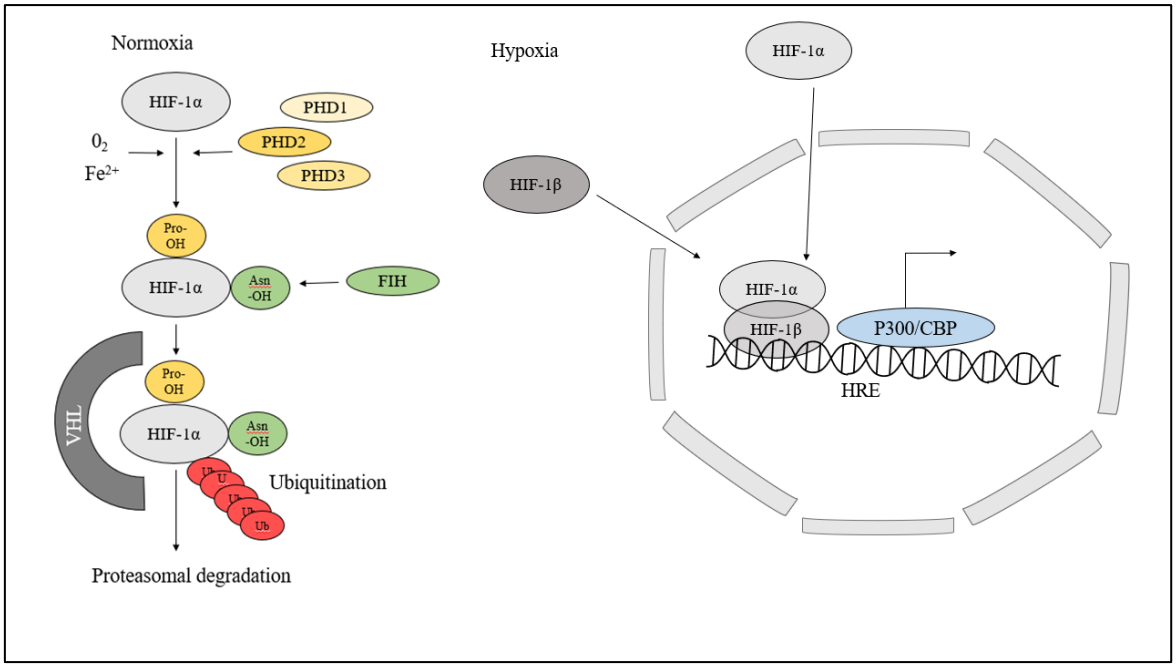


Figure *17*. A schematic of HIF-1 regulation in hypoxic and normoxic conditions.

Prolyl hydroxylase domain proteins (PHDs)

Prolyl-hydroxylase domain (PHDs) enzymes were discovered around a decade after HIF (Bruick and McKnight 2001; Ivan et al. 2001; Jaakkola et al. 2001). They belong to a family of iron (Fe^{2+}) and 2-oxoglutarate (2-OG) dependent oxygenases, spanning over 70 members. All PHD isoforms have a low affinity towards oxygen, between 2-10 above the physiological PO_2, making them sensitive to changes in oxygen concentration and consequently precise oxygen sensors (Rabinowitz 2013).

Three mammalian isoforms of PHDs have been described: PHD1 (EGLN2), PHD2 (EGLN1) and PHD3 (EGLN3), all of which are capable of hydroxylating HIF-α subunits (Bruick and McKnight 2001; Epstein et al. 2001). PHDs have a high homology at the C terminal but not N terminal, and show different tissue expression pattern as well as subcellular localization (Jokilehto and Jaakkola 2010). PHD2 is the most abundantly expressed HIF hydroxylase and its expression is almost ubiquitous, while PHD1

and PHD3 are mostly found in the testes and in the heart, respectively (Wong et al. 2013). Intracellularly, PHD1 locates to the nucleus, PHD2 to cytoplasm and PHD3 is evenly distributed in both (Metzen et al. 2003). They show different affinities towards different HIF isoforms. PHD1 primarily hydroxylates HIF2α in conditions of normoxia, PHD2 hydroxylates HIF-1α in normoxia, and PHD3 shows the highest affinity toward HIF-2α in hypoxia (Appelhoffl et al. 2004). PHD3 is also a target of HIF-1α, which provides a negative regulation of HIF-2α (Rabinowitz 2013).

Unsurprisingly, different PHD isoforms exert different functions in physiology and development, with PHD2 being the main oxygen sensor. PHD1 and PHD3 knockout mice (KO) are viable, while PHD2 KO is embryonically lethal due to vascular and placental defects (Takeda et al. 2006). PHD1 KO mice show reduced tolerance to exercise and a shift towards glycolytic cellular energetics in skeletal muscle and liver. They also show increased hepatocytes proliferation and decreased mammary gland proliferation (Katschinski 2009; Mollenhauer et al. 2012). PHD3 KO mice on the other hand show changes in the central nervous system innervation and decreased resting blood pressure (Bishop et al. 2008). Due to embryonic lethality of PHD2 KO mice, only conditional KO can be employed to study the role of this protein *in vivo*. A somatic deletion of PHD2 at 6 weeks led to elevation of VEGF-A in the serum, increased angiogenesis, over 200-fold increase in EPO levels, and severe polycythaemia (Takeda, Cowan, and Fong 2007). Hematopoietic progenitor cells in liver and spleen were also elevated (Minamishima et al. 2008; Takeda et al. 2008).

Regulation of PHDs activity

PHDs are regulated at several levels, from transcription to their enzymatic activity and while hypoxia is the major inhibitor of their activity, other factors play a role as well.

Intermediates of Krebs cycle (the tricarboxylic acid - TCA) like isocitrate, fumarate and succinate inhibit PHDs catalytic activity, as they compete with 2-oxogluatare for the binding to the active site (Selak et al. 2005; Wong et al. 2013). That competitive inhibition is also a mechanism of action of several PHDs inhibitors used in research, like dimethyloxalylglycine (DMOG).

In prostate cancer PHD2 has been found to be inhibited by microRNA. HIF-1α promotes the transcription of mir182, which in turn inhibits PHD2, providing a positive feedback loop and strengthening HIF signalling (Y. Li et al. 2015).

PHD2 is also positively regulated by HIF itself, as its promoter contains a HRE region. That leads to increased PHD2 levels under hypoxic conditions, which ensures a quick degradation of HIF after re-oxygenation (D'Angelo et al. 2003).

PHDs can be pharmacologically inhibited and are of considerable research and clinical interest for the treatment of anaemia due to chronic kidney disease (CKD), ischaemia, and neurodegenerative disorders. Most of the currently investigated PHD inhibitors (PHIs) are 2-oxoglutarate antagonists, raising safety concerns due to lack of specificity towards PHDs (Chan et al. 2016; Maxwell and Eckardt 2016). Here I have identified a selective small molecule PHD2 inhibitor, IOX2, which is able to increase SMN protein levels and improve the phenotype of severe SMA mice.

Results

Selective PHD2 inhibitor, IOX2, increases SMN protein levels in SMA patient-derived fibroblasts.

From the library of small epigenetic probes (**Table *2*; Figure *5***), IOX2 has shown to increase SMN protein levels in patient fibroblasts, and therefore I decided to further characterize this molecule. IOX2 (chemical structure: **Figure *18*A**) is an inhibitor of PHD2, the primary enzyme hydroxylating HIF-α, scheduling it for degradation. I tested the effect of several IOX2 concentration on *SMN2* transcripts levels and SMN protein in type II patient-derived fibroblasts (**Figure *18*B-F**). IOX2 had no effect on FL, Δ7, or total *SMN2* transcript levels in the cells and resulted in an up to 2-fold increase in SMN protein level that did not reach significance, probably due to high variation between experimental replicates. Despite it not reaching significance, I decided to treat SMA mice with IOX2 to confirm or rule out its effect on SMN protein.

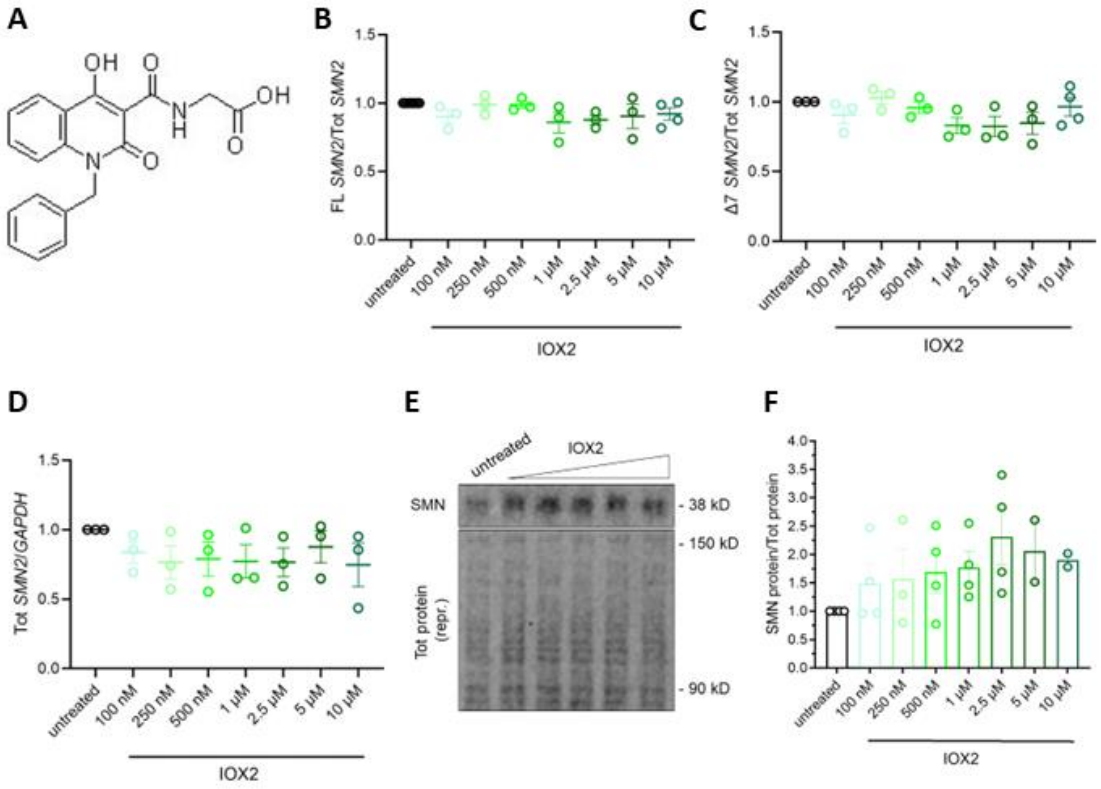


Figure *18*. Effect of IOX2 on SMN transcript and protein.

A, Chemical structure depiction of IOX2 (PubChem CID: 54685215). **B**, FL *SMN2* and **C,** Δ7 *SMN2* transcript levels relative to Tot *SMN2* upon treatment with increasing concentrations of IOX2 are expressed as fold change compared to untreated SMA fibroblasts and normalized to one. Each dot represents a biological replicate (n = 3-4). **D**, Tot *SMN2* transcript levels relative to *GAPDH* are expressed as fold change compared to untreated SMA fibroblasts and normalized to one. Each dot represents a biological replicate (n = 3-4). **E**, Western blot shows SMN protein levels upon treatment with increasing IOX2 concentrations (top). A representative section of total protein stain, used for protein normalization, is shown (bottom). Size in kilodalton is indicated on the right. **F**, Quantification of SMN protein levels related to total protein is shown. Each dot represents a biological replicate (n = 3). **B-D,F,** Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons.

Oral administration of IOX2 improves the phenotype of SMA mice alone and in synergy with nusinersen.

Next, I evaluated the efficacy and tolerability of IOX2 treatment *in vivo* in a severe preclinical model of SMA (Hsieh-Li et al. 2000) (**Figure *19***). Daily oral administration of IOX2 or vehicle (0.5 % DMSO in saline solution) was performed in SMA mice from P0 to P10. A range of doses was tested (0.5, 1, 2 and 5 mg/kg), and treatment with both 0.5 mg/kg and 1 mg/kg resulted in significant increase in survival time, with the 0.5 mg/kg dose achieving the best effect (median: 10 days), compared to both untreated (median: 7) and vehicle-treated (median: 6 days) mice ($P < 0001$). Mice treated with 0.5 and 2 mg/kg also showed an improvement in the disease-associated weight loss. 5 mg/kg of IOX2 has failed to significantly improve survival or weights of mice and therefore I have not tried doses higher than 5 mg/kg. No obvious toxicity was observed.

Based on those positive results I endeavoured to determine whether, like with MS023, combinatorial treatment with nusinersen can have synergistic effects on mice survival, weights, and *SMN2* transcripts and SMN protein levels.

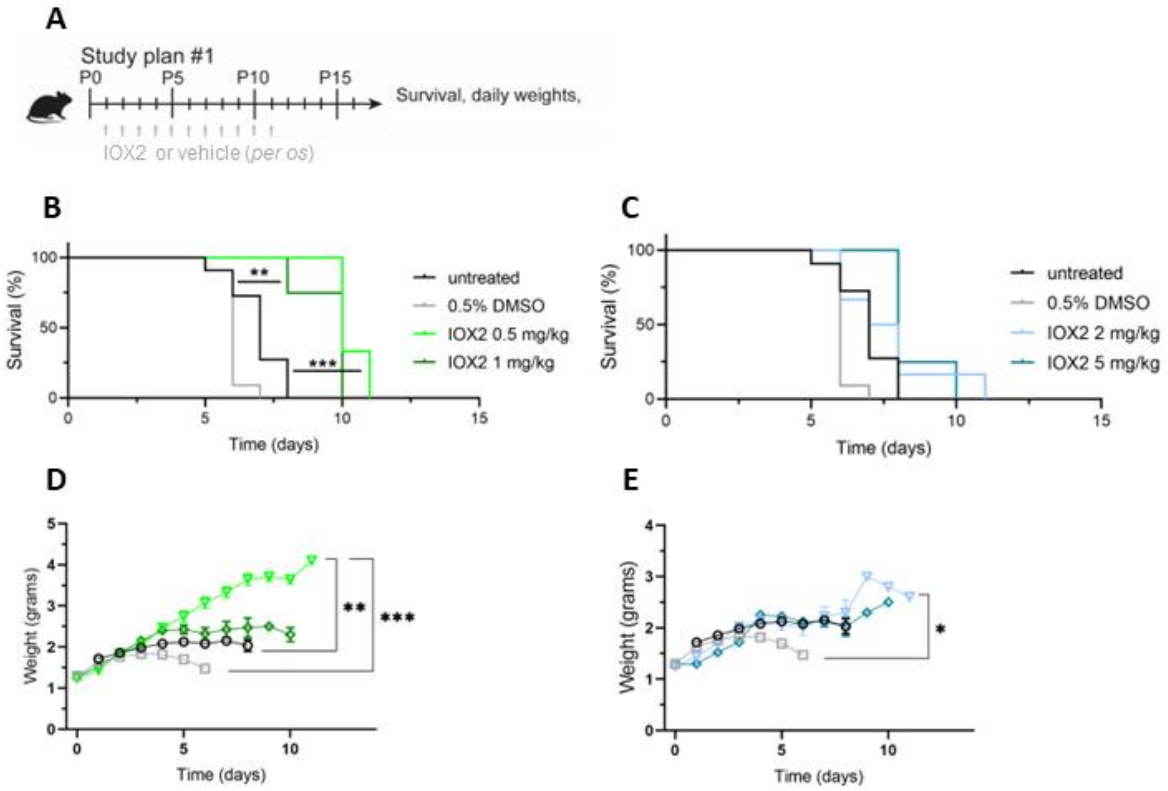


Figure *19*. Oral administration of IOX2 improves the phenotype of SMA mice.
A, Diagram of study design; Mice were treated daily with oral administrations of IOX2 (0.5 m/kg) or vehicle (0.5 % DMSO in saline) from postnatal day 0 (P0) using a Hamilton syringe. **B,** Kaplan-Meier survival estimation of untreated (n = 11), treated with vehicle (n = 11), and 0.5 mg/kg IOX2 (n = 9) and 1 mg/kg IOX2 (n = 4) SMA mice. **C,** Kaplan-Meier survival estimation of untreated (n = 11), treated with vehicle (n = 11), and 2 mg/kg IOX2 (n = 6) and 5 mg/kg IOX2 (n = 4) SMA mice. **D,E,** Body weights of untreated, IOX2- (0.5, 1, 2 and, 5 mg/kg) and vehicle-treated SMA mice from postnatal day 0 are shown. **B-E**, Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

SMN2 transcript and SMN protein levels upon IOX2, nusinersen and combinatorial treatment

As with MS023, I postulated that a combinatorial treatment of IOX2 with nusinersen would synergistically lead to improved therapeutic benefit. In order to test this hypothesis SMA mice were treated at P0 with a single subcutaneous administration of 30 mg/kg nusinersen, a minimum effective dose previously shown to extend the SMA mice lifespan (Hammond et al. 2016), alone or in combination with oral 0.5 mg/kg IOX2 from P1 to P6 (**Figure *20***). Spinal cord, brain, skeletal muscle, kidney and liver were harvested at P7. Treatment with IOX2 significantly increased the levels of FL *SMN2*/Tot *SMN2* ratio in all harvested tissues compared to untreated animals, but on a molecular level, combinatorial approach provided no added benefit compared to nusinersen alone (**Figure *20*B-D**). Changes in Δ7 *SMN2*/Tot *SMN2* ratio were observed only in the liver, and were decreased upon nusinersen and the combination treatment, but not IOX2 alone. No changes to Tot *SMN2*/*Polj* and *Gapdh* were observed in spinal cord, kidney and liver. A slight increase in the Tot *SMN2* was observed in the brain upon nusinersen administration and in skeletal muscle upon nusinersen and combination therapy compared to IOX2 treated animals, but not untreated.

Levels of SMN protein were significantly increased in the spinal cord and brain of treated animals upon IOX2 treatment alone, and on molecular level, there was no additional benefit of the treatments when used in combination (**Figure *20*E-J**). Surprisingly, no changes in protein were observed in skeletal muscle, kidney and liver upon any of the treatments. No increase in SMN protein levels was observed in mice treated with nusinersen at P0, which was surprising and not consistent with previous results (Hammond et al. 2016).

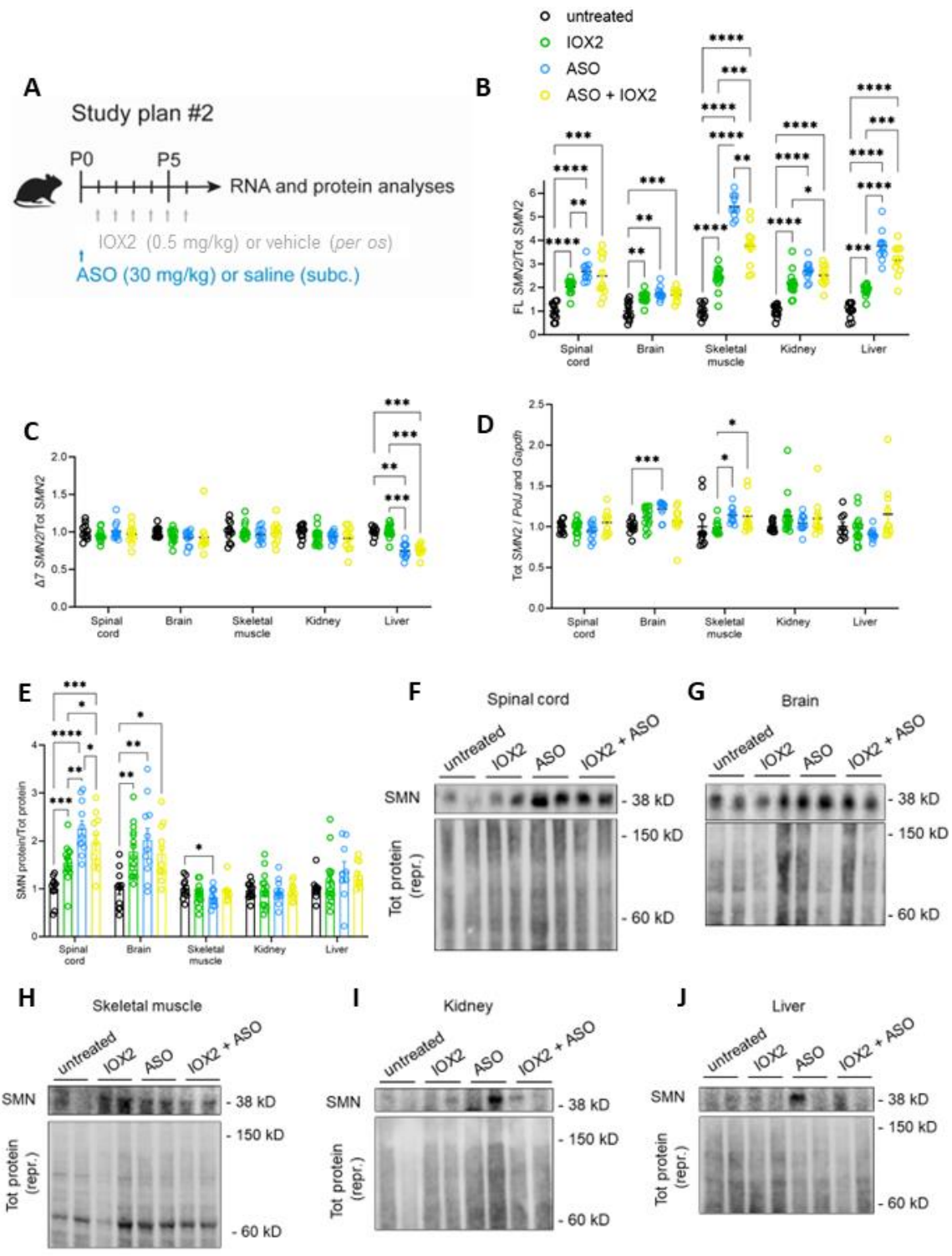


Figure *20*. Effects of the combinatorial treatment with IOX2 and ASO on SMN levels in mice.

A, Diagram of study design: At P0 SMA mice were injected subcutaneously with *SMN2*-targeting ASO (30 mg/kg) or saline; from P1 mice were treated daily with oral administrations of IOX2 (0.5 m/kg) or vehicle (0.5 % DMSO in saline) using a Hamilton

syringe until P6. At P7, mice were sacrificed and tissues collected for analysis.
B, FL *SMN2* and **C**, *Δ7 SMN2* transcript levels relative to Tot *SMN2* in spinal cord, brain, skeletal muscle, kidney and liver of treated SMA mice compared to vehicle-treated SMA mice, normalized to one. Each dot represents a biological replicate (n = 10-12).
D, Tot *SMN2* transcript levels relative to *PolJ* and *Gapdh* in spinal cord, brain, skeletal muscle, kidney and liver of treated SMA mice compared to vehicle-treated SMA mice, normalized to one. Each dot represents a biological replicate (n = 10-12). **E**, Quantification of SMN protein levels related to total protein is shown. Each dot represents a biological replicate (n = 10-14). **F,** Western blot shows SMN protein levels upon the indicated treatments in **F**, spinal cords, **G**, brain, **H**, skeletal muscle, **I**, kidney and **J**, liver of SMA mice (top). A representative section of total protein stain, used for protein normalization, is also shown (bottom). Size in kilodalton is indicated on the right. **B-C,** Data are represented as mean ± s.e.m. and compared with a one-way ANOVA test with multiple comparisons (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001)

Combinatorial treatment of IOX2 and nusinersen shows synergistic effects on survival

In parallel to tissue collection I have carried out a survival study to determine if there are any positive effects on survival and weights upon combinatorial treatment with nusinersen and IOX2 (**Figure *21*A**). As expected, treatment with IOX2 or nusinersen alone prolongs lifespan of mice (median survival 10 and 19.5 days respectively) compared to untreated (median: 6 days, $P \leq 0.0001$). Likewise, combinatorial therapy significantly improved survival of mice in comparison to nusinersen alone (median: 126 days, $P \leq 0.0001$), with several mice still being alive for over 300 days and at the time of writing the thesis (**Figure *21*B**). No differences in weights between the ASO-treated and the IOX2- and ASO-treated groups were observed (**Figure *21*C**).

In conclusion, I have identified IOX2, a PHD2 inhibitor to significantly increase SMN protein levels in spinal cord and brain of SMA mice and to improve their lifespan significantly, alone or in combination with a currently approved therapy – nusinersen. The mechanism *via* which inhibition of PHD2 leads to elevation of SMN protein in mice remains to be established and is the object of future work.

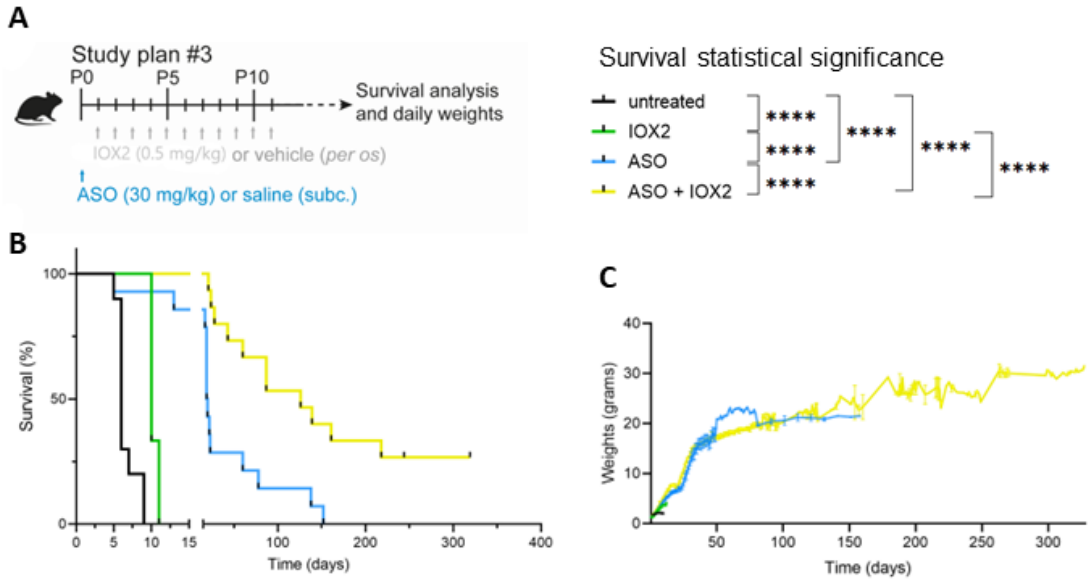


Figure *21*. Effect of combinatorial treatment with IOX2 and ASO on survival of SMA mice.

A, Diagram of study design: At P0 mice were injected subcutaneously with *SMN2*-targeting ASO (30 mg/kg) or saline. From P1 mice were treated daily with oral administrations of IOX2 (0.5 m/kg) or vehicle (0.5 % DMSO in saline) using a Hamilton syringe until P12. Mice were weighed daily until they reached their humane end point. **B**, Kaplan-Meier survival estimation of SMA mice treated with *SMN2*-targeting ASO (n = 15), IOX2 (n = 9), *SMN2*-targeting ASO and IOX2 (n =14), and vehicle (n = 23). **C**, Body weights of SMA mice from postnatal day 0 are shown. **B-C** Data are represented as mean ± s.e.m. and compared with a one way ANOVA test with multiple comparisons (****$P \leq 0.0001$).

Discussion, conclusions, future directions

I have identified IOX2, a small molecule epigenetic probe and inhibitor of prolyl hydroxylase domain 2 (PHD2), a primary regulator of HIF mediated hypoxia signalling, to upregulate SMN protein levels *in vitro* and *in vivo* and prolong survival of severe SMA mice, alone, and in combination with nusinersen. I have observed a discrepancy in *SMN2* transcripts levels *in vitro* (no change) and *in vivo* (increase of FL *SMN2*/Tot *SMN2* in all tested tissues). As mentioned before discrepancies between *SMN2* RNA and protein from the same materials have been observed before in the context of SMA, which may explain the observation of SMN protein increase *in vitro*, but not RNA. Further studies are needed to address this discrepancy.

Combinatorial treatment with IOX2 and nusinersen showed a significant improvement in survival of treated mice, despite no obvious synergy on a molecular level in terms of SMN transcripts and protein, suggesting that other SMN-independent mechanisms may be implicated.

IOX2 was first described in 2013 (Chowdhury et al. 2013) as a selective PHD2 inhibitor (IC_{50} – 22 nM). Chemically, it is a N-benzyl derivative of 4-hydroxy-2-oxo-1,2-dihydroquinoline glycinamide analogues (4HQs). Its selectivity against several other members of the 2-OG family, human histone methyl lysine demethylases (KDMs), has been determined, showing that IOX2 selectively targets PHD compared to KDMs and FIH (Chowdhury et al. 2013). IOX2 is also inactive against a panel of 55 receptors and ion channels at 10μM. At the moment no negative control for IOX2 is available. In the study characterizing IOX2, it has been found that it inhibits HIF-1α prolyl hydroxylation in several human cell lines: renal carcinoma (RCC4) cell line lacking VHL and RCC4 cells expressing VHL, embryonic kidney (293T), bone osteosarcoma (U2OS). No change to asparagine hydroxylation at position 803 have been observed, confirming no activity

towards FIH. IOX2 is not only more selective, but also a more potent inhibitor of PHD2 compared to routinely used DMOG in cell-based assays. However, even assuming excellent selectivity towards PHD2 against other members of the 2-OG family, influence on other proteins besides HIF cannot be ruled out. PHDs specificity is still controversial, with some studies reporting selectivity, and lack of activity towards non-HIF substrates (Cockman et al. 2019), and others providing evidence towards PHD activity on inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), p53, forkhead box O3 (FOXO3a) and other proteins (Strowitzki, Cummins, and Taylor 2019).

The molecular mechanism by which PHD2 inhibition leads to SMN protein increase is currently unknown. It has been found that prolyl hydroxylation increases the stability of several proteins, one of them being hnRNPA1 (Stoehr et al. 2016). Therefore, one can speculate, that inhibiting PHD2 may decrease hnRNPA1 stability and expression levels, which ultimately results in increased exon 7 inclusion. Further experiments are necessary to prove this hypothesis.

Hypoxia and SMA

Hypoxia is not a widely studied phenomenon in the context of SMA, and little is known of its impact on SMA tissues and patients. The research so far points to existence of hypoxia in patients and mouse models, possibly due to decreased vasculature density or impaired oxygenation.

Necrosis at the tips of fingers and toes has been observed in severe cases of SMA, together with thrombotic occlusion of small vessels (Rudnik-Schöneborn et al. 2010). Around a 50 % decrease in vascular architecture has been observed in two SMA mice models compared to controls (Somers et al. 2016). One study have found that MRI from SMA patients is similar to individuals who experienced a hypoxic-ischaemic brain injury

prenatally (Harding et al. 2015). Finally, patients with mild SMA show decreased oxygenation of muscles upon exercise (Montes et al. 2021).

On a molecular level it has been shown that hypoxia can induce exon 7 skipping in *SMN2* pre-mRNA in several human cell lines, from neuronal to fibroblasts, and lead to lower levels of SMN proteins in those cells (Bebee et al. 2012). Bebee and colleagues also reported that Δ7 SMA mice subjected to hypoxia show a growth defect, while in hyperoxic conditions SMA mouse improved their motor function, assessed by righting reflex. No changes to survival and SMN protein levels in the skeletal muscle have been observed when comparing normoxic, hypoxic and hyperoxic conditions (Bebee et al. 2012).

Several tissues of early symptomatic Taiwanese SMA mice have been found to be hypoxic, including spinal cord, brain and kidney and have shown an increase in glucose uptake, a common metabolic change in response to hypoxia (Hernandez-Gerez et al. 2020; Somers et al. 2016). An inverse correlation between SMN protein levels and vulnerability to hypoxia has been observed in wild type and SMN KD NSC-34 cells, where cells with low SMN levels where more vulnerable to cell death than dose with higher levels (Hernandez-Gerez et al. 2020).

Interestingly, Zhong and colleagues report that SMN protein itself is protective in cardiomyocytes subjected to hypoxia (Zhong, Song, and Song 2020). In this study hypoxia, as expected, led to an increase of HIF-1α protein levels, but also a decrease in SMN levels. The authors did not look at FL and Δ7 transcript levels, but the observed decline in SMN protein may be due to increased exon skipping under hypoxic conditions, as reported above by Bebee and colleagues. Overexpression of SMN *via* a viral vector resulted in a decrease of proapoptotic (Bax) and an increase in antiapoptotic (Bcl-2)

protein levels, which improved survival of cardiomyocytes (Zhong, Song, and Song 2020), suggesting protective properties of SMN in hypoxic conditions.

Changes in HIF transcript levels have also been observed in SMA. Levels of *Hif3a* transcripts have been reported to be elevated in SMA mice tissues (Zhenxi Zhang et al. 2008), which can constitute a cellular adaptive response to hypoxia. *Hif3a* upregulation is consistent with the RNA sequencing data from results chapter 3, where I observed an over 2-fold increase in levels of that transcript in spinal cords of SMA mice, compared to wild type. That upregulation was not corrected by nusinersen alone, but was by combinatorial therapy with nusinersen and MS023.

Overall, it appears that SMA leads to tissue hypoxia due to impaired vasculature and improper oxygenation. On a molecular level, it is plausible that SMN protein deficiency leads to tissue hypoxia, which in turn favours exon skipping and results in even less full length SMN protein, creating a vicious circle. I report that blocking PHD2 increases SMN protein levels and improves the phenotype, while Zhong and colleagues report that SMN protein overexpression itself can ameliorate cell death in hypoxic conditions. To my knowledge, this is the first report that inhibiting PHD2 can increase SMN protein levels and ameliorate SMA mice phenotype.

Respiratory care is an important aspect of multidisciplinary care of SMA patient and correct oxygenation has a profound impact on the quality of life. The question arises whether proper ventilatory support and tissue oxygenation can also increase SMN protein levels in SMA patients.

Protective properties of PHD inhibition in hypoxic tissues have been reported before, in chronic kidney diseases (CKD), ischaemia and reperfusion conditions, neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis. Anaemia due to chronic kidney diseases is an

indication, where small molecule PHD inhibitors are in the most advanced stages of clinical research and have entered the market in some countries.

PHD inhibitors in other human diseases

Chronic kidney disease

Small molecule prolyl hydroxylase domain inhibitors (PHIs) are of great clinical interest for the treatment of anaemia due to chronic kidney disease (CKD). Since kidney are the main organs producing erythropoietin, their failure leads to decreased levels of erythropoietin (EPO) and anaemia. Currently it is managed by erythropoiesis-stimulating agents (ESAs), which have several major disadvantages, like immunological response and increasing the risk of cardiovascular events (Kaushik and Yaqoob 2013; Macdougall et al. 2015; Pfeffer et al. 2009; Singh et al. 2006). Small molecules are not only more cost-effective, more stable and easier to store and administer, but they solve the issue of antibody response against ESAs or native EPO of patients. Moreover, there is hope they are going to have a better cardiovascular safety profile and lastly, PHI are not only going to enhance EPO levels, but can potentially have a beneficial effect on angiogenesis and vasodilatation. In preclinical development, healthy animals and animals with renal impairment have shown an increase in erythropoietin levels and haematocrit, without serious safety concerns, after treatment with small molecule PHIs (Flamme et al. 2014; Hsieh et al. 2007). At least six PHI, roxadustat, daprodustat, vadadustat, enarodustat and FG-2216/IOX3, are in clinical trials (Maxwell and Eckardt 2016) and five of them, with the exception of FG-2216 are approved in Japan (Hirota 2021). In the US, roxadustat and vadadustat were not approved by the FDA based on safety concerns, and daprodustat is currently under review (Drugs.com 2022).

Except for chronic kidney disease, PHI are also a promising target in the context of ischaemia, since HIF promotes angiogenesis, vasodilatation, glucose uptake and decreases the production of reactive oxygen species (ROS), and in neurodegenerative disorders.

Ischaemic stroke and neurodegenerative diseases

Central nervous system (CNS) is the most sensitive to the lack of oxygen. Hypoxia-induced changes of mitochondrial dysfunction and oxygen metabolism can have detrimental effects on neuronal structure and function, resulting in cell death (Azevedo et al. 2020; Kumari et al. 2020). Hippocampus is extremely sensitive to hypoxia and cell death resulting from hypoxia in the hippocampus is the main source of neurological deficits (Gordan et al. 2012). Damage in the hippocampus leads to a decline in cognitive function and has a detrimental effect on the quality of life (Mitroshina et al. 2021). Hypoxia has different effects on the body depending on the location, intensity and duration. In the CNS, activation of HIF can have a neuroprotective response and restore cellular functions affected by hypoxia, but HIF appears to have a complex effect on the brain. Early after ischaemia (<24h) it promotes cell death (Barteczek et al. 2017; Chai et al. 2014), but after more than 4 days, it promotes cell survival by limiting the infarct size (Baranova et al. 2007; Li et al. 2019). My results are in line with those findings, since the hypoxia in SMA tissues seems to be a chronic, and not an acute event, I observed that upregulating HIF activity led to improved phenotype and increased survival. In fact, PHD inhibitors have shown positive effects in the model of chronic hypoperfusion of the brain (Gruneberg et al. 2016). In that experiment, mice subjected to PHD suppression have exhibited enhanced neurogenesis in the hippocampus, and better cognitive function.

The main source of hypoxic brain stress is ischaemic stroke. In the post ischaemic period, the inactivation of HIF caused impaired learning and decreased neurogenesis, as well as more extensive brain damage and shorter survival (Devience et al. 2017). After stroke, the oxygenation of nerve cells in affected areas is severely impaired, leading to decreased glucose metabolism, neuronal apoptosis and neurological disorders (Zhou et al. 2017). Upregulating HIF-1α after traumatic brain injury (TBI) increases glucose transporters, GLUT1 and GLUT23, which in turn improved glucose uptake by the hypoxic neurons and can reduce cell death (Wu et al. 2018).

HIF pathways are being pursued as therapeutic targets in several neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS).

Alzheimer's disease

Alzheimer's disease is an adult onset neurodegenerative condition impairing cognitive abilities and memory. It is the most common neurodegenerative disorder and affects over 50 % of people over the age of 80 (Bonda et al. 2011). There are several features of AD that suggest that HIF activation can be a target for this disease. For example, cardiovascular risks correlate with AD (Leszek et al. 2020) and one of AD features is impaired blood flow in the brain and a dysfunction of the neurovascular unit, potentially leading to impaired oxygenation (Ahmad et al. 2020). It has been shown that physical exercise can decrease AD risks *via* enhancing brain oxygen supply (De la Rosa et al. 2020). Finally, AD patients show decreased levels of glucose transporters, aerobic glycolysis and lower HIF-1α levels and the drop in HIF-1α levels facilitates Tau phosphorylation and neurofilament formation, hallmarks of AD (Merelli et al. 2018). Considering all of the above, activating HIF pathway can be neuroprotective and is pursued as a therapeutic target for AD. Increasing HIF levels or activity has slowed down

cognitive decline and AD progression, and M30, a HIF inducer, has been studied in AD mice, where it exerted neuroprotective functions (Zhang et al. 2011). Similarly, adenoviral delivery of HIF-1α to hippocampal AD neurons in culture decreased apoptosis (Chai et al. 2014).

Parkinson's disease

Parkinson's disease (PD) is another adult onset neurodegenerative disorder, caused by degeneration of dopaminergic neurons. The exact mechanisms of the disease is to be elucidated but oxidative stress and mitochondrial dysfunction contribute to PD pathology (Burbulla et al. 2017; Hauser and Hastings 2013). Current therapies provide symptoms alleviations but offer no neuroprotection, therefore vast research efforts are being invested in drug discovery in PD (Jankovic and Tan 2020). HIF-1 is essential for the differentiation and survival of dopaminergic neurons, and decreased HIF-1 expression causes neuronal death in the development of PD (Mehrabani et al. 2020), suggesting that upregulation of HIF-1 is a potential therapeutic target. It has been reported that activation of HIF-1 can provide neuroprotection *in vivo* and *in vitro*, *via* the upregulation of EPO and VEGF, as EPO itself has antioxidant and anti-inflammatory properties (Feng et al. 2014; Semenza 2014; Strowitzki, Cummins, and Taylor 2019).

PHD inhibitors have been studied in the context of PD *in vitro* and *in vivo* – in several cell cultures models: SHSY5Y cells (Johansen et al. 2010), PC12 cells (Aimé et al. 2020), and primary cultures of nerve cells (Johansen et al. 2010), where they led to an increased expression of tyrosine hydroxylase, resulting in elevation of dopamine levels. *In vivo*, HIF PHD inhibitor reduced the loss of tyrosine hydroxylase positive neurons in substantia nigra and ameliorated behavioural patterns in mice (Mitroshina et al. 2021).

PHD inhibitors may also correct mitochondrial dysfunction, once of the hallmarks of PD. *In vitro* studies have shown they can normalize mitochondrial membrane potential

and oxygen consumption, as well as decrease the amount of reactive oxygen species (Zhang et al. 2018).

Amyotrophic lateral sclerosis

Tissue hypoxia is observed in ALS, probably due to changes in vasculature, impaired blood flood and decreased EPO and VEGF levels (Miyazaki et al. 2012). Excessive ROS production is a hallmark of some subtypes of the disease, further leading to cell death (Tafani et al. 2016). It has been suggested that impaired response to hypoxia can be responsible for cell death in ALS (Kim et al. 2013). Interestingly, HIF is elevated before clinical onset of ALS and downregulated in later stages of the disease (Nomura et al. 2019). Not surprisingly, increasing HIF signalling *in vivo* led to less hypoxic damage, and less motor neuron loss (Nomura et al. 2019). *In vitro* a PHI (FAE) improved neuronal survival *via* upregulation of GLUT and VEGF (Wiesner et al. 2013). However, contrary to the above, Tada and colleagues have reported that downregulating of HIF-1α improved survival and motor function in ALS animal models (Tada et al. 2019).

Conclusions

I have identified IOX2, a small molecule potent and selective PHD2 inhibitor to increase SMN protein levels and improve severe SMA mice survival, alone and in combination with nusinersen. The results are in line with evidence of hypoxia and SMA, but to my knowledge this is the first time to be reported, that blocking PHD2 can upregulate SMN protein levels and improve phenotype *in vivo.* The next step would be to identify the molecular mechanism *via* which PHD2 inhibition leads to SMN elevation and understand other, potentially SMN-independent activity, that mediate the beneficial effect observed in the SMA mouse model.

Discussion

Epigenetic screening of *SMN2* modulators

The primary aims of the project were to identify novel small molecule(s) targeting epigenetic mechanisms involved in SMA pathology, characterize their mechanisms of action and evaluate the therapeutic potential in SMA animal models alone and in combination with nusinersen. To this end, I have performed a cell-based screen in SMA type II-patient derived fibroblasts carrying three copies of the *SMN2* gene, using a collection of 54 chemical probes from the Structural Genomics Consortium (SGC) collection (**Table *2***). In the screen I have identified type I PRMT inhibitor MS023 to increase FL *SMN2* levels and concomitantly decrease Δ7 *SMN2* levels, as well as elevate SMN protein levels by 1.6 fold in human patient fibroblasts (**Figure *4***, **Figure *5***). Several other compounds, including bromodomain inhibitors (BAY-299, BI-9564, JQ1), lysine demethylase inhibitors (GSK-J1, GSK-LSD), and prolyl hydroxylase domain 2 (PHD2) inhibitor, IOX2, also determined a ≥1.5 fold increase in SMN protein without affecting mRNA levels, suggesting a direct or indirect effect on SMN protein regulation (**Figure *5***). Interestingly PRMT5 inhibitors GSK591 and LLY-283 and bromodomain inhibitor I-BRD9 slightly reduced FL *SMN2* without affecting Δ7 *SMN2* levels, with GSK591 treatment also resulting in reduction of SMN protein, supporting a mechanism of transcriptional repression (**Figure *4***, **Figure *5***). Type I PRMTs catalyse asymmetric dimethylation (ADMA), while PRMT5 catalyses symmetric dimethylation (SDMA), and it has been shown that ADMA or SDMA on the same arginine residue can have opposing effect on the protein binding properties. PRMT1 – the main enzyme of type I family, and PRMT5 have also been found to scavenge each other's substrates in the absence of one or the other enzyme (Dhar et al. 2013). Therefore, it is likely that asymmetric and symmetric dimethylation can exert opposite effects on SMN protein regulation and,

consequently, its levels. PRMTs are involved in several critical biological functions (Blanc and Richard 2017) and represent a promising therapeutic target for many human diseases from cancer to neurodegeneration, with eight PRMT inhibitors attaining clinical trial testing in human cancers (Guccione and Richard 2019; Hwang et al. 2021; Yang and Bedford 2013). Altogether the direct effect of PRMT type I inhibition on *SMN2* exon 7 inclusion and the potential for clinical impact of this class of molecules, was a reason to further investigate MS023 as a therapeutic agent for SMA. Another molecule characterized further but to a lesser extend is PHD2 inhibitor, IOX2, due to its effect on SMN protein levels, which has been discussed in Results chapter 4. Therefore the main discussion is going to focus on MS023.

Type I PRMT inhibition promotes exon 7 inclusion in *SMN2* pre-mRNA by decreasing hnRNPA1 binding

I then sought to investigate the mechanism *via* which PRMT inhibition leads to inclusion of *SMN2* exon 7, and showed that knockdown of individual PRMTs inhibited by MS023 or the combination of two has no effect on SMN transcript and protein levels (**Figure *6***). This suggests a high degree of functional overlap between these enzymes, which is widely established and has been observed before (Wei et al. 2021). I then showed that MS023 decreases the binding between *SMN2* pre-mRNA and hnRNPA1, a splicing factor and a major negative regulator of exon 7 inclusion (Bose et al. 2008; Cartegni et al. 2006; Chen et al. 2008; Hua et al. 2008; Kashima et al. 2007; Kashima and Manley 2003; Kashima, Rao, and Manley 2007; Singh et al. 2013; Xiao et al. 2012) (**Figure *7***). hnRNPA1 has several binding sites across *SMN2* pre-mRNA, two of them are located withing the ISS-N1 sequence. One binding site is specific to *SMN2*, as it is created by the C to T substitution between *SMN1* and *SMN2* genes. Nusinersen acts *via* blocking

hnRNPA1 binding to ISS-N1. I postulate that MS023 treatment increases the MMA and decreases the ADMA of hnRNPA1, leading to a decreased binding to *SMN2* pre-mRNA, making more *SMN2* pre-mRNA available for nusinersen. *In vivo,* in the CSF nusinersen increases SMN protein levels by around 2-fold (Geary, Yu, and Levin 2001; Gidaro and Servais 2019), meaning there is still a substantial fraction of *SMN2* pre-mRNA that is 'resistant' to nusinersen treatment and continues to produce truncated SMN. hnRNPA1 has been described as a PRMT1 target in the 1990s (Rajpurohit, Paik, and Kim 1994) and has several arginine methylation sites. Methylation of arginine (and other PTMs of hnRNPA1) have been shown to decrease their binding with RNA and are regarded as inhibitory (Bekenstein and Soreq 2013). There are two studies describing the effect of PRMT type I inhibition on hnRNPA1 arginine methylation at specific residues. In both cases, treatment with MS023, or its analogue GSK3368712, resulted in increased MMA (Fong et al. 2019; Noto et al. 2020). Fong and colleagues also observed a decrease in ADMA (as expected), while Noto did not discriminate between ADMA and SDMA, but rather observed a decrease in dimethylation (DMA) overall. Therefore I proposed, that upon MS023, hnRNPA1 methylation pattern changes, with less ADMA and more MMA, which leads to its decreased binding to *SMN2* pre-mRNA and disinhibits exon 7 inclusion. However, since *SMN2* exon 7 splicing is regulated by at least 40 different proteins and MS023 affects methylation of nine of them (hnRNPA1, hnRNP U, hnRNPA2/B1, HuR, RBM10, Sam68, SFRS9, TRA2B, U2AF1) (Fong et al. 2019), it cannot be excluded that other splicing factors are also affected by MS023 (directly or indirectly) and contribute to exon 7 inclusion observed upon treatment (Singh et al. 2018).

Oral administration of MS023 improves the phenotype of SMA mice alone and in synergy with nusinersen.

Administration of 2 mg/kg of MS023 improved the lifespan and weights of severe SMA mice, alone and in combination with nusinersen (**Figure *9***, **Figure *13***). On its own, MS023 upregulated SMN transcripts and protein levels in skeletal muscle and spinal cords, the tissues mostly affected by the disease and located both in the CNS and periphery, showing a favourable distribution of MS023 across the body (**Figure *10***). Interestingly, mice treated with a high dose (40 mg/kg) of MS023 showed shorter lifespan and lower weights than untreated SMA mice, hinting that with this dose the therapeutic window has been surpassed (**Figure *9*B,D**). Combinatorial treatment with MS023 and a low dose of nusinersen showed a dramatic, nearly five-fold, increase in survival in comparison to nusinersen alone, suggesting a synergistic effect of those therapies (**Figure *13***). One can potentially envision a situation, when burdensome nusinersen injections are less frequent upon combination with a small, orally available molecule, that can be taken daily at home. Inhibiting type I PRMTs is investigated in the context of cancer, which raises questions over their safety and cytotoxic properties. I have not observed any safety concerns at 2 mg/kg (or 5 mg/kg). In the cancer models, mice are treated with 60 mg/kg or 160 mg/kg of MS023 (intraperitoneally), doses 30 and 80-fold higher than used in this study, which probably explains the difference (Fong et al. 2019; Zhu et al. 2019). It is reassuring that low doses can be used to effectively upregulate SMN protein, with much lower probability of side effects and toxicity.

Transcriptomic analysis of SMA mouse spinal cords treated with single and combinatorial therapies reveals molecular signature of combinatorial treatment

In order to understand the molecular underpinnings of the added benefit provided by the combinatorial treatment, I performed bulk transcriptomic analysis in P7 spinal cords of SMA mice treated with MS023 only, nusinersen only, and MS023 in combination with nusinersen, compared to wild type and SMA littermates, following the treatment paradigm described above (**Figure *11*A**).Between untreated and control animals, I have identified 5509 significantly differentially expressed genes, of which 2874 were upregulated, and 2635 were downregulated (**Figure *15*A**). The majority of dysregulated genes were corrected by nusinersen alone. Combinatorial treatment was able to rescue 359 (6.5 % out of 5509) additional genes that were not corrected upon treatment with nusinersen alone, and their correction may be responsible for increase in lifespan. Hallmark analysis of those 359 genes revealed enrichment of TNF-α signalling, together with several other immune-related pathways, including the interferon response and complement activation, overall suggesting that therapeutic targeting of neuroinflammation is key to achieving a beneficial effect in SMA. Interestingly, astrocyte dysfunction and chronic microglia activation have been observed early in SMA and other neurological conditions (Eikelenboom et al. 2002; Heneka, Kummer, and Latz 2014; Sargsyan, Monk, and Shaw 2005; Vukojicic et al. 2019) and their contribution to neuronal dysfunction and death is abundantly supported (Martin et al. 2017; Mcgivern et al. 2013; Rindt et al. 2015; Zhou, Feng, and Ko 2016). Neither of treatment regimens (MS023 alone, nusinersen alone, combination) was able to rescue 403 (7.3 %) of genes (‘super resistant’ genes). Gene ontology of those genes shows enrichment in vascular endothelial growth factor receptor activity, transmembrane receptor protein tyrosine kinase activity, ATPase-coupled cation transmembrane transporter

activity, growth factor binding, amongst others pathways. From 5509 transcripts dysregulated in SMA, only six were 'overcorrected' upon combinatorial MS023 and nusinersen treatment. Four transcripts, *heat shock protein 5 (Hspa5), fructosamine 3 kinase (Fn3k), insulin-like growth factor binding protein 2 (Igfbp2) and X-box binding protein 1 (Xbp1*) changed from downregulated to upregulated, while two, *calcium channel, voltage-dependent, T type, alpha 1H subunit (Cacna1h) and chromodomain helicase DNA binding protein 2 (Chd2)* changed from downregulated to upregulated. In all cases the overcorrection was modest, 0.41 fold change at most. Treatment with nusinersen alone led to overcorrection of eight distinct transcript.

I also analysed alternative splicing events, given the role of SMN in RNA biogenesis and spliceosomal proteins assembly, and the observation of increased mis-splicing events upon SMN depletion (Bäumer et al. 2009; Custer et al. 2016; Doktor et al. 2017; Huo et al. 2014; Zhenxi Zhang et al. 2008, 2013). Even though 2 mg/kg of MS023 treatment did not provide a complete rescue of the animals and resulted in 50 % increase in survival, the splicing profile of mice treated with MS023 alone was almost entirely corrected (368 events corrected out of 446). The same was true for a low dose of nusinersen (347/446), which prolonged the lifespan by around 150 %. Combinatorial therapy provided minimal additive effect (370/446) with 14 unique splicing events corrected (**Figure *16*** and **Table *3***), four of which are involved in neuronal function. That indicates that mis-splicing has a low threshold for normalization in SMA and is a poor predictor of treatment response.

Overall, MS023, an inhibitor of type I PRMTs provided a good upregulation of SMN *in vitro* and *in vivo*, and a prolonged lifespan of severe SMA mice, alone and in combination with nusinersen. A dose used in the study was much lower (up to 80 times lower) than doses used in cancer mouse models, decreasing the probability of side effects

and toxicity. RNA sequencing revealed correction of several pathways upon combinatorial treatment only, many of which related to neuroinflammation, which might be responsible for the increase in survival.

PRMT inhibitors in preclinical and clinical development

PRMTs are involved in several critical biological functions (Blanc and Richard 2017) and represent a promising therapeutic target for many human diseases from cancer to neurodegeneration. Efforts to develop small molecule, potent, selective and cell-permeable PRMTs inhibitors has yielded great results. Inhibitors from the SGC library screened in this study are underlined.

Type I PRMT inhibitors

Due to high sequence conservation in the SAM binding pocket between type I PRMTs, no specific PRMT1 inhibitors are available. MS023 is substrate competitive, pan-type I inhibitor targeting PRMT1, 3, 4, 6 and 8, and acting in nanomolar concentrations (Eram et al. 2016). Its analogue, GSK3368715, has been tested in a phase I study in participants with solid tumours and diffuse large B-cell lymphoma (DLBCL). The study evaluated the safety and pharmacokinetic–pharmacodynamic properties of the compound and included a preliminary assessment of its therapeutic activity. Sadly, it was terminated in May 2022, according to clinicaltrials.gov, due to unfavourable benefit/risk ratio, with no further details, while the sponsor's press release states the reason for termination was 'prioritisation within the synthetic lethal portfolio' (NCT03666988) (clinicaltrials.gov n.d.; GlaxoSmithKline 2022).

Several PRMT inhibitors specific for individual type I family members have been reported. SGC707, a cell-active PRMT3 inhibitor feasible for animal studies is an allosteric compound, SAM non-competitive and substrate non-competitive (Kaniskan et al. 2018; Siarheyeva et al. 2012). Currently no clinical indication has been identified for PRMT3 inhibition.

Several selective PRMT4 inhibitors have been identified: TP-064 (Nakayama et al. 2018), GSK3536023 and the further optimized GSK3359088 (Drew et al. 2017). They are suitable for animal studies and show efficiency in reducing haematological malignancies, including multiple myeloma and AML, and also breast cancer (Drew et al. 2017; Lu Wang et al. 2016). These compounds are yet to reach the clinical stage.

There are currently two PRMT6 specific inhibitors: EPZ020411 (Mitchell et al. 2015), and SGC6870 (Shen et al. 2021) while MS049 inhibits PRMT4 and PRMT6 (Shen et al. 2016).

None of the more specific type I PRMTs inhibitors that I have tested (SGC707, TP-064, MS049) showed any effect on SMN transcript or protein levels, suggesting a functional overlap between members of type I family. Only MS023, a pan-type I inhibitor increased FL *SMN2* and SMN protein levels, an effect mediated by PRMT inhibition, as MS094, a negative control for MS023 without PRMT inhibition activity, failed to affect SMN protein levels in patient fibroblasts (**Figure *8***).

Type II PRMTs inhibitors

Efforts to identify a selective PRMT5 inhibitors have been successful and include numerous compounds. EPZ015666 and similar compounds (for example, GSK3326595 and GSK591) bind PRMT5 in its substrate-binding pocket and make an additional contact with its SAM- binding pocket (Chan-Penebre et al. 2015; Gerhart et al. 2018). Development of SAM-competitive inhibitors of PRMT5 has also been successful and resulted in generation of LLY-283 (Bonday et al. 2018) and JNJ- 64619178 (Brehmer et al. 2017). In this study, GSK591 and LLY-283 resulted in a significant decrease of FL *SMN2* and GSK591 treatment decreased SMN protein levels, suggesting that inhibition of different types of arginine methylation can have opposing results on SMN

protein. It would be interesting to explore what the effect of PRMT5 inhibition on hnRNPA1 methylation levels is, or if the mechanism is completely independent of that identified.

Type III PRMTs inhibitors

Currently one inhibitor of type III PRMTs has been reported. SGC3027 is a cell-permeable prodrug that is converted into SGC8158 in cells. SGC8158 is a potent and selective PRMT7 inhibitors which has been linked to solid and haematological tumours (Robak and Robak 2019; Szewczyk et al. 2020). SGC3027 had no effect on SMN transcript or protein levels in the screen.

PRMT inhibitors in clinical trials

As mentioned before, PRMTs are heavily involved in regulation of splicing. As dysregulation of splicing is a pathological hallmark of several cancers, targeting splicing for cancer treatment is currently a growing area of research. Whole genome sequencing analysis of patients with myelodysplastic syndrome (MDS) revealed that 45-85 % of those individuals have mutations in genes encoding for components of the splicing machinery (Yoshida et al. 2011). Cancer cells are able to tolerate only partial dysregulation of splicing, so inhibiting PRMTs can lead to additional splicing defects that a cell cannot sustain and preferential killing of cancer cells. Fong and colleagues have shown that inhibition of type I PRMTs with MS023 and PRMT5 with GSK591 leads to widespread changes in splicing and preferentially kills splicing factor mutant leukaemia cells (Fong et al. 2019). Profound changes in splicing upon PRMT type I inhibition and exacerbated by PRMT5 inhibition have been confirmed in pancreatic cell model by a different group (Fedoriw et al. 2019).

Due to its cytotoxic effects stemming from disruption of fundamental processes like splicing, PRMTs are being pursued as cancer therapies, with several PRMT inhibitors in clinical trials (**Table *4***) (clinicaltrials.gov 2022).

	Intervention	Mode of action	Condition	NCT
1	GSK3326595	PRMT5 inhibitor	Breast cancer	NCT04676516
2	JNJ-64619178	PRMT5 inhibitor	Neoplasms, solid tumour Non-Hodgkin Lymphoma Myelodysplastic Syndromes	NCT03573310
3	AMG 193	PRMT5 inhibitor	Advanced MTAP-null Solid Tumours	NCT05094336
4	PF-06939999	PRMT5 inhibitor	Advanced and metastatic Solid Tumours	NCT03854227
5	PRT811	PRMT5 inhibitor	Advanced Solid Tumour Recurrent Glioma CNS Lymphoma	NCT04089449
6	PRT543	PRMT5 inhibitor	Relapsed/Refractory Advanced Solid Tumours Relapsed/Refractory Diffuse Large B-cell Lymphoma Relapsed/Refractory Myelodysplasia Relapsed/Refractory Myelofibrosis Adenoid Cystic Carcinoma Relapsed/Refractory Mantle Cell Lymphoma Relapsed/Refractory Acute Myeloid Leukaemia Refractory Chronic Myelomonocytic Leukaemia	NCT03886831
7	TNG908	PRMT5 inhibitor	Locally Advanced Solid Tumour	NCT04794699
8	MRTX1719	PRMT5•MTA complex inhibitor	Mesothelioma Non-Small Cell Lung Cancer Malignant Peripheral Nerve Sheath Tumors Solid Tumor Pancreatic Adenocarcinoma Advanced Solid Tumor	NCT05245500

Table *4*. PRMT inhibitors in clinical trials

(clinicaltrials.gov 2022)

Final conclusions

In this study I have identified two small molecules able to increase SMN protein levels and improve survival of SMA mice, and focused on PRMT type I inhibitor, MS023, to understand its mechanism of action and whether it works in synergy with nusinersen. It has shown a good SMN restoration on a molecular level, comparable to currently approved risdiplam, and a good safety profile, with best efficacy at a dose tens of times lower than used in cancer animal models. RNA sequencing revealed correction of neuroinflammation upon combinatorial treatment only, which might be responsible for the increase in survival. In the future it will be interesting to validate the correction of neuroinflammation upon MS023 treatment, for examples in iPSC SMA models. Current SMA therapy is extremely costly and available to the minority of the SMA population worldwide. The largest number of patients is currently treated with nusinersen, which only targets the CNS, when it is established that for best long-term improvement, both peripheral and central SMN rescue is necessary. Nusinersen administration is burdensome and becomes more difficult with time. It requires well trained clinicians and hospitalization, adding to the healthcare burden. Risdiplam, the first at home therapy for SMA, is convenient, but still extremely costly and for this reason will remain unavailable to many patients. Extensive efforts are underway to optimise currently available therapies and develop new ones. MS023 falls under the category of non-gene targeted *SMN2* splicing modifiers and presents an option for a combinatorial therapy, possibly leading to less frequent nusinersen injections and whole body SMN increase. PRMTs involvement has been described in SMA, with PRMT4 being upregulated in SMA tissues of mice. PRMT5 is intimately connected with SMN in biogenesis of spliceosome. MS023, as a type I inhibitor, and therefore a PRMT4 inhibitor, has the potential to correct

defects caused by the upregulation of PRMT4, without affecting PRMT5 function, which belongs to type II PRMTs.

MS023 is an openly available, not protected by patent small molecule, that has a potential to decrease frequency of nusinersen administration and consequently, hospital administrations and the cost of treatment. The mechanism of action IOX2, a second small molecule identified in the screen, remains to be determined. While more studies need to be carried out, MS023 has the potential to progress to the next stages of pre-clinical development, with the ultimate hope to improve the quality of life of individuals living with SMA and their families.

Material and methods

Small molecules library

The library of small molecules was obtained from the Structural Genomic Consortium in Oxford. Upon reception, the molecules were diluted in DMSO to 10 mM, aliquoted and stored at -20°C for the duration of the study. All the compounds, their targets and doses used in the study are listed in **Table *5***.

Small molecule	Target protein family	Specific Targets	Concentration used
GSK8814	Bromodomains	ATAD2, ATAD2B	10 µM
BAZ2ICR	Bromodomains	BAZ2A, BAZ2B	10 µM
GSK2801	Bromodomains	BAZ2A, BAZ2B	10 µM
BAY299	Bromodomains	BRD1, TAF1	10 µM
JQ1(+)	Bromodomains	BRD2, BRD3, BRD4, BRDT (BET)	10 µM
PFI1	Bromodomains	BRD2, BRD3, BRD4, BRDT (BET)	10 µM
IBRD9	Bromodomains	BRD9	10 µM
BI9564	Bromodomains	BRD9, BRD7	10 µM
LP99	Bromodomains	BRD9, BRD7	10 µM
TP472	Bromodomains	BRD9, BRD7	10 µM
GSK6853	Bromodomains	BRPF1	10 µM
NI57	Bromodomains	BRPF1, BRPF2, BRPF3	10 µM
OF1	Bromodomains	BRPF1, BRPF2, BRPF3	10 µM
PFI4	Bromodomains	BRPF1B	10 µM
NVSCER21	Bromodomains	CECR2	1 µM
ICBP112	Bromodomains	CREBBP, EP300	10 µM
SGCCBP30	Bromodomains	CREBBP, EP300	10 µM
SGC0946	Methyltransferase	DOT1L	10 µM
A395	Methyl Lysine Binder	EED	10 µM
UNC0642	Methyltransferase	EHMT2 (G9a), EHTM1 (GLP)	2 µM
GSK343	Methyltransferase	EZH2	10 µM
UNC1999	Methyltransferase	EZH2	5 µM
A366	Methyltransferase	G9a, GLP	10 µM
UNC0638	Methyltransferase	G9a, GLP	2 µM

Small molecule	Target protein family	Specific Targets	Concentration used
BAY876	Glucose transporter	GLUT1	10 µM
GSKJ1	Lysine Demethylase	JMJD3, UTX, JARID1B	10 µM
UNC1215	Methyl Lysine Binder	L3MBTL3	10 µM
GSKLSD1	Lysine Demethylase	LSD1	10 µM
T26c	Matrix metalloproteinase	MMP-13	10 µM
GSK864	Dehydrogenase	Mutant isocitrate dehydrogenase 1	10 µM
BAY678	Serine Proteases	Neutrophil Elastase	10 µM
A485	Acetyltransferase	p300, CBP	10 µM
GSK484	Arginine deiminases	PAD-4	10 µM
NVSPAK11	Serine/threonine-protein kinase	PAK1	10 µM
IOX1	Lysine Demethylase	pan-2-OG	10 µM
BSP	Bromodomains	pan-Bromodomain	10 µM
LMOSES	Bromodomains	PCAF bromodomain	10 µM
GSK4027	Bromodomains	PCAF, GCN5	10 µM
IOX2	2OG	PHD2	10 µM
SGC707	Methyltransferase	PRMT3	10 µM
TP064	Methyltransferase	PRMT4	10 µM
MS049	Methyltransferase	PRMT4,6	10 µM
GSK591	Methyltransferase	PRMT5	10 µM
LLY283	Methyltransferase	PRMT5	10 µM
SGC3027	Methyltransferase	PRMT7	5 µM
MS023	Methyltransferase	PRMTs type I	10 µM
PFI2	Methyltransferase	SETD7	10 µM
PFI3	Bromodomains	SMARCA,PB1	10 µM
PFI5	Methyltransferase	SMDY2	5 µM
BAY598	Methyltransferase	SMYD2	10 µM
BAY6035	Methyltransferase	SMYD3	10 µM
A196	Methyltransferase	SUV420H1/H2	10 µM
OICR9429	WD40	WDR5	10 µM

Table *5.* Library of small molecule epigenetic inhibitors obtained from the Structural Genomic Consortium used in the screen, their specific targets and the concentration established in the viability assay and used in the initial screen.

Tissue culture

General tissue culture conditions

Cells were grown in a humidified incubator at 37°C with 5 % CO2. SMA type II patient fibroblasts (GM03813) were obtained from Coriell Institute. SMA patient fibroblasts carry 3 copies of *SMN2* and homologous deletion of *SMN1* exons 7 and 8.

The cells were maintained in Gibco Dulbecco's Modified Eagle Medium GlutaMAX (DMEM) supplemented with 10 % Gibco foetal bovine serum (Gibco) and 1x Gibco Antibiotic-Antimycotic (PSA: Penicillin, Streptomycin and Amphotericin B, Sigma).

Determination of the highest non-toxic concentration of epigenetic regulators in SMA patient fibroblasts

MTS Cell Proliferation Assay Kit (Abcam) was used to determine the highest non-toxic concentration of small molecules in SMA patient fibroblasts. This colorimetric method is based on the reduction of MTS tetrazolium compound by viable, metabolically active cells to generate a coloured formazan. The reaction is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes. The formazan dye is quantified by measuring the absorbance at 490-500 nm.

Briefly, 3,000 cells/well were plated in triplicates in a 96-well plate in half of the final volume of medium (100 µL). After 6 h, compounds of interest were diluted to 2x final concentration in 100 µL of medium and added in a range of concentrations (1 µM to 10 µM). 1 µM of staurosporine was used as a positive control for cell death. After 44 h, 20 µL of MTS reagent was added to each well. 4 h later, the absorbance was measured at 490 nm on Clariostar plate reader. The concentration was deemed

non-toxic if the absorbance was insignificantly different from the absorbance of untreated cells, the cells looked viable after visual inspection and the confluency of treated cells was comparable to untreated via visual inspection.

Effect of small molecules on the expression of *SMN2* transcripts levels

SMA patient fibroblasts were plated in triplicates, in 12-well plates, at 50,000 cells per well in 500 µL of medium. After 6 h, compounds of interest were diluted in 500 µL of medium to 2x final concentration and added to the cells. After 48 h of incubation RNA was extracted for qPCR analysis.

Effect of small molecules on the expression of SMN protein

SMA patient fibroblasts were plated in duplicates or triplicates in 6-well plates, at 100,000 cells per well in 1 mL of medium. After 6 h compounds of interest were diluted in 1 mL of medium to 2x final concentration and added to the cells. Total incubation time was 72 h of and medium with a small molecule was changed once approximately after 36 h.

Effect of PRMT siRNAs on expression of SMN transcript and protein

siRNAs for PRMT1, 3, 4, 6 and 8 were obtained from Darmacon. Briefly, in a 6 well plate, 400 µL Optimem was mixed with siRNA (to a final concentration of 25 nM) and with 2 µL lipofectamine RNAiMAX (Invitrogen) and left to incubate.
In the meantime, cells were trypsinized and 100,000 cells were diluted in 600 µL Optimem (Gibco), to a final volume of 1 ml. After 6 h, 1 mL of complete Gibco medium was added. After 48 h cells RNA was collected, after 72 h protein was collected.

Crosslinking and Immunoprecipitation assay (CLIP)

Crosslinking and immunoprecipitation assay (CLIP) is an assay used to study RNA binding proteins and their corresponding RNAs. In this study CLIP was employed to determine the changes in binding between hnRNPA1 and FL *SMN2* transcript upon MS023 treatment.

Tissue culture. Briefly, for each condition, four 150 cm^2 cell dishes of human patient fibroblasts were treated with 100 nM, 250 nM and 1 µM of MS023 for 48 h.

Crosslinking and digestion. After the incubation, the medium was aspirated, cells were washed with ice cold PBS and kept on ice. Cells were subjected to 150 mJ/cm^2 254 nM UV light in a Stratalinker UV Crosslinker. The cells were then scraped, pelleted and resuspended in NP-40 lysis buffer supplemented with protease inhibitors.

Antibody binding. Magnetic Dynabeads Protein G (10003D, Thermofisher) and hnRNPA1 antibody (4B10, Santa Cruz) in PBS Tween were used to immunoprecipitate hnRNPA1 for 10 minutes in room temperature, followed by three washes with 200 µL NP-40 lysis buffer.

DNA and protein digestion. Pellets were incubated with 20 units of RNAse-free DNAse (Thermofisher) in 37 °C for 15 minutes. 700 µL of NP-40 buffer was added and supernatant was aspirated. Next, pellets were incubated with 0.1 % SDS and 0.5 mg/mL of proteinase K (Roche) in 55 °C for 15 minutes. After supernatant collection, supernatant was placed in a new tube.

RNA extraction, cDNA and qPCR. RNA was isolated using acidic phenol Trizol (Invitrogen) and ethanol precipitation, and cDNA was generated using an ABI High Capacity cDNA Reverse Transcription Kit (Invitrogen) as described above. A qPCR reaction using Power SYBR Green Master Mix (Life Technologies) was performed and analysed on an Applied Biosystems StepOnePlusTM real-time PCR system (Life

Technologies). FL *SMN2* and *GAPDH* transcripts were amplified using gene-specific primers, which are available in **Table *6***.

Nuclear / cytoplasmic fractionation

Preparation of nuclear and cytoplasmic extracts from SMA patient-derived fibroblasts was performed using a NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermofisher), as per manufacturer's instructions. Briefly, cells were treated with increasing concentrations of MS023 and harvested after 72 h of incubation. Cells were trypsinized and pelleted, the supernatant was aspirated. Ice cold CER I buffer was added to the supernatant, followed by vortexing and 10-minute incubation on ice. Next, ice cold CER II buffer was added to the cells, followed by vortexing, and 1-minute incubation on ice. After 5-minute centrifugation at maximum speed the supernatant containing the cytoplasmic extract was transferred to a new tube. Ice cold NER was added to the pellet, vortexed and incubated for 40 minutes with vortexing every 10 minutes. Next, the sample was centrifuged at a maximum speed for 10 min and transferred to a new tube. Protein concentration was determined as described above and Western blot was performed as described, the antibodies used were: histone 3 (Cell Signalling), vinculin (Sigma).

Animal work

Conditions and husbandry

All animal procedures were carried out at the Biomedical Services Building, University of Oxford. Procedures were authorised by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 and by the University of Oxford ethics committee (PPL no: PDFEDC6F0, PIL no: IF5D73644).

Taiwanese mouse model

All experiments were performed on SMA-like mouse (*Mus musculus*) strain FVB.Cg-Smn1tm1HungTg(SMN2)2Hung/J – the 'Taiwanese' model (*Smn*$^{-/-}$;*hSMN2*$^{+/-}$), generated as presented in **Figure *22***. The affected Taiwanese mice have a deletion of the mouse *Smn* exon 7 (mice have only one *Smn* gene) and carry one copy of human *SMN2* (Gogliotti et al. 2010; Hsieh-Li et al. 2000). It is a severe model of SMA, that typically starts showing symptoms around postnatal day 5 (P5) and reaches the humane end point between P7 and P9. The humane end point is reached when animal loses at least 15 % of body weight, loses weight for two consecutive days or is not able to right itself for 30 seconds. Typically, half of the litter is affected (mutant animals) and the other half is not (heterozygous animals).

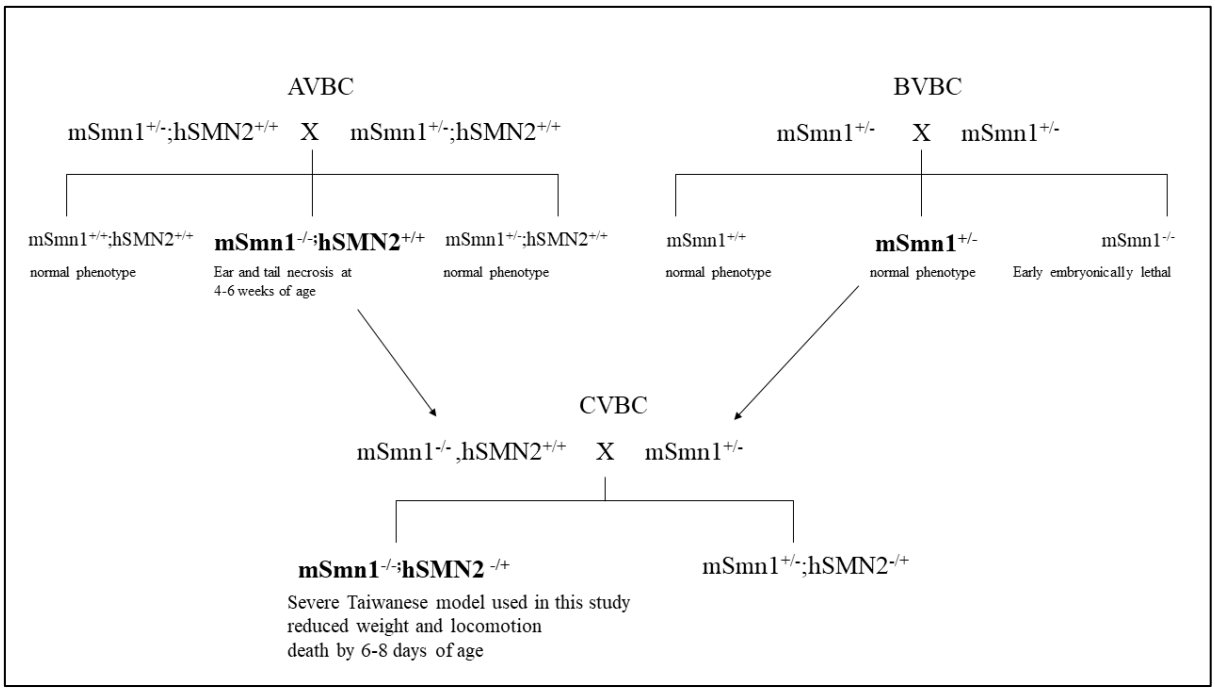


Figure *22*. A schematic of Taiwanese model generation

Genotyping

Mouse tails or ear clips were used for genotyping animals used. Briefly, tissue samples were incubated in 200 µL of DirectPCR Lysis Reagent (Mouse Tail) (Viagen Biotech, 102-T) and 5 µL of proteinase K overnight at 65°C. Following that the temperature was increased to 100°C for 10 mins. The samples were centrifuged for 1 min at 8 000 x g. 2.5 µL of each sample was added to PCR tube with 24 µL of master mix containing ddH20, smn1 primers (primer 1 - AGC CTG AAG AAC GAG ATC AGC, primer 2 - GCG TTG ATG GCA TTT CAC TA, primer 3 - TGC TCG TGG AAG TCA GTG AG) (10 µM), Taq DNA polymerase, DreamTaq™ Green Buffer (10X) (Thermo scientific, B71) and dNTPs (2 µM). PCR reaction was run on a thermocycler. PCR products were run on 2 % agarose gel with 0.05 µL/mL ethidium bromide at 120 V for ~75 mins. Gel was visualised under UV light.

Administration of MS023 and IOX2

To test the effect of selected small molecules on survival and weights of the mice, daily oral administration of MS023 or IOX2 from P0 using a Hamilton syringe (Hamilton) were performed. Small molecules were diluted in 0.5 % DMSO and 0.9 % saline and administered at a volume of 5 µL/g of body weight. Each pup was scruffed and a syringe with a blunt end was introduced into their mouth.

MS023. Doses of 0, 1, 2, 5 and 40 mg/kg of MS023 were administered.

IOX2. Doses of 0, 0.5, 1, 2 and 5 mg/kg of MS023 were administered.

Administration of nusinersen

Nusinersen (sequence:

U*sC*sA*sC*sU*sU*sU*sC*sA*sU*sA*sA*sU*sG*sC*sU*sG*sG*s, where ‘S’ is

phosphorothioate backbone and '*' is a 2'MOE Chemistry) was diluted in 0.9 % saline and given at 20 ul/g body weight, *via* subcutaneous injection, once at P0, in a dose of 0 or 30 mg/kg.

Welfare observations and culling

Weights were recorded and overall health was assessed daily. Mice aged P0–10 were sacrificed by decapitation. Cervical dislocation was used to sacrifice mice older than P10. Both males and females were randomly used. Tissues for RNA and protein isolation were placed in cryovials and snap-frozen in liquid nitrogen.

Blinding, randomization, confounders,

Since the pups were treated daily from P0 and identifying and marking individual pups between P0 and P7 poses a high risk of misidentifying an animal, in this study a litter constitutes an experimental unit, all mice in the litter are subjected to the same treatment. Only litters between 7 and 11 pups have been used, to correct for average weight (mice in smaller litters tend to be bigger and live longer and the opposite is true for litters of 12 and above), treatment was allocated randomly to a litter before it was born. All the experimental units treated are included in the analysis. Personnel performing daily weights and welfare checks for combinatorial treatment were blinded, researcher performing oral administration, injections and data analysis was not blinded. For survival analysis, the humane end point is reached upon 15 % weight loss from a maximum weight or when the mouse is not able to right itself for 30s. Tissues were harvested either at P7 (SMA mice) or on the day the last SMA mouse was culled (control animals).

qPCR and Western Blots

RNA extraction using Maxwell simplyRNA kit

RNA extraction from mouse tissues was performed using Maxwell® RSC simplyRNA Kit (Promega, UK) following the manufacturer's instructions. Approximately 30 μg of tissue was used. Prior to extraction the tissues were homogenized in 200 μL of homogenization solution containing 5 μL of proteinase K and 15 μL of thioglycerol using Precellys tubes. Next, samples were incubated for 10 min at 60°C. 200 μL of Lysis Buffer was added to the homogenate. 400 μL of homogenate was added to well #1 of the respective cartridges. Cartridges were prepared in the meantime according to manufacturer's instruction. RNA was extracted using simplyRNA tissue program.

RNA concentration was measured using Nanodrop ND 1000 spectrophotometer (ThermoFisher, UK) and sample purity was assessed by calculating A260/A280 and A260/A230 ratios for each sample. A sample is considered pure if the A260/A280 ratio is around ~2 and an A260/A230 ratio >1.8. Samples with ratios that fell substantially below these criteria were re-extracted using the method described above. Samples were stored at -80°C.

cDNA generation

cDNA was generated from 500 ng (cells) or 1 μg (tissues) of RNA using the ABI High Capacity cDNA Reverse Transcription Kit (Invitrogen, cat. no 4368813) following the manufacturer's instructions. 20 μL RT reaction were carried out and random primers were used. Samples were incubated for 10 minutes at 25°C to allow primer extension and then cDNA was reverse transcribed for 2 hours at 37°C. The reaction was terminated at 80°C for 15 minutes.

Quantitative PCR using SYBR green reagents

After diluting cDNA to final concentration of 10 ng/μL, a qPCR reaction using Power SYBR Green Master Mix or Fast SYBR Green Master Mix (Life Technologies) was performed and analysed on an Applied Biosystems StepOnePlusTM real-time PCR system (Life Technologies) (Pfaffl 2001). Universal cycling conditions were used (95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds, 60°C for 1 minute).
qPCR efficiencies were corrected for using the LinReg software. FL *SMN2*, Δ7 *SMN2*, Tot *SMN2*, *GAPDH*, *PolJ* and *Gapdh* transcripts were amplified using gene-specific primers. Primers were obtained from IDT, their sequences and primer location are available in **Table *6***.

Primer name	**Primer sequence (5′ – 3′)**	**Location**
Full length SMN forward primer	GCT TTG GGA AGT ATG TTA ATT TCA	Exon 6
Full length SMN reverse primer	CTA TGC CAG CAT TTC TCC TTA ATT	Exon 7-8
Delta 7 SMN forward primer	ACT TAC TAT CAT GCT GGC TG	Exon 6
Delta 7 SMN reverse primer	CCA GCA TTT CCA TAT AAT AGC C	Exon 6-8
Total SMN forward primer	GCG ATG ATT CTG ACA TTT GG	Exon 2a
Total SMN reverse primer	GGA AGC TGC AGT ATT CTT CT	Exon 2b
mouse GAPDH forward primer	AAAGGGTCATCATCTCCGCC	Exon 4
mouse GAPDH reverse primer	ACTGTGGTCATGAGCCCTTC	Exon 4-5
mouse PolJ forward primer	ACC ACA CTC TGG GGA ACA TC	
mouse PolJ reverse primer	CTC GCT GAT GAG GTC TGT GA	
human GAPDH forward primer	ACA TCG CTC AGA CAC CAT	
human GAPDH reverse primer	TGT AGT TGA GGT CAA TGA AGG G	

Table *6*. The sequences of primers used throughout the study and their location in the transcript.

Detection of SMN protein by Western Blotting

Cells or tissues were lysed in Pierce RIPA Buffer (Thermo Scientific, cat. no. 89900) with complete mini proteinase inhibitors (Roche). Protein concentration was determined using a colorimetric Pierce BCA Protein Assay Kit (Thermo Scientific, cat.no: 23227) against

bovine serum albumin standards. Western Blot was performed using 10 to 15 µg (cells) or 25 to 30 µg (tissues) of total protein. Protein was separated on 10 % Novex Tris-Glycine gels (Thermo Scientific) and transferred to polyvinylidene fluoride membranes. The membrane was incubated in 0.0005 % Fast Green FCF (Merck KGaA) stain for 15 min. Fast Green was used as a control of transfer efficiency and loading control. Subsequently, membrane was incubated with a primary monoclonal anti human SMN antibody (Millipore) in 4°C, overnight. An IRDye® 800CW goat anti-mouse IgG (LI-COR Biosciences, Lincoln, NE), was used as a secondary antibody for 1 h. Membranes were imaged on Li-Cor Odyssey® FC imager and analysed with Image StudioTM software (LI-COR Biosciences, Lincoln, NE).

For protein quantification from animal tissues, power calculation was performed before the experiment using GPower. Assumptions: Mean group 1 (control): 1, mean group 2 (MS023): 1.5, SD group 1: 0.3, SD group 2: 0.3, $\alpha = 0.05$, power (β) = 0.8. Minimum number of mice per group: 6, actual power: 0.85.

RNA sequencing

Transcriptomic analysis was performed on P7 spinal cords of mice in 5 treatment groups, 4 replicates in each group: untreated SMA mice, SMA mice treated with nusinersen alone, SMA mice treated with MS023 alone, SMA mice treated with nusinersen and MS023 and untreated control animals. RNA isolation was performed using Maxwell® RSC simplyRNA Kit (Promega, UK) following the manufacturer's instructions. RNA quality evaluation (yield, purity and integrity), cDNA library construction and Illumina sequencing were performed by NovoGene (Cambridge). The DEGs and alternatively spliced transcripts between the two samples were identified if they had a adjusted p value of ≤ 0.05. A hierarchical clustering analysis of DEGs was performed using MEV software.

Gene ontology pathway analyses were performed using geneontology.org to analyse the gene enrichment of the DEGs only corrected by the combination and super resistant ones.

Collection and preparation. RNA quantification and integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation for Transcriptome sequencing. Total RNA was used as input material for the RNA sample preparations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends *via* exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 370~420 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform, coverage of 25 million reads, and 150 bp paired-end reads were generated.

Quality control. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads

containing adapter, reads 1 containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Reads mapping to the reference genome. Mus Musculus (GRCm38/mm10) reference genome was used, index of the reference genome was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5

Novel transcripts prediction. The mapped reads of each sample were assembled by StringTie (v1.3.3b) (Pertea et al. 2015) in a reference-based approach.

Quantification of gene expression level. Feature Counts v1.5.0-p3 was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated.

Differential expression analysis. Differential expression analysis was performed using the DESeq2 R package (1.20.0), Benjamini-Hochberg-adjusted P-values reported. Corrected P-value of 0.05 and absolute foldchange of 2 were set as the threshold for significantly differential expression.

AS analysis. rMATS (4.1.0) software was used to analysis the AS event.

Data analysis and visualisation

GraphPad Prism software was used to prepare graphs and calculate standard errors of the mean (SEMs) where experiments were performed with replicates. When more than two groups were compared, a one-way analysis of variance (one-way ANOVA) with Dunnett's correction for multiple comparisons was used. Results were considered significant at a confidence of 95 % ($p < 0.05$). Biorender and Power Point were used to prepare cartoons and diagrams. Multiple Experimental Viewer (MEV) was used to generate the heatmap in **Figure *15*B**. Gene Set Enrichment Analysis (GSEA) was used for hallmark analysis in **Figure *15*C** and **D**.

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Appendix 1 – Genes corrected by combinatorial treatment but not nusinersen alone (transcriptomics)

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
Gene name	Gene id	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (> 0.05) corrected
39142	ENSMUSG00000026977	-0.24084	0	0.00034	-0.19496	0	0.04635	-0.15909	0	0.10110
0610012G03Rik	ENSMUSG00000107002	0.19619	1	0.00702	0.23866	1	0.04287	0.21758	1	0.15106
2410021H03Rik	ENSMUSG00000100572	-1.12632	0	0.00005	-0.87897	0	0.01455	-0.68176	0	0.06215
2610021A01Rik	ENSMUSG00000091474	-0.26979	0	0.01969	-0.29503	0	0.04772	-0.08527	0	0.71912
2700049A03Rik	ENSMUSG00000034601	-0.38073	0	0.00939	-0.44693	0	0.01093	-0.23471	0	0.27396
2900026A02Rik	ENSMUSG00000051339	-0.38215	0	0.00001	-0.30410	0	0.01422	-0.19464	0	0.42808
2900055J20Rik	ENSMUSG00000071860	-1.15828	0	0.01091	-1.11349	0	0.03871	-0.95474	0	0.11291
4930412M03Rik	ENSMUSG00000051008	-1.47819	0	0.01507	-1.38388	0	0.04508	-0.97940	0	0.20309
4930447C04Rik	ENSMUSG00000021098	-0.70066	0	0.00013	-0.53670	0	0.01837	-0.59956	0	0.05157
6330403K07Rik	ENSMUSG00000018451	0.26927	1	0.00000	0.24669	1	0.02654	0.14513	1	0.28537
6330403L08Rik	ENSMUSG00000075585	0.47093	1	0.00001	0.24010	1	0.04224	0.14116	1	0.42217
A230057D06Rik	ENSMUSG00000109394	-0.24524	0	0.00198	-0.26599	0	0.00562	-0.21706	0	0.11712
A330102I10Rik	ENSMUSG00000086363	-0.36459	0	0.00071	-0.26248	0	0.02526	-0.16610	0	0.28015
Aamp	ENSMUSG00000006299	0.18465	1	0.00129	0.19849	1	0.03136	0.12962	1	0.22569
Abhd17a	ENSMUSG00000003346	0.16613	1	0.00404	0.22283	1	0.02154	0.21000	1	0.10208
AC149090.1	ENSMUSG00000095041	0.46467	1	0.00000	0.21759	1	0.00562	0.18897	1	0.20108
Actr1b	ENSMUSG00000037351	0.15858	1	0.00881	0.20898	1	0.00425	0.14751	1	0.10651
Adap1	ENSMUSG00000056413	0.20216	1	0.00105	0.18864	1	0.01751	0.11301	1	0.37722
Adgrl3	ENSMUSG00000037605	-0.26937	0	0.00001	-0.31860	0	0.00893	-0.31968	0	0.05387
Adgrl4	ENSMUSG00000039167	-0.46455	0	0.00220	-0.46757	0	0.01751	-0.35759	0	0.06426
Adgrv1	ENSMUSG00000069170	-0.61963	0	0.00174	-0.64164	0	0.01470	-0.24128	0	0.54642
Adora2a	ENSMUSG00000020178	-1.39756	0	0.00000	-0.56161	0	0.04040	-0.46416	0	0.18950
Adrm1	ENSMUSG00000039041	0.26164	1	0.00002	0.21371	1	0.01189	0.13742	1	0.20195

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
Gene name	Gene id	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (> 0.05) corrected
Afdn	ENSMUSG00000068036	-0.20883	0	0.00706	-0.24043	0	0.00434	-0.20006	0	0.13227
Agt	ENSMUSG00000031980	2.26890	1	0.00000	0.47370	1	0.00000	0.24396	1	0.14463
Ahcyl1	ENSMUSG00000027893	0.34867	1	0.00000	0.24992	1	0.00278	0.08706	1	0.55707
AI837181	ENSMUSG00000047423	0.38203	1	0.00000	0.24325	1	0.04748	0.19629	1	0.08450
Ak1	ENSMUSG00000026817	0.21831	1	0.01503	0.23957	1	0.02120	0.24147	1	0.07419
Alas2	ENSMUSG00000025270	-0.59300	0	0.00011	-2.06076	0	0.00041	-0.88723	0	0.21046
Aldoc	ENSMUSG00000017390	0.63361	1	0.00000	0.29155	1	0.00001	0.20309	1	0.06501
Aplp1	ENSMUSG00000006651	0.24776	1	0.00097	0.21505	1	0.02217	0.13989	1	0.48876
Apoe	ENSMUSG00000002985	0.30846	1	0.00000	0.27321	1	0.02180	0.18349	1	0.22366
App	ENSMUSG00000022892	0.25305	1	0.00003	0.15705	1	0.01838	0.12695	1	0.19910
Arap3	ENSMUSG00000024451	-1.01878	0	0.00000	-0.31212	0	0.03824	-0.24278	0	0.16373
Arhgef5	ENSMUSG00000033542	-1.25867	0	0.00000	-0.50786	0	0.02526	-0.35836	0	0.19877
Arl6ip4	ENSMUSG00000029404	0.33646	1	0.00002	0.27264	1	0.01715	0.23210	1	0.05032
Ascc3	ENSMUSG00000038774	-0.32966	0	0.00011	-0.31273	0	0.02544	-0.16930	0	0.36659
Ash1l	ENSMUSG00000028053	-0.13424	0	0.04297	-0.31595	0	0.00548	-0.24562	0	0.05966
Aspm	ENSMUSG00000033952	-2.20430	0	0.00000	-0.54125	0	0.04712	-0.29806	0	0.38268
Atoh7	ENSMUSG00000036816	0.85112	1	0.02843	0.89005	1	0.04635	0.49306	1	0.48536
Atp1b2	ENSMUSG00000041329	0.15855	1	0.01698	0.18131	1	0.03771	0.08156	1	0.51892
Atp5j	ENSMUSG00000022890	0.14062	1	0.03216	0.17125	1	0.03747	0.20804	1	0.22014
Atp6v0d1	ENSMUSG00000013160	0.16631	1	0.00217	0.19352	1	0.04959	0.15288	1	0.07860
Atp6v1b2	ENSMUSG00000006273	0.31698	1	0.00000	0.21426	1	0.01995	0.18205	1	0.07532
Atp6v1d	ENSMUSG00000021114	0.21345	1	0.00222	0.25288	1	0.03370	0.23500	1	0.05811
Auh	ENSMUSG00000021460	0.21647	1	0.00711	0.27316	1	0.01295	0.24060	1	0.20825
Axin2	ENSMUSG00000000142	-0.36340	0	0.00038	-0.25722	0	0.04637	-0.20170	0	0.26907
Azin2	ENSMUSG00000028789	0.30276	1	0.00398	0.29351	1	0.03468	0.26925	1	0.05555

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
Gene name	Gene id	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (> 0.05) corrected
B3gat3	ENSMUSG00000071649	0.39188	1	0.00000	0.27136	1	0.00800	0.21987	1	0.07703
B830012L14Rik	ENSMUSG00000098202	-0.87572	0	0.00120	-1.14175	0	0.00013	-0.53909	0	0.14620
Bcl2l2	ENSMUSG00000089682	0.29999	1	0.00083	0.23239	1	0.02949	0.19545	1	0.19944
Bin1	ENSMUSG00000024381	0.23826	1	0.00042	0.20183	1	0.04879	0.15021	1	0.35822
Bnip3	ENSMUSG00000078566	0.23611	1	0.00040	0.17730	1	0.03536	0.15531	1	0.32064
Borcs6	ENSMUSG00000045176	0.24513	1	0.03957	0.27469	1	0.04996	0.17901	1	0.31154
C1qa	ENSMUSG00000036887	0.76630	1	0.00000	0.29298	1	0.02544	0.24679	1	0.11823
Cacng7	ENSMUSG00000069806	0.33113	1	0.00000	0.25968	1	0.00379	0.22026	1	0.16333
Calm3	ENSMUSG00000019370	0.28031	1	0.00000	0.19306	1	0.04945	0.14770	1	0.13762
Cand2	ENSMUSG00000030319	-0.83828	0	0.00000	-0.33519	0	0.00617	-0.23401	0	0.28015
Capn2	ENSMUSG00000026509	0.29735	1	0.00000	0.16596	1	0.03374	0.10764	1	0.22901
Cartpt	ENSMUSG00000021647	0.46958	1	0.03904	0.52621	1	0.03423	-0.20079	0	0.60375
Cbarp	ENSMUSG00000035640	0.45181	1	0.00000	0.21082	1	0.04445	0.18967	1	0.07042
Ccdc92	ENSMUSG00000037979	0.20156	1	0.00596	0.28044	1	0.00960	0.29383	1	0.08435
Ccndbp1	ENSMUSG00000023572	0.28289	1	0.00001	0.26857	1	0.01208	0.19232	1	0.18578
Cd248	ENSMUSG00000056481	-0.95701	0	0.00000	-0.65256	0	0.00521	-0.24998	0	0.62801
Cd38	ENSMUSG00000029084	0.72922	1	0.00000	0.51495	1	0.00821	0.37479	1	0.08656
Cd46	ENSMUSG00000016493	-0.47722	0	0.00543	-0.49350	0	0.02812	-0.43357	0	0.07560
Cd81	ENSMUSG00000037706	0.18309	1	0.00479	0.20917	1	0.04135	0.11159	1	0.59096
Cdh5	ENSMUSG00000031871	-1.33256	0	0.00013	-0.66213	0	0.01341	-0.35445	0	0.67376
Cdh6	ENSMUSG00000039385	-0.19170	0	0.03726	-0.33276	0	0.02867	-0.22032	0	0.07153
Cdip1	ENSMUSG00000004071	0.29494	1	0.00001	0.20962	1	0.00242	0.16845	1	0.20108
Cdk5rap2	ENSMUSG00000039298	-0.40225	0	0.00014	-0.29849	0	0.02603	-0.19965	0	0.18998
Cebpb	ENSMUSG00000056501	0.69045	1	0.00023	0.67275	1	0.00478	0.30290	1	0.37301
Cend1	ENSMUSG00000060240	0.20195	1	0.01267	0.26898	1	0.00279	0.26266	1	0.07394

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
Gene name	Gene id	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (≤0.05)	log2 FC	up (1)/ down (0)	padj (> 0.05) corrected
Cep192	ENSMUSG00000024542	-0.39709	0	0.00119	-0.32784	0	0.00657	-0.25464	0	0.10431
Chd9	ENSMUSG00000056608	-0.16269	0	0.01483	-0.29115	0	0.01039	-0.22971	0	0.09838
Chga	ENSMUSG00000021194	0.29456	1	0.00000	0.30093	1	0.00106	0.27607	1	0.05323
Chmp4b	ENSMUSG00000038467	0.15116	1	0.00406	0.14148	1	0.03321	0.10372	1	0.30354
Ciita	ENSMUSG00000022504	-1.45435	0	0.00000	-0.61134	0	0.02416	-0.37456	0	0.33350
Cirbp	ENSMUSG00000045193	0.64643	1	0.00000	0.26846	1	0.03202	0.13706	1	0.26975
Clba1	ENSMUSG00000037594	0.31757	1	0.01956	0.34404	1	0.03058	0.27801	1	0.13762
Cnbp	ENSMUSG00000030057	0.17572	1	0.00046	0.16165	1	0.04338	0.12308	1	0.24003
Coq10a	ENSMUSG00000039914	0.33983	1	0.00012	0.25507	1	0.01595	0.22374	1	0.06714
Coq8a	ENSMUSG00000026489	0.50333	1	0.00040	0.33816	1	0.04339	0.30874	1	0.12109
Cox4i1	ENSMUSG00000031818	0.22952	1	0.00000	0.22518	1	0.00414	0.20594	1	0.17165
Cpeb1	ENSMUSG00000025586	0.43563	1	0.00001	0.34539	1	0.01398	0.28080	1	0.14856
Ctnna3	ENSMUSG00000060843	-1.92123	0	0.00000	-0.50478	0	0.00984	-0.58677	0	0.05811
Ctsb	ENSMUSG00000021939	0.10836	1	0.03862	0.16157	1	0.02459	0.17300	1	0.05874
Ctsf	ENSMUSG00000083282	0.50237	1	0.00000	0.22568	1	0.02162	0.16180	1	0.16694
Cxcl14	ENSMUSG00000021508	0.47760	1	0.00000	0.35477	1	0.00987	0.29847	1	0.22901
Cyp2d22	ENSMUSG00000061740	0.85913	1	0.00000	0.41254	1	0.03586	0.38670	1	0.17012
D7Ertd443e	ENSMUSG00000030994	1.26474	1	0.00112	0.97064	1	0.04161	0.61795	1	0.40760
Dapk3	ENSMUSG00000034974	0.22136	1	0.00493	0.26881	1	0.02848	0.11667	1	0.45994
Dazap2	ENSMUSG00000000346	0.25768	1	0.00001	0.19010	1	0.03514	0.18024	1	0.19985
Dbf4	ENSMUSG00000002297	-0.89987	0	0.00000	-0.42016	0	0.04759	-0.35886	0	0.19775
Dcbld2	ENSMUSG00000035107	-0.15335	0	0.04347	-0.20150	0	0.04094	-0.15306	0	0.32183
Dcc	ENSMUSG00000060534	-0.62593	0	0.00000	-0.69140	0	0.01180	-0.53673	0	0.07054
Ddx47	ENSMUSG00000030204	0.30828	1	0.00017	0.23053	1	0.04237	0.11932	1	0.46308
Dmd	ENSMUSG00000045103	-0.54536	0	0.00000	-0.32060	0	0.04803	-0.31949	0	0.07237

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Dmxl2	ENSMUSG00000041268	-0.21618	0	0.00002	-0.24433	0	0.04670	-0.19427	0	0.15766
Dnah11	ENSMUSG00000018581	-0.95366	0	0.00073	-0.59451	0	0.00911	-0.40523	0	0.42307
Dnah2	ENSMUSG00000005237	-0.35880	0	0.03957	-0.41890	0	0.00922	-0.18252	0	0.73043
Dnah6	ENSMUSG00000052861	-0.76293	0	0.00018	-0.46691	0	0.01082	-0.30278	0	0.49234
Dnah9	ENSMUSG00000056752	-0.37210	0	0.01159	-0.49625	0	0.00106	-0.36750	0	0.20839
Dock7	ENSMUSG00000028556	-0.24060	0	0.00092	-0.29784	0	0.00172	-0.27876	0	0.10000
Dohh	ENSMUSG00000078440	0.38210	1	0.00134	0.36451	1	0.03256	0.18604	1	0.51080
Dok7	ENSMUSG00000044716	0.28644	1	0.00703	0.36597	1	0.01191	0.27021	1	0.12465
Dynlrb1	ENSMUSG00000047459	0.16680	1	0.00505	0.23477	1	0.00617	0.17985	1	0.20138
Dzip3	ENSMUSG00000064061	-0.39272	0	0.00000	-0.20582	0	0.02414	-0.17137	0	0.12987
Ecsit	ENSMUSG00000066839	0.23137	1	0.00691	0.24666	1	0.02526	0.16451	1	0.40864
Eef2	ENSMUSG00000034994	0.34050	1	0.00000	0.13455	1	0.02325	0.10336	1	0.08760
Egr1	ENSMUSG00000038418	0.82765	1	0.00000	0.77655	1	0.00085	0.47555	1	0.13446
Ei24	ENSMUSG00000062762	0.16701	1	0.00631	0.17792	1	0.04764	0.10675	1	0.32618
Eif6	ENSMUSG00000027613	0.17765	1	0.00880	0.18788	1	0.04243	0.13981	1	0.16499
Emc4	ENSMUSG00000027131	0.24435	1	0.00296	0.27046	1	0.01331	0.23932	1	0.08068
Enho	ENSMUSG00000028445	0.21418	1	0.00080	0.25184	1	0.03478	0.18409	1	0.18920
Ep400	ENSMUSG00000029505	-0.17838	0	0.00285	-0.23984	0	0.03563	-0.22088	0	0.05504
Ephb4	ENSMUSG00000029710	-0.97314	0	0.00000	-0.48723	0	0.03699	-0.29979	0	0.32618
Eral1	ENSMUSG00000020832	0.26016	1	0.02078	0.29296	1	0.03489	0.21433	1	0.21021
F3	ENSMUSG00000028128	0.78371	1	0.00000	0.69495	1	0.00000	0.31718	1	0.28626
F8a	ENSMUSG00000078317	0.28152	1	0.00862	0.28631	1	0.04867	0.27371	1	0.05704
Fabp7	ENSMUSG00000019874	-1.55488	0	0.00004	-0.21544	0	0.01613	-0.17644	0	0.16824
Fam124b	ENSMUSG00000043230	-1.80685	0	0.00002	-1.25973	0	0.00617	-0.83355	0	0.15106
Frem2	ENSMUSG00000037016	-1.25737	0	0.00000	-0.56537	0	0.00557	-0.32924	0	0.31709

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Fzd2	ENSMUSG00000050288	-0.41834	0	0.03122	-0.41995	0	0.04408	-0.27761	0	0.41977
Gabrb2	ENSMUSG00000007653	-0.45832	0	0.00005	-0.48186	0	0.02148	-0.34306	0	0.13462
Gadd45g	ENSMUSG00000021453	0.39093	1	0.00099	0.47217	1	0.00740	0.07271	1	0.81859
Gde1	ENSMUSG00000033917	0.20429	1	0.00173	0.24674	1	0.00712	0.20889	1	0.13448
Ggt7	ENSMUSG00000027603	0.22569	1	0.00015	0.24584	1	0.01015	0.13894	1	0.32292
Glul	ENSMUSG00000026473	1.05581	1	0.00000	0.26529	1	0.00028	0.12351	1	0.33900
Gm14377	ENSMUSG00000109501	-1.45588	0	0.00035	-0.99646	0	0.01848	-0.56126	0	0.27583
Gm14399	ENSMUSG00000090093	-0.77644	0	0.04736	-0.97794	0	0.03767	-0.75577	0	0.12742
Gm17477	ENSMUSG00000084917	-2.13074	0	0.00002	-1.34390	0	0.00664	-0.89371	0	0.13227
Gm26811	ENSMUSG00000097816	-0.99039	0	0.00050	-0.86046	0	0.00775	-0.55991	0	0.14349
Gm27206	ENSMUSG00000098889	-1.18718	0	0.01965	-1.29350	0	0.03057	-0.86473	0	0.23669
Gm3227	ENSMUSG00000114306	-1.60664	0	0.00600	-1.46444	0	0.04879	-1.25802	0	0.11164
Gm36992	ENSMUSG00000103435	-2.01399	0	0.00370	-1.61914	0	0.01980	-1.28211	0	0.08737
Gnas	ENSMUSG00000027523	0.21753	1	0.00011	0.20261	1	0.04126	0.15124	1	0.12963
Golga2	ENSMUSG00000002546	0.36010	1	0.00000	0.18482	1	0.01594	0.12183	1	0.28921
Gorasp2	ENSMUSG00000014959	0.20413	1	0.00361	0.21094	1	0.03396	0.17064	1	0.11926
Got1	ENSMUSG00000025190	0.28226	1	0.00360	0.24512	1	0.02507	0.25029	1	0.11542
Got2	ENSMUSG00000031672	0.26437	1	0.00000	0.20125	1	0.01049	0.17894	1	0.12101
Gpr146	ENSMUSG00000044197	0.47397	1	0.00001	0.30501	1	0.03374	0.12635	1	0.64225
Gpx4	ENSMUSG00000075706	0.12827	1	0.03062	0.20909	1	0.01613	0.18042	1	0.13076
Gypa	ENSMUSG00000051839	-1.15071	0	0.01693	-2.23930	0	0.00000	0.56101	1	0.86184
Hcn2	ENSMUSG00000020331	0.30517	1	0.00000	0.23349	1	0.01227	0.22383	1	0.10179
Hdac3	ENSMUSG00000024454	0.21838	1	0.00010	0.21443	1	0.04040	0.18347	1	0.06449
Helz	ENSMUSG00000020721	-0.14763	0	0.03252	-0.32607	0	0.00775	-0.22184	0	0.12465
Hes5	ENSMUSG00000048001	-0.56254	0	0.00463	-0.60357	0	0.02230	-0.44078	0	0.11030

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Hic1	ENSMUSG00000043099	-1.47517	0	0.00508	-0.64801	0	0.00190	-0.18837	0	0.69727
Hif3a	ENSMUSG00000004328	2.77941	1	0.00000	0.49286	1	0.04635	-0.25786	0	0.37514
Hook1	ENSMUSG00000028572	-0.17404	0	0.02756	-0.25050	0	0.04759	-0.21531	0	0.06872
Hoxb13	ENSMUSG00000049604	-3.27637	0	0.04556	-4.59470	0	0.00566	-0.51914	0	0.93435
Htra1	ENSMUSG00000006205	1.26524	1	0.00000	0.43069	1	0.00023	0.30414	1	0.05179
Id2	ENSMUSG00000020644	0.38313	1	0.00000	0.30699	1	0.00090	0.11423	1	0.52629
Idh3b	ENSMUSG00000027406	0.17646	1	0.00597	0.19974	1	0.02109	0.19102	1	0.12196
Igfbp5	ENSMUSG00000026185	-0.48622	0	0.02039	-0.47227	0	0.00297	-0.01500	0	0.99076
Igsf10	ENSMUSG00000036334	-0.40153	0	0.00020	-0.41809	0	0.01751	-0.29252	0	0.21537
Igsf8	ENSMUSG00000038034	0.32383	1	0.00303	0.34356	1	0.00177	0.27623	1	0.08666
Il17rd	ENSMUSG00000040717	-0.39163	0	0.00026	-0.42438	0	0.00059	-0.38941	0	0.06426
Inka2	ENSMUSG00000048458	-0.85323	0	0.00158	-0.36192	0	0.00729	-0.00907	0	0.98069
Inpp5a	ENSMUSG00000025477	0.35122	1	0.00000	0.24045	1	0.01836	0.13854	1	0.24295
Iqgap1	ENSMUSG00000030536	-0.44013	0	0.00001	-0.32595	0	0.04135	-0.22999	0	0.34267
Isg20	ENSMUSG00000039236	-0.90576	0	0.01030	-1.66544	0	0.00259	-0.62236	0	0.49491
Iws1	ENSMUSG00000024384	-0.26922	0	0.00005	-0.22461	0	0.03320	-0.17659	0	0.08731
Jph3	ENSMUSG00000025318	0.37887	1	0.00000	0.18016	1	0.01995	0.16486	1	0.10997
Jund	ENSMUSG00000071076	0.34677	1	0.00001	0.28148	1	0.02692	0.10521	1	0.44904
Kat6a	ENSMUSG00000031540	-0.16139	0	0.00164	-0.27456	0	0.02824	-0.29051	0	0.05759
Kcmf1	ENSMUSG00000055239	0.31228	1	0.00000	0.19343	1	0.01345	0.12478	1	0.32075
Klc2	ENSMUSG00000024862	0.32955	1	0.00000	0.26437	1	0.01112	0.22861	1	0.08436
Klhdc3	ENSMUSG00000063576	0.38195	1	0.00000	0.26347	1	0.00106	0.16748	1	0.05811
Knl1	ENSMUSG00000027326	-2.05738	0	0.00000	-0.86446	0	0.01021	-0.53220	0	0.13813
Lgmn	ENSMUSG00000021190	0.23132	1	0.00019	0.26457	1	0.00090	0.21595	1	0.21275
Limk1	ENSMUSG00000029674	0.22787	1	0.00006	0.17801	1	0.03514	0.21110	1	0.12483

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Lmo3	ENSMUSG00000030226	0.60307	1	0.00000	0.25676	1	0.04214	0.10285	1	0.62291
Lonp1	ENSMUSG00000041168	0.14683	1	0.01290	0.19877	1	0.01694	0.10935	1	0.27249
Lpp	ENSMUSG00000033306	-0.39911	0	0.00026	-0.46377	0	0.03411	-0.32740	0	0.20118
Lrch3	ENSMUSG00000022801	-0.44474	0	0.00000	-0.31142	0	0.02750	-0.14197	0	0.34657
Lypd6b	ENSMUSG00000026765	0.40848	1	0.00007	0.27075	1	0.04127	0.24109	1	0.13691
Lyst	ENSMUSG00000019726	-0.17827	0	0.02131	-0.31975	0	0.00562	-0.20022	0	0.22040
Map1s	ENSMUSG00000019261	0.25278	1	0.00091	0.20670	1	0.04161	0.13837	1	0.28626
Map2	ENSMUSG00000015222	-0.13113	0	0.00231	-0.22649	0	0.00449	-0.20226	0	0.09320
Mapk8ip1	ENSMUSG00000027223	0.21991	1	0.00000	0.16511	1	0.03369	0.10787	1	0.18766
Mbtps2	ENSMUSG00000046873	-0.19711	0	0.04529	-0.27140	0	0.02952	-0.21264	0	0.12393
Mdc1	ENSMUSG00000061607	-0.39458	0	0.00000	-0.28780	0	0.00466	-0.15798	0	0.16658
Megf6	ENSMUSG00000057751	-0.26596	0	0.03717	-0.33314	0	0.04795	-0.36992	0	0.06101
Mest	ENSMUSG00000051855	-0.42672	0	0.00000	-0.38556	0	0.00013	-0.19165	0	0.55206
Mex3a	ENSMUSG00000074480	-0.29457	0	0.02856	-0.43273	0	0.01155	-0.35967	0	0.05817
Mir1188	ENSMUSG00000080406	-0.96357	0	0.00005	-0.65218	0	0.01730	-0.42882	0	0.18954
Mir341	ENSMUSG00000070101	-1.05854	0	0.00006	-0.73834	0	0.02180	-0.61136	0	0.13646
Mki67	ENSMUSG00000031004	-2.13213	0	0.00012	-0.53338	0	0.00614	-0.12664	0	0.78740
Mlf2	ENSMUSG00000030120	0.13381	1	0.00917	0.18044	1	0.01434	0.11378	1	0.29316
Mmp28	ENSMUSG00000020682	-1.27124	0	0.00000	-0.71173	0	0.01837	-0.61282	0	0.20696
Mpi	ENSMUSG00000032306	0.29740	1	0.00023	0.29173	1	0.00693	0.18906	1	0.25458
Mt1	ENSMUSG00000031765	1.98118	1	0.00000	0.24547	1	0.00568	0.13322	1	0.48581
Mt3	ENSMUSG00000031760	0.27310	1	0.00005	0.30412	1	0.00572	0.15424	1	0.59910
mt-Tl1	ENSMUSG00000064340	0.66960	1	0.00075	0.66181	1	0.03489	0.37255	1	0.30298
Naalad2	ENSMUSG00000043943	-1.21612	0	0.00008	-1.05010	0	0.00294	-0.54412	0	0.27249
Ncor1	ENSMUSG00000018501	-0.11908	0	0.04263	-0.27155	0	0.00031	-0.20551	0	0.10319

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Ndufa13	ENSMUSG00000036199	0.16534	1	0.02804	0.28852	1	0.00103	0.25371	1	0.08359
Ndufa4	ENSMUSG00000029632	0.14297	1	0.01711	0.17698	1	0.03421	0.14386	1	0.36409
Ndufs7	ENSMUSG00000020153	0.34656	1	0.00000	0.33471	1	0.00305	0.26200	1	0.13173
Ndufv1	ENSMUSG00000037916	0.26856	1	0.00000	0.20560	1	0.02507	0.18751	1	0.06318
Ndufv2	ENSMUSG00000024099	0.21015	1	0.00044	0.24063	1	0.00378	0.26535	1	0.07340
Nectin3	ENSMUSG00000022656	-0.51523	0	0.00000	-0.31124	0	0.03425	-0.21444	0	0.11821
Nfkbia	ENSMUSG00000021025	0.48040	1	0.00000	0.36937	1	0.00304	0.09511	1	0.65878
Nin	ENSMUSG00000021068	-0.36379	0	0.00043	-0.31538	0	0.02304	-0.31268	0	0.09174
Nop53	ENSMUSG00000041560	0.58651	1	0.00000	0.24760	1	0.00440	0.12073	1	0.30239
Npas1	ENSMUSG00000001988	0.53882	1	0.00003	0.43018	1	0.00586	0.35285	1	0.06990
Nrcam	ENSMUSG00000020598	-0.23319	0	0.00000	-0.21399	0	0.03468	-0.20242	0	0.05889
Nrip2	ENSMUSG00000001520	0.42406	1	0.00101	0.37544	1	0.04923	0.31508	1	0.05646
Nrsn1	ENSMUSG00000048978	0.44777	1	0.00000	0.28511	1	0.00906	0.23151	1	0.11223
Nsd2	ENSMUSG00000057406	-0.21640	0	0.00094	-0.26305	0	0.00893	-0.23344	0	0.12329
Nudt9	ENSMUSG00000029310	0.51365	1	0.00000	0.27748	1	0.01039	0.18929	1	0.16810
Nutf2-ps1	ENSMUSG00000071497	-1.15099	0	0.00077	-0.94068	0	0.03649	-0.53688	0	0.69439
Nwd2	ENSMUSG00000090061	-0.23664	0	0.01295	-0.36637	0	0.00976	-0.34040	0	0.05032
Nynrin	ENSMUSG00000075592	-0.22602	0	0.01612	-0.38054	0	0.04635	-0.28983	0	0.15751
Oaz2	ENSMUSG00000040652	0.21118	1	0.00031	0.19465	1	0.02627	0.13925	1	0.07259
Obscn	ENSMUSG00000061462	-0.70577	0	0.00349	-0.79589	0	0.00116	-0.62706	0	0.05515
Ociad1	ENSMUSG00000029152	0.14825	1	0.01180	0.18419	1	0.04259	0.15877	1	0.11889
Omg	ENSMUSG00000049612	0.48135	1	0.00008	0.34622	1	0.00655	0.32906	1	0.15480
Otub1	ENSMUSG00000024767	0.32389	1	0.00000	0.29499	1	0.00004	0.22796	1	0.07149
Oxa1l	ENSMUSG00000000959	0.29032	1	0.00000	0.20762	1	0.02600	0.16613	1	0.11668
Pag1	ENSMUSG00000027508	-0.26159	0	0.00003	-0.18856	0	0.02894	-0.15771	0	0.29110

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Pak3	ENSMUSG00000031284	-0.29667	0	0.00000	-0.26813	0	0.02242	-0.23713	0	0.09480
Pcdh1	ENSMUSG00000051375	-0.28060	0	0.00071	-0.16338	0	0.03321	-0.11713	0	0.38346
Pcdhga12	ENSMUSG00000102428	-0.55548	0	0.00218	-0.46106	0	0.01553	-0.30939	0	0.30239
Pcgf3	ENSMUSG00000033623	-0.15905	0	0.01099	-0.20901	0	0.03136	-0.20088	0	0.07852
Pcmt1	ENSMUSG00000019795	0.21714	1	0.00013	0.24854	1	0.00209	0.20214	1	0.07629
Pcsk2os2	ENSMUSG00000087115	-1.13393	0	0.03343	-1.56510	0	0.01180	-0.40946	0	0.65442
Pde5a	ENSMUSG00000053965	-0.57390	0	0.00038	-0.54396	0	0.00761	-0.31136	0	0.30239
Pdgfa	ENSMUSG00000025856	0.21623	1	0.00558	0.24309	1	0.01439	0.23645	1	0.08910
Pdk2	ENSMUSG00000038967	0.23346	1	0.02780	0.31822	1	0.04867	0.23704	1	0.25409
Pds5b	ENSMUSG00000034021	-0.27101	0	0.00000	-0.19431	0	0.02350	-0.17162	0	0.23357
Pear1	ENSMUSG00000028073	-0.88363	0	0.00022	-0.58036	0	0.01059	-0.38048	0	0.41653
Per1	ENSMUSG00000020893	1.30205	1	0.00000	0.62017	1	0.00000	0.08939	1	0.66892
Pgbd5	ENSMUSG00000050751	0.22947	1	0.00563	0.26511	1	0.01411	0.22425	1	0.06318
Phlda3	ENSMUSG00000041801	0.18158	1	0.04888	0.37963	1	0.00116	0.35419	1	0.08609
Pim3	ENSMUSG00000035828	0.23981	1	0.00236	0.26920	1	0.00502	0.20119	1	0.36977
Pink1	ENSMUSG00000028756	0.55821	1	0.00000	0.24446	1	0.00096	0.18773	1	0.11206
Pip4p1	ENSMUSG00000035953	0.25067	1	0.00014	0.21863	1	0.03942	0.17185	1	0.24322
Pitpna	ENSMUSG00000017781	0.19205	1	0.00008	0.15030	1	0.04886	0.12651	1	0.17229
Plag1	ENSMUSG00000003282	-0.70723	0	0.00528	-0.62219	0	0.03833	-0.39629	0	0.34519
Pnck	ENSMUSG00000002012	0.14409	1	0.01338	0.25950	1	0.04803	0.18694	1	0.06061
Polr2j	ENSMUSG00000039771	0.26877	1	0.03066	0.34522	1	0.02242	0.17562	1	0.43648
Ppip5k2	ENSMUSG00000040648	-0.15730	0	0.03478	-0.21566	0	0.02472	-0.20232	0	0.05625
Ppp1r12a	ENSMUSG00000019907	-0.27086	0	0.00000	-0.22109	0	0.00945	-0.15049	0	0.08140
Ppp1r9b	ENSMUSG00000038976	0.29961	1	0.00000	0.15032	1	0.02006	0.09883	1	0.29294
Ppp2r2cos	ENSMUSG00000087487	-2.24242	0	0.00038	-1.88613	0	0.00932	-1.04686	0	0.21998

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
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Ppp5c	ENSMUSG00000003099	0.15839	1	0.01742	0.20921	1	0.02369	0.15194	1	0.08381
Pqbp1	ENSMUSG00000031157	0.29237	1	0.00004	0.27210	1	0.04635	0.22267	1	0.05711
Prkn	ENSMUSG00000023826	0.63549	1	0.00000	0.36272	1	0.02988	0.21944	1	0.24108
Prrx1	ENSMUSG00000026586	-1.00257	0	0.00000	-0.40874	0	0.03729	-0.18667	0	0.50030
Psmc2	ENSMUSG00000028932	0.23458	1	0.00058	0.17423	1	0.03164	0.19763	1	0.09149
R3hcc1	ENSMUSG00000034194	0.28813	1	0.00019	0.29224	1	0.00480	0.18190	1	0.20704
Rab1b	ENSMUSG00000024870	0.14025	1	0.02393	0.20114	1	0.04297	0.16097	1	0.12652
Rab4a	ENSMUSG00000019478	0.38798	1	0.00000	0.23665	1	0.02472	0.13590	1	0.42022
Ralgapa1	ENSMUSG00000021027	-0.34312	0	0.00000	-0.27423	0	0.01436	-0.22612	0	0.08586
Rdm1	ENSMUSG00000010362	-1.13473	0	0.00000	-0.73813	0	0.00246	-0.45706	0	0.12715
Rfx7	ENSMUSG00000037674	-0.35890	0	0.00000	-0.24858	0	0.01540	-0.20205	0	0.06830
Rgs6	ENSMUSG00000021219	0.23513	1	0.01186	0.26899	1	0.02214	0.33955	1	0.07589
Rnd2	ENSMUSG00000001313	0.16731	1	0.03828	0.24738	1	0.00430	0.20081	1	0.33342
Rnf126	ENSMUSG00000035890	0.38284	1	0.00000	0.25731	1	0.01980	0.20098	1	0.08587
Rnf208	ENSMUSG00000044628	0.19604	1	0.00195	0.24661	1	0.03508	0.19565	1	0.21964
Rnf220	ENSMUSG00000028677	0.29911	1	0.00008	0.20999	1	0.01107	0.15298	1	0.48305
Rpgrip1l	ENSMUSG00000033282	-0.44171	0	0.00000	-0.23113	0	0.04074	-0.12161	0	0.44783
Rrp1	ENSMUSG00000061032	0.19164	1	0.00046	0.19414	1	0.02176	0.15756	1	0.20316
Rsad2	ENSMUSG00000020641	-1.19808	0	0.00020	-1.96483	0	0.00000	-0.93671	0	0.14349
Rsl24d1	ENSMUSG00000032215	0.22257	1	0.03696	0.25276	1	0.04445	0.19127	1	0.41147
Rtn3	ENSMUSG00000024758	0.21383	1	0.00003	0.19092	1	0.00599	0.14804	1	0.08845
Rtraf	ENSMUSG00000021807	0.20841	1	0.00192	0.21431	1	0.01887	0.18391	1	0.23223
S1pr3	ENSMUSG00000067586	-0.74711	0	0.00078	-0.51523	0	0.00418	-0.27706	0	0.56061
Sap30l	ENSMUSG00000020519	0.48907	1	0.00000	0.23297	1	0.03466	0.24850	1	0.07394
Saraf	ENSMUSG00000031532	0.21311	1	0.00003	0.20483	1	0.00775	0.16423	1	0.09534

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
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Sars	ENSMUSG00000068739	0.13318	1	0.00539	0.18471	1	0.02992	0.13365	1	0.25556
Scml2	ENSMUSG00000000037	-0.66491	0	0.00111	-0.49397	0	0.04081	-0.32310	0	0.36912
Scn1b	ENSMUSG00000019194	0.39113	1	0.00005	0.39788	1	0.00012	0.40895	1	0.06318
Sdc4	ENSMUSG00000017009	1.01682	1	0.00000	0.56934	1	0.00000	0.26531	1	0.07261
Sdhd	ENSMUSG00000000171	0.17495	1	0.00128	0.19597	1	0.03396	0.15547	1	0.05433
Sesn3	ENSMUSG00000032009	-0.11392	0	0.04111	-0.25497	0	0.00554	-0.20836	0	0.12958
Sfpq	ENSMUSG00000028820	-0.48789	0	0.00000	-0.22868	0	0.04161	-0.16266	0	0.08672
Shank1	ENSMUSG00000038738	-0.17173	0	0.00372	-0.19069	0	0.03829	-0.20042	0	0.14349
Shisa8	ENSMUSG00000096883	0.57643	1	0.00000	0.30574	1	0.04408	0.18488	1	0.18603
Sirt3	ENSMUSG00000025486	0.17506	1	0.04428	0.21014	1	0.04635	0.20345	1	0.06698
Slc1a3	ENSMUSG00000005360	-0.33018	0	0.00000	-0.26812	0	0.00026	-0.15622	0	0.42266
Slit2	ENSMUSG00000031558	-0.30239	0	0.00000	-0.20226	0	0.04537	-0.12275	0	0.30489
Slitrk2	ENSMUSG00000036790	-0.32887	0	0.00000	-0.23279	0	0.02111	-0.17216	0	0.12594
Slitrk4	ENSMUSG00000046699	-0.41747	0	0.00000	-0.43628	0	0.00000	-0.23892	0	0.07817
Slitrk6	ENSMUSG00000045871	-0.48190	0	0.00927	-0.61808	0	0.00346	-0.20751	0	0.51866
Smarcad1	ENSMUSG00000029920	-0.25128	0	0.00121	-0.23459	0	0.04158	-0.18005	0	0.14232
Smox	ENSMUSG00000027333	0.87613	1	0.00000	0.30617	1	0.00042	0.02638	1	0.92254
Snap25	ENSMUSG00000027273	0.34838	1	0.00000	0.27175	1	0.00533	0.24826	1	0.07350
Sned1	ENSMUSG00000047793	-0.96768	0	0.00000	-0.47270	0	0.00975	-0.31899	0	0.55045
Sod1	ENSMUSG00000022982	0.23186	1	0.00001	0.18190	1	0.03488	0.18971	1	0.15513
Sorbs2	ENSMUSG00000031626	-0.28758	0	0.00004	-0.23477	0	0.00557	-0.19841	0	0.06436
Sorbs2os	ENSMUSG00000085440	-0.93392	0	0.04905	-1.11134	0	0.03199	-0.60098	0	0.29110
Sox7	ENSMUSG00000063060	-1.94020	0	0.00000	-0.50510	0	0.02478	-0.42197	0	0.09106
Sp4	ENSMUSG00000025323	-0.34793	0	0.00198	-0.39215	0	0.00554	-0.26672	0	0.16673
Spta1	ENSMUSG00000026532	-1.01453	0	0.01789	-1.97452	0	0.00000	0.71978	1	0.74426

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
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Srrm4os	ENSMUSG00000086219	-0.94271	0	0.00018	-0.75132	0	0.00899	-0.63507	0	0.07167
St6gal2	ENSMUSG00000024172	-0.65563	0	0.00000	-0.33751	0	0.00046	-0.24682	0	0.11083
St8sia4	ENSMUSG00000040710	-0.40507	0	0.00021	-0.42868	0	0.02118	-0.37161	0	0.09149
Stag2	ENSMUSG00000025862	-0.27528	0	0.00012	-0.32435	0	0.03116	-0.18257	0	0.55788
Sult4a1	ENSMUSG00000018865	0.20405	1	0.00034	0.27570	1	0.00209	0.19864	1	0.14097
Svep1	ENSMUSG00000028369	-0.92139	0	0.00000	-0.47661	0	0.00085	-0.48953	0	0.16206
Swt1	ENSMUSG00000052748	-0.43949	0	0.01453	-0.42312	0	0.03482	-0.05459	0	0.90253
Syn3	ENSMUSG00000059602	-0.26337	0	0.00849	-0.42307	0	0.00299	-0.34314	0	0.22316
Syne2	ENSMUSG00000063450	-0.60981	0	0.00000	-0.37344	0	0.00417	-0.37552	0	0.09166
Syngr3	ENSMUSG00000007021	0.15339	1	0.01605	0.27189	1	0.02006	0.19518	1	0.05067
Tbc1d7	ENSMUSG00000021368	0.26571	1	0.03548	0.34815	1	0.02544	0.34432	1	0.05161
Tcea2	ENSMUSG00000059540	0.64244	1	0.00000	0.31478	1	0.04450	0.26407	1	0.05295
Tead2	ENSMUSG00000030796	-1.01588	0	0.00000	-0.53250	0	0.00427	-0.28109	0	0.58886
Tent5c	ENSMUSG00000044468	-0.53103	0	0.01755	-1.53158	0	0.00000	-0.38866	0	0.71363
Tfpt	ENSMUSG00000006335	0.37231	1	0.00014	0.31346	1	0.03374	0.21017	1	0.18171
Th	ENSMUSG00000000214	1.01227	1	0.00963	0.86669	1	0.04539	0.82334	1	0.06020
Thap2	ENSMUSG00000020137	-0.46448	0	0.00005	-0.39735	0	0.03374	-0.15477	0	0.41026
Thy1	ENSMUSG00000032011	0.25160	1	0.01825	0.36708	1	0.00141	0.35522	1	0.06157
Tmem160	ENSMUSG00000019158	0.29636	1	0.00021	0.34721	1	0.00439	0.26244	1	0.07354
Tmem240	ENSMUSG00000084845	0.43492	1	0.00000	0.22685	1	0.03730	0.22776	1	0.09320
Tmem91	ENSMUSG00000061702	0.28936	1	0.00001	0.26275	1	0.04803	0.20399	1	0.06995
Tnpo1	ENSMUSG00000009470	-0.15043	0	0.03651	-0.28881	0	0.02187	-0.20454	0	0.05861
Tnrc18	ENSMUSG00000039477	-0.15236	0	0.02553	-0.27592	0	0.00008	-0.29568	0	0.05439
Top2a	ENSMUSG00000020914	-2.44170	0	0.00005	-0.43087	0	0.04829	-0.17209	0	0.21397
Tpgs1	ENSMUSG00000020308	0.31115	1	0.00008	0.32670	1	0.02748	0.25345	1	0.06086

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
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Tprgl	ENSMUSG00000029030	0.25072	1	0.00021	0.16913	1	0.04635	0.14071	1	0.20473
Tra2a	ENSMUSG00000029817	-0.79626	0	0.00003	-0.20377	0	0.03902	-0.11998	0	0.19355
Triobp	ENSMUSG00000033088	-0.33231	0	0.00109	-0.27642	0	0.03368	-0.19248	0	0.14549
Trnp1	ENSMUSG00000056596	0.19278	1	0.03430	0.24278	1	0.04635	0.29214	1	0.12944
Tsc22d3	ENSMUSG00000031431	0.80236	1	0.00000	0.32672	1	0.00519	0.27204	1	0.12741
Tspan3	ENSMUSG00000032324	0.12920	1	0.02887	0.18897	1	0.04164	0.17810	1	0.14594
Tssc4	ENSMUSG00000045752	0.54332	1	0.00000	0.30035	1	0.00439	0.18064	1	0.41592
Tubg1	ENSMUSG00000035198	0.23760	1	0.00262	0.21940	1	0.04594	0.20998	1	0.07924
Uaca	ENSMUSG00000034485	-1.06105	0	0.00000	-0.43724	0	0.03321	-0.29182	0	0.33050
Uba6	ENSMUSG00000035898	-0.70958	0	0.00000	-0.36987	0	0.00688	-0.20896	0	0.06777
Uchl5	ENSMUSG00000018189	0.27825	1	0.00376	0.25777	1	0.04900	0.13210	1	0.48503
Ufc1	ENSMUSG00000062963	0.21410	1	0.01653	0.21393	1	0.02584	0.10632	1	0.46879
Uggt2	ENSMUSG00000042104	-0.28769	0	0.00059	-0.26830	0	0.01340	-0.25332	0	0.16274
Urm1	ENSMUSG00000069020	0.18011	1	0.01275	0.22854	1	0.03059	0.12570	1	0.54908
Usp43	ENSMUSG00000020905	-1.01869	0	0.00194	-0.99009	0	0.01049	-0.61658	0	0.26023
Utp20	ENSMUSG00000004356	-0.34351	0	0.00028	-0.23632	0	0.03594	-0.22895	0	0.05418
Vdac1	ENSMUSG00000020402	0.18412	1	0.00032	0.19716	1	0.00748	0.15107	1	0.17109
Vgf	ENSMUSG00000037428	0.47064	1	0.00000	0.31206	1	0.02205	0.09527	1	0.64665
Vmp1	ENSMUSG00000018171	0.16975	1	0.00888	0.17438	1	0.04537	0.19922	1	0.28830
Vtn	ENSMUSG00000017344	-0.94263	0	0.00138	-0.55405	0	0.01912	-0.38276	0	0.46395
Wbp2	ENSMUSG00000034341	0.29447	1	0.00000	0.27970	1	0.00008	0.20331	1	0.08737
Ypel3	ENSMUSG00000042675	0.45026	1	0.00000	0.27547	1	0.02006	0.23626	1	0.12253
Zbtb26	ENSMUSG00000050714	-0.37007	0	0.00385	-0.38250	0	0.00869	-0.24377	0	0.16774
Zfp322a	ENSMUSG00000046351	-0.22014	0	0.00296	-0.22628	0	0.01489	-0.05205	0	0.77139
Zfp36l1	ENSMUSG00000021127	-0.46014	0	0.00015	-0.48511	0	0.00346	-0.25392	0	0.23901

		UNTREATED VS CONTROL			NUSINERSEN VS CONTROL			MS023 + NUSINERSEN VS CONTROL		
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Zfp397	ENSMUSG00000024276	-0.46545	0	0.00000	-0.31686	0	0.04109	-0.21449	0	0.07635
Zfp407	ENSMUSG00000048410	-0.25625	0	0.04611	-0.34667	0	0.00617	-0.22540	0	0.15094
Zfp442	ENSMUSG00000068130	-1.34705	0	0.00000	-0.83015	0	0.03551	-0.40448	0	0.37697
Zfp568	ENSMUSG00000074221	-0.53566	0	0.00034	-0.54019	0	0.01936	-0.14705	0	0.60895
Zfp60	ENSMUSG00000037640	-0.38966	0	0.00000	-0.22348	0	0.01115	-0.15623	0	0.14909
Zfp644	ENSMUSG00000049606	-0.30165	0	0.00000	-0.28374	0	0.04803	-0.17443	0	0.07589
Zfp873	ENSMUSG00000061371	-0.37598	0	0.04013	-0.45007	0	0.04846	-0.23505	0	0.44783
Zfp882	ENSMUSG00000089857	-0.76964	0	0.00012	-0.61517	0	0.00434	-0.33595	0	0.16310
Zfp992	ENSMUSG00000070605	-0.63615	0	0.00471	-0.52486	0	0.04032	-0.41823	0	0.19897
Zfyve16	ENSMUSG00000021706	-0.53732	0	0.00000	-0.35827	0	0.00038	-0.20023	0	0.07199
Zyx	ENSMUSG00000029860	0.19133	1	0.00344	0.17015	1	0.04829	0.13809	1	0.25030

Table *7.* Genes corrected by combinatorial treatment but not nusinersen alone (transcriptomics)