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Peripheral Spinal Muscular Atrophy Pathology During Human Development

Georg Lindner

Department of Genetics

and Microbiology

Faculty of Biosciences

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Peripheral Spinal Muscular Atrophy Pathology During Human Development

Thesis Director	Thesis Director	Student
Dr. Eduardo Tizzano	Dra. Elena Martínez	Georg Lindner

Institute: Hospital Vall d'Hebron, Vall d'Hebron Institut de Investigación

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LIST OF ABBREVIATIONS

Spinal muscular atrophy	-	SMA
Motor neuron	-	MN
Survival motor neuron	-	SMN
Axonal-SMN	-	a-SMN
Messenger ribonucleic acid	-	mRNA
Ribonucleic acid	-	RNA
Small nuclear ribonucleoproteins	-	snRNPs
Neuromuscular junction	-	NMJ
Ras homolog gene family, member A	-	RhoA
Rho-associated kinase	-	ROCK
C-Jun NH2-terminal kinase	-	JNK
Mitogen-activated protein kinases	-	MAPK
Knockout	-	KO
Plastin-3	-	PLS-3
Neurocalcin delta	-	NCALD
Antisense oligonucleotide	-	ASO
induced pluripotent stem cells	-	iPSC
Real-time quantitative polymerase chain reaction	-	qPCR
Multiplex Ligation-dependent Probe Amplification	-	MLPA
Next-Generation Sequencing	-	NGS
Neurofilament light chains	-	NfL
Micro-RNAs	-	miRNA
Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders	-	CHOP-INTEND
The Hammersmith Functional Motor Scale – Expanded	-	HFMSE
Revised Upper Limb Module	-	RULM
6-Minute Walk Test	-	6MWT
Blood-brain barrier	-	BBB
Onasemnogene abeparvovec	-	OA

Adeno-associated virus serotype 9	-	AAV9
Ubiquitin-like modifier activating enzyme 1	-	UBA1
Intrathecal	-	IT
Monoclonal antibody	-	mAb
Histone deacetylase	-	HDAC
Postnatal	-	P
Dorsal root ganglion	-	DRG
Deoxyribonucleic acid	-	DNA
Acetylcholine receptor	-	AChR
Myosin heavy chain	-	MHC
Immunohistochemistry	-	IHC
Room temperature	-	RT
Red blood cells	-	RBC
Glycophorin-A	-	GYPA
Liver sinusoid endothelial cells	-	LSECs
Interstitial cells of Cajal	-	ICC
Collagen IV	-	Col4
Disease-modifying therapy	-	DMT
Choline acetyltransferase	-	ChAT
Chorionic villus sampling	-	CVS
Enzyme replacement therapy	-	ERT
In utero gene therapy	-	IUGT
Neuropathic Gaucher disease	-	nGD
Human factor IX	-	hF.IX
Integration-deficient lentiviral vector	-	IDLV
Green Fluorescent Protein	-	GFP
Umbilical cord blood sampling	-	PUBS
U.S. Food and Drug Administration	-	FDA
Laser Capture Microdissection	-	LCM

ABSTRACT

This doctoral thesis explores the developmental and systemic impact of a neurodegenerative disease called spinal muscular atrophy (SMA). SMA is traditionally viewed as a motor neuron disease, caused by mutations in the SMN1 gene, leading to reduced levels of survival motor neuron (SMN) protein. Going beyond the neuromuscular system, this work focuses on the effects of SMN deficiency on peripheral organ development, including the liver, pancreas, intestine, heart, lung, kidney, and lymphoid tissues. These aspects of the disease in peripheral organs have never been previously investigated and constitute new information necessary to better understand the developmental aspects of SMA.

To achieve this aim, autopsy material from SMA fetuses, newborns and infants genetically confirmed for SMN1 bi-allelic deletions and with one or two SMN2 copies (predicted to develop type I SMA disease) together with age-matched controls were obtained from Vall d'Hebron University Hospital The thesis focuses on a spatiotemporal analysis of tissue development, examining specific regions of each organ using targeted antibodies through immunopathological histochemistry and immunofluorescence techniques.

The findings provide pathological evidence of particular early, organ-specific defects, demonstrating that SMA extends beyond motor neurons to multiple organ systems during development. The here provided results supports the hypothesis of SMA as a multi-organ disease, emphasize the importance of early diagnosis and intervention, and advocate for investigation of therapeutic strategies that address systemic dysfunction in addition to SMN restoration.

In conclusion, this thesis advances understanding of SMA's complexity and supports the development of more comprehensive clues for follow-up and treatment approaches. It highlights the importance for personalized medicine and contributes to the optimization of SMA management strategies aimed at improving survival and quality of life for affected individuals.

I. INTRODUCTION

1. SMA Clinical and Historical Overview

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder primarily defined by the degeneration and functional loss of alpha motor neurons (MNs) in the anterior horn of the spinal cord. It is the second most common autosomal recessive inherited condition, with a global incidence ranging from 1 in 6,000 to 1 in 10,000. The global carrier frequency is estimated to be between 1 in 32 and 1 in 72 [1].

1.1 Historical Background and Discovery

SMA was first described by Guido Werdnig in 1891 who reported two cases of hereditary progressive muscle dystrophy [2]. At the same time, Johann Hoffmann independently documented seven cases of children exhibiting progressive muscle weakness and early death. Their observations laid the foundation for SMA which first became known as Werdnig-Hoffmann disease, now recognized as SMA type I [3]. Interestingly their observed cases nowadays more closely resemble type II SMA, yet the name remained. In the early 20th century both Beevor and Sylvestre described more severe forms with very early onset, now more resembling SMA type I [4], [5]. The question for why the early infantile form of SMA has become known as Werdnig–Hoffmann disease is likely due to historical confusion with "amyotonia congenita," which had identical pathological findings to the cases described by Werdnig and Hoffmann [6]. Physicians therefore referred to SMA that develops in early infancy as “amyotonia congenita” or “Werdnig–Hoffmann disease”. “Amyotonia congenita” was later discarded as a sole clinical entity as this symptom was present in various conditions, yet the link between early infantile SMA and “Werdnig–Hoffmann disease” remained.

In 1964 Dubowitz described the intermediate phenotype of SMA in detail, but most importantly the variation of severity among affected siblings within the same family, suggesting phenotypic variation in severity of the same basic disease process [7]. Ultimately SMA type II was referred to as Dubowitz Disease.

The mild phenotype was first described by Kugelberg and Welander in 1956, where 12 patients in their 40s – 50s were documented. Initially those patients were diagnosed with muscular dystrophy, however electromyography and muscle biopsy showed evidence of lower motor neuron damage, suggesting muscular disorder as secondary pathology. Their medical history showed that the age of onset ranged from 2 to 17 years, indicating a long period of independent walking and a more favorable and slower disease progression [8]. Type III SMA is now also called Kugelberg-Welander disease.

For almost a century, SMA was classified solely based on clinical symptoms and age of onset categorizing into chronic SMA (intermediate and mild SMA) and acute SMA (severe SMA). Then, in the 1990s multiple researchers discovered disease loci in SMA patients could be mapped to the same

region on chromosome 5q13, where large-scale deletions were reported [9], [10], [11], [12]. In 1995 the responsible gene for SMA was identified and termed the survival motor neuron (*SMN*) gene [13]. At the same time the almost identical *SMN2* gene was also discovered. This led to a breakthrough in diagnosis and classification of SMA. Genetic testing for *SMN1* deletions or mutations became the gold standard, replacing invasive muscle biopsies. SMA was definitively recognized as an autosomal recessive monogenic disorder, and research shifted toward understanding how the lack of SMN protein leads to MN degeneration. The identification of *SMN1* and *SMN2* not only clarified the biological basis of SMA but also laid the groundwork for therapeutic innovation.

1.2 Classification and Subtypes (SMA Types 0–4)

SMA classification is based on age of onset of symptoms and the highest degree of motor function achieved (Table 1). It can be classified into 3 main phenotypes, however some outliers on the extremes of the spectrum subclassification of SMA type I (Type 0 SMA) and SMA type III (Type IV SMA) were introduced. Regardless of the classification, manifestation and differences in degrees of symptoms can vary in patients. However, the scheme remains relevant and provides useful clinical and prognostic information.

Type 0 SMA is the rarest of SMA forms [14]. It is associated with prenatal onset of signs/symptoms such as decreased fetal movement. Patients suffer from arthrogryposis multiplex congenita, as well as profound hypotonia and respiratory stress soon after birth. Congenital heart defect is also a feature of most type 0 cases. Their life expectancy is the lowest of all SMA types with less than 6 months of survival. Some patients are also severely affected by the disease during the first days of life, which is classified as type IA [15].

Typical type IB SMA is the most common form with more than 50% of recorded SMA cases. Disease onset starts in the first 3 months of life and patients will never be able to sit, stand and walk due to severe muscle weakness. Mortality in these babies usually occurs before 2 years after birth due to respiratory muscle dysfunction and ultimately respiratory failure [16].

The type II SMA phenotype is presented with delayed motor developmental milestones and impaired motor skills. While being able to sit upright and even stand using leg braces, they are unable to walk independently and remain wheelchair bound their entire life. They usually suffer from kyphoscoliosis and if untreated, also from restrictive lung disease. Their cough and airway secretion clearing are progressively compromised. Even though the majority may survive into adulthood, high supportive management is required as gastrointestinal (GI) and respiratory complications will increase [17].

Type III SMA patients are able to walk independently during early life. They show profound symptom heterogeneity and patients are often misdiagnosed with myopathy or muscular dystrophy. The distribution pattern of muscle weakness is similar to type I and II SMA, however disease progression is

much slower. Since muscle weakness will progressively increase, specifically in the leg muscles, at some point in life, the need of a wheelchair might be required [18].
Type IV SMA is considered to be the mildest form of SMA as symptoms usually arise in the second decade of life. The comorbidities resemble type III SMA, with mostly weakness of lower extremities. They have an average lifespan and the ability to walk throughout adulthood [18].

Patients with type 0 disease may have one *SMN2* copy whereas most type I cases have 2 *SMN2* copies. Patients with type II may have generally 3 *SMN2* copies whilst long-term walkers usually have 4 copies [19]. While classification-based severity has clinical advantages, it is not always sufficient to provide prognostic information to classify severity of SMA. Furthermore, the prediction of the phenotype using *SMN2* copies is not 100% accurate. Especially for patients with type II and 3 SMA, the parameters of MN loss are not well established, yet are still of high importance to determine their specific therapeutic window for treatment.

Type	Age of Onset	Highest Physiological Function	Age of Death	*SMN2* Gene Copies
0	Prenatal	Respiratory support	<1 month	1
I	0 – 6 months	Never sit	<2 years	2
II	< 18 months	Never stand	>2 years	3
III	>18 months - 3 years	Stand alone	Adult	3 to 4
IV	>21 years	Stand alone	Adult	4 or more

Table 1: Overview SMA classification including their specific characteristics

1.3 Natural History and Disease Progression

SMA is a progressive condition marked by the gradual loss of motor neurons, leading to increasing muscle weakness and atrophy. The natural history varies significantly between types but generally follows a pattern of declining motor function. In more severe forms, such as types 0 and I, the disease progresses rapidly, often leading to early mortality due to respiratory failure. In milder types, such as types III and IV, progression may be slow, and life expectancy remains relatively normal, though quality of life may be impaired [20].
Before disease-modifying treatments, management was purely supportive, focusing on respiratory care, nutritional support, orthopedic interventions, and physical therapy. However, recent advancements have dramatically altered the natural course of SMA. The approval of treatments like nusinersen (Spinraza®), onasemnogene abeparvovec-xioi (OA, Zolgensma®), and risdiplam (Evrysdi®) have shifted the outlook for patients [21], [22]. These therapies aim to increase SMN protein levels and slow or even halt motor

neuron loss. Early diagnosis, particularly through newborn screening, is now essential, as pre-symptomatic treatment has shown significantly better outcomes [23].

2. Genetics and Molecular Pathophysiology

SMA is an autosomal recessive genetic disorder, primarily caused by a mutation in the *SMN1* gene. An individual must inherit two faulty copies of the *SMN1* gene, one from each parent, for SMA to manifest. Carriers with only one faulty gene and one intact gene typically do not show symptoms.

2.1. The *SMN1* and *SMN2* Genes: Structure, Function, and Mutations

Both the *SMN1* and *SMN2* gene produce the SMN protein which is essential for maintenance and function of motor neurons [13]. The *SMN1* gene, located on chromosome 5q13, is primarily responsible for producing the full-length (294 amino acids, 38 kDa), functional SMN protein. It comprises of nine exons and generates transcripts that include exon 7 [24].

In contrast, the SMN2 gene is a centromeric copy, which is nearly identical in sequence to SMN1 and differs by 15 nucleotide changes called paralogue sequence variants [25], most notably the C→T transition at the 6th position in exon 7 [26]. It is proposed that this nucleotide alteration disrupts an exonic splicing enhancer and promote the gain of a silencer ultimately leading to the exclusion of exon 7 in 90% of *SMN2* transcripts. The resulting truncated SMNΔ7 protein is unstable and rapidly degraded, rendering it mostly non-functional [27] (Fig. 1). SMNΔ7 is not entirely non-functional and can associate with full-length SMN and other binding partners, as has been shown in the SMNΔ7 SMN mouse model [28]. This association can improve its stability and its potential function.

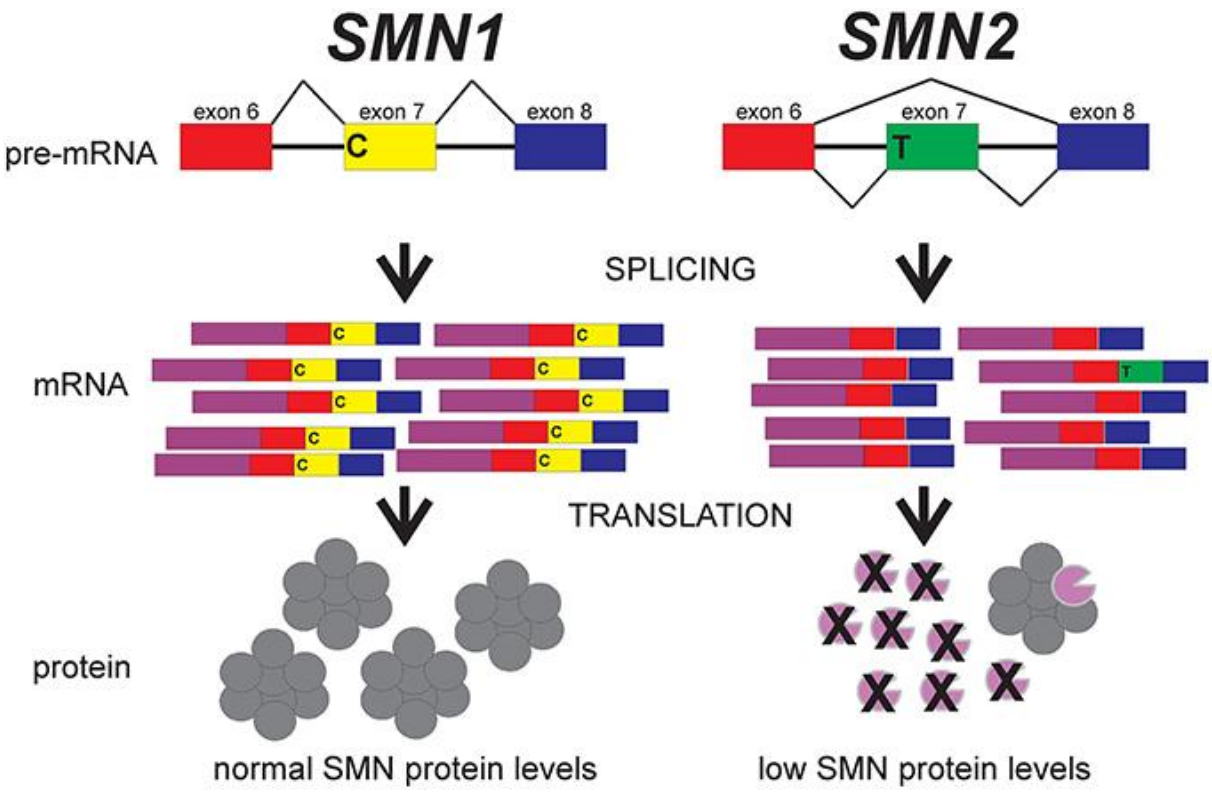


Figure 1: The effect of the C-to-T transition in exon 7 between *SMN1* and *SMN2* on splicing from Butchbach, 2016

Other isoforms of SMN also exist, such as the SMN6B protein which can be translated from both the *SMN1* and *SMN2* gene by inclusion of an Alu sequence following exon 6, as an alternative [30]. SMN6B is present in both the nucleus and the cytosol, making it is twice as stable as SMNΔ7, however its physiological function is not fully understood. Another prominent isoform is SMNΔ5, which can be found in the central nervous system (CNS) and muscle cells, yet again its physiological function remains to be uncovered [31].

There is also an axonal-SMN (a-SMN), which is produced by exclusion of intron 3 during splicing and hypothesized to play a role in axogenesis [32]. Due to an intron 3 stop codon, a-SMN messenger ribonucleic acid (mRNA) is shorter than full-length SMA and only encodes a protein of around 19kDa. It is proposed that a-SMN expression is enhanced in brain and spinal cord during development, yet declines during maturation [32].

2.2. Molecular Mechanisms of Motor Neuron Degeneration

Degeneration of MNs in SMA primarily results from insufficient levels of functional SMN protein, which plays a crucial role in ribonucleic acid (RNA) processing and transport, particularly in the assembly of small nuclear ribonucleoproteins (snRNPs) required for pre-mRNA splicing [33]. Lack of full-length SMN impairs axonal mRNA transport and local translation, both of which are essential for maintaining synaptic integrity and neuromuscular junction (NMJ) function. Disruption of these processes leads to NMJ denervation and synaptic loss, ultimately resulting in MN cell death [34].

Furthermore, defects in cellular functions might result in activation of intracellular stress signaling pathways, that mediate SMA neurodegeneration. While the exact mechanisms remain to be determined, two major signaling pathways have been identified as contributors to neurodegeneration: the Ras homolog gene family, member A (RhoA)/ Rho-associated kinase (ROCK) signaling pathway and the c-Jun NH2-terminal kinase (JNK) signaling pathway [34].

The RhoA/ROCK pathway plays a central role in regulating cytoskeletal dynamics necessary for neuronal growth, stability, and function [35]. ROCK activity and its subsequent downstream targets (profilin IIa, cofilin, lim kinases, myosin regulatory light chain, and myosin light chain phosphatase) showed alterations across multiple human diseases [36], [37]. Low levels of SMN, which interact with both ROCK and profilin, resulted in hyperactivation of ROCK/profilin complexes, leading to abnormal phosphorylation of downstream targets [38]. It is to note though that, while SMN deficiency results in ROCK activation, the modulation of its downstream targets appear to be inconsistent, across the multiple different cell and animal models used [39], [40].

The JNK pathway has also recently been shown to be involved in SMA pathogenesis by activating two upstream kinase modules, ASK1/MKK4/JNK3 and MEKK1/MKK7/JNK3 in both SMAΔ7 mice and SMA patients [41]. JNK is a member of the mitogen-activated protein kinases (MAPKs) and is critical regulator of apoptosis [42]. In SMA specifically, the three isoforms JNK1, 2, 3 showed an increased phosphorylation and especially the JNK3 isoform, predominantly expressed in the brain, mediated neurodegeneration [41]. A JNK3-SMNΔ7 knockout (KO) mouse model showed reduced MN loss, improved muscle development and motor function and increased overall survival [41]. Furthermore a JNK-inhibitor peptide, which was administered to SMNΔ7 mice postnatally delayed MN death and decreased inflammation in spinal cord, ameliorated skeletal muscle innervation and significantly increased lifespan [43]. These mouse model studies highlight the JNK-pathway as a promising non-SMN therapeutic target for mitigating MN cell death and progressive skeletal muscle atrophy.

Another MAPK, the ERK/ELK-1 pathway negatively regulates *SMN2* gene expression in the spinal cord and its inhibition resulted in AKT/CREB pathway activation, subsequently increasing SMN expression and significantly enhanced neuroprotection [44]. Activation of the AKT/CREB pathway improves transcription of *SMN2*, which can be further stimulated by N-methyl-D-aspartate receptors [44]. Studies in mouse models of SMA have shown that activating this pathway can lead to improved motor neuron survival and function.

In addition, several genetic and molecular modifiers have been identified that further influence severity and progression of SMA. One such modifier is Plastin-3 (PLS-3), a calcium-dependent actin-bundling protein, important for axonogenesis by increasing F-actin levels [45]. Overexpression of PLS-3 rescued axon length and outgrowth defects associated in MNs of SMA mouse embryos and in zebrafish models [46]. More recently PLS-3 overexpression restored the ability of clustering calcium channel Cav2.2

protein in growth cones of SMN-deficient motoneurons, suggesting that PLS-3 is required for the proper targeting and function of presynaptic transmembrane proteins [47].

The Zinc Finger Protein 1 has also been proposed as an emerging modifier of SMA [48] as overexpression in SMA mice led to an increase of *SMN2* expression and rescued severe to moderate SMA [48]. Furthermore >3-fold overexpression did not cause any adverse effects in control and SMA mice [48], highlighting its potential for stand-alone or combinational therapy.

In addition, neuronal calcium sensor Neurocalcin delta (NCALD) reduction also acts as a protective SMA modifier, previously demonstrated in five asymptomatic *SMN1*-deleted individuals carrying only four *SMN2* copies [49]. Furthermore, NCALD knockdown also effectively ameliorates SMA-associated pathological defects across species, including worm, zebrafish, and mouse [49]. In induced pluripotent stem cells (iPSC), NCALD-antisense oligonucleotide(ASO) treatment improved neuronal activity and growth cone maturation of SMA MNs, emphasizing its protective effect [50].

Genetic and molecular modifiers underscore the complex interplay of pathways involved in SMA and support the development of combination therapies that go beyond SMN replacement to include modulation of secondary targets.

3. Diagnosis and Biomarkers for Spinal Muscular Atrophy

SMA is a disorder with ~60% of cases having a life expectancy of less than two years. Therefore, early and accurate diagnosis for SMA is imperative for initiating effective treatment and improving patient outcomes. Advancements in genetic testing, biomarker identification, and clinical assessments have improved significantly in the last decades and enhanced diagnostic precision and disease monitoring. Recent advancements in biomarker identification for SMA and newborn screening (NBS) enable prompt intervention and may further improve long-term outcomes for affected children.

3.1 Newborn Screening and Early Detection

NBS for SMA is becoming increasingly implemented globally, with currently 9 countries regularly testing for SMA, allowing for presymptomatic diagnosis and early intervention. Early detection of SMA is crucial as studies show that treatment before the onset of symptoms results in significantly better outcomes as seen in the 5-year follow-up of the NURTURE trial [51]. NBS involves collecting a blood sample on a Guthrie card, followed by genetic testing. The rapid identification of affected infants enables initiation of gene therapy and/or other treatments during the optimal therapeutic window.

3.2 Genetic Testing Methodologies

Genetic testing is the gold standard for SMA diagnosis, with three methods being commonly applied. Firstly, real-time quantitative polymerase chain reaction (qPCR) is highly sensible and specific in testing for SMA. qPCR can identify deletions in exon 7 and exon 8 of the *SMN1* gene, as well as conversions in the *SMN2* gene [52]. Furthermore qPCR can also be applied for carrier testing, to identify individuals who might carry a mutated version of *SMN1* [53]. This is especially important for families planning on conceiving a child and might help with decision-making. qPCR is also inexpensive allowing for high-throughput screening.

The gold-standard method, multiplex Ligation-dependent Probe Amplification (MLPA) is a powerful technique that can accurately detect homozygous deletions of the SMN1 gene, or even partial deletion which might not be detected by more conventional methods, but also determine the SMN2 copy number [54].

Sanger sequencing is traditionally employed to detect point pathogenic variants in SMN1 [55]. Lately, Next-Generation Sequencing (NGS) allows for comprehensive sequencing of SMN1 to detect point mutations and intragenic variants [56].

3.3 SMA Biomarkers

The most direct and relevant biomarkers for SMA are SMN protein levels and *SMN2* mRNA splicing patterns for monitoring disease progression and treatment efficacy [57]. Although SMN expression in whole blood samples can be readily measured, SMN protein in blood is unstable and many studies show that expression levels do not correlate with disease severity or motor gain functions [57], [58], [59], [60], [61]. However, other candidate biomarkers are currently being investigated. Further biomolecular candidates include neurofilament light chains (NfL) as higher levels of NfLs indicate greater axonal degradation and a more severe phenotype [62], [63]. Additionally, creatinine and creatinine kinase levels, which are markers for muscle damage, can be elevated in more progressive SMA [64].
Apart from SMN2 copy number, expression of other non-SMN genes may act as a genetic biomarker. A potential candidate can be PLS-3, which has been shown to correlate with disease severity in children [65]. However, it may not be a useful genetic marker [66].

Gene transcription and splicing regulators can also be candidates. Micro-RNAs (miRNA) showed involvement in SMA pathogenesis [67] and long non-coding RNAs might impact gene expression, including *SMN2* activation [68]. Methylation of *SMN2* impacts its expression and may be a way to measure SMN protein production [69]. Furthermore, genome-wide methylation patterns and methylation of genes other than *SMN1* and *SMN2* may correlate with disease severity [59].
Other, less direct biomarkers are magnetic resonance imaging (MRI) from muscle to determine severity, atrophy and treatment response in SMA [70], and electrophysiological parameters such as electrical impedance myography to measure compound muscle action potential (CMAP) to indicate the degree of muscle response to [71]. Furthermore motor unit number estimation (MUNE) by stimulus of MN's can be applied, as both CMAP and MUNE decline with increased SMA disease severity [72].

3.4 Clinical Scales for Assessment

Clinical assessment scales complement genetic and molecular diagnostics by measuring motor function and disease progression.
Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) is a standardized clinical assessment tool designed for infants with SMA type I, to assess motor skills such as head control, trunk and limb movement, antigravity strength, postural control and spontaneous movement [73].
The Hammersmith Functional Motor Scale – Expanded (HFMSE) is designed for children with SMA types 2 and 3, with greater motor abilities than those assessed by CHOP INTEND, evaluating gross motor skills and ambulatory ability [74]. The HFMSE is often used in clinical trials and real-world practice to measure response to treatment with FDA-approved drugs for SMA.

Complementary to HFMSE, the Revised Upper Limb Module (RULM) assesses upper limb function, more specifically in non-ambulatory SMA patients [75], and the 6-Minute Walk Test (6MWT) to measure fatigue and functional decline [76].

These scales are essential for both clinical trials and routine patient monitoring, offering objective measures of treatment response.

4. Current and Emerging Therapies for Spinal Muscular Atrophy

Therapeutic strategies have dramatically evolved in recent years, offering improved outcomes and quality of life for individuals with SMA. SMN-dependent therapeutic approaches have demonstrated significantly improved disease progression in several types of SMA, particularly in type I SMA patients. In less severe SMA types the outcome of treatment is often to stabilize and halt progression of motor dysfunction. There are also other promising treatment approaches, focused on non-SMN-targets in tissues outside the CNS, which are currently in clinical trials.

4.1 Antisense Oligonucleotides: Nusinersen

Nusinersen (Spinraza®) was the first FDA-approved treatment for SMA and represents a landmark in ASO therapy. Nusinersen targets the ISS-N1 motif in intron 7 of *SMN2* mRNA to include exon 7, thereby promoting production of full-length SMN protein [77]. For maximal efficacy, nusinersen has to be administered intrathecally at an early neonatal stage.

Clinical trials such as ENDEAR and CHERISH were the first to demonstrate remarkable motor milestone achievements and reduced disease progression [21], [78]. To add, a recent long-term study demonstrated continuous efficacy and safety of nusinersen in a large proportion of adult patients with SMA [79]. Despite its success, nusinersen is unable to penetrate the blood-brain barrier (BBB) and therefore periodic intrathecal injections need to be performed.

4.2 Gene Replacement Therapy: Onasemnogene Abeparvovec

As an alternative to *SMN2* upregulation, onasemnogene abeparvovec (OA; Zolgensma®) is a one-time gene therapy, via single intravenous infusion, approved for SMA in children under two years of age. OA is an adeno-associated virus serotype 9 (AAV9) vector driven by the ubiquitous and stable CBA promotor to deliver a functional copy of the *SMN1* gene for permanent restoration [22]. AAV9 is able to cross the BBB during early development, therefore allowing transgene delivery to both peripheral tissues and the CNS, where MNs are targeted. Given that SMN is ubiquitously produced, this is expected to provide additional benefits.

Although OA showed a higher efficacy and response rate than nusinersen, limitations are still present. Major adverse events include, immune responses to the viral vector, hepatotoxicity and thrombocytopenia [80].

4.3 Splicing Modifiers: Risdiplam

Risdiplam (Evrysdi®) is an orally administered small molecule that promotes exon 7 inclusion in *SMN2* transcripts to increase production of functional SMN protein. Unlike nusinersen, risdiplam can systemically deliver the drug to non-neuronal organs, offering potential benefits in peripheral organ systems affected by SMA, such as skeletal muscle [81]. Approved for patients aged two months and older, risdiplam has shown effectiveness in improving or maintaining motor function in both infants and adults with SMA [82]. Its oral administration has expanded access to treatment, especially for patients who do not qualify as candidates for intrathecal injection.

Branaplam was another oral splicing modifier that showed early promise in enhancing *SMN2* splicing, however its development has encountered setbacks, including concerns about potential off-target effects and toxicity [83].

4.4 SMN-Independent Pathways

Beyond SMN-targeted approaches, emerging therapies focus on addressing the broader pathology of SMA through small molecules, neuroprotective agents, and combinatorial strategies.

Given that present therapies are not able to provide a full cure, therapeutics that target SMN-independent pathways are also under investigation. While neuroprotective agents have shown no clear and convincing results in clinical trials [84], myostatin inhibitors have become promising. Myostatin is a hormone that inhibits skeletal muscle growth. Overexpression of the endogenous myostatin antagonist follistatin has shown increased skeletal muscle mass in mice, as well as improved muscle strength and motor function [85], [86]. Other SMN-independent pathway therapies under investigation include ubiquitin-like modifier activating enzyme (UBA1) targeting, which is disrupted in SMA. AAV9-UBA1 therapy managed to increase UBA1 levels as well as increase survival and motor function in the Taiwanese mouse model [87]. Both upregulation of the PLS-3 gene [88], [89] as well as NCALD act as protective modifiers by restoring endocytosis [90], a cellular mechanism affected in several SMA mouse models. Other preclinical trials of SMN-independent pathways, summarized in Chilcott *et al.,* 2022 [91] resulted in variable increases in survival rate in different SMA mouse models.

4.5 Combination Therapy

Combination therapy for patients is currently being explored in multiple clinical trials. One clinical trial called RESPOND, which is currently in phase 4, uses combination therapy to assess the clinical outcomes of intrathecal (IT) injection treatment with nusinersen in patients ranging from 3 to 36 months, who were previously treated with OA. Preliminary data (presented at the US MDA conference, Orlando, March 6, 2024) highlighted that the addition of nusinersen resulted in a reduction of NfL levels, a

biomarker that may indicate stabilization of axonal injury/degeneration, as well as further improvement in motor function. ASCEND is a Phase 3b, open-label study of higher dose nusinersen in patients with later-onset SMA who were previously treated with risdiplam and have remaining unmet needs, in which patient groups range from ≥5 and ≤30 months of age at screening. TOPAZ is a phase 2 clinical study using apitegromab, a fully human anti-proMyostatin monoclonal antibody (mAb) of the immunoglobulin G4/lambda isotype that binds to human pro/latent myostatin with high affinity, administered intravenously every 4 weeks in 2 to 21-year individuals with SMA types 2 or 3 for whom nusinersen treatment was initiated at least 6 months prior. SAPPHIRE (NCT05156320) is a phase 3 clinical study to assess the safety and efficacy of apitegromab in improving motor function as an adjunctive therapy in patients with later-onset SMA who were previously treated with either risdiplam or nusinersen. The age range for eligible participants is 2 to 21 years old at screening. ONYX (NCT05626855) is an open-Label extension study that evaluates the long-term safety and efficacy of apitegromab in patients with type II and type III SMA who have previously completed TOPAZ or SAPPHIRE in a timeframe of up to 6 years. JEWELFISH is an open-label study to investigate the safety, tolerability, and pharmacodynamics of risdiplam in adult and pediatric patients, who will receive multiple doses of risdiplam orally once daily for 24 months [92]. The age range can differ and eligible participants must have had previous treatment with nusinersen, olesoxime or OA. MANATEE (NCT05115110) is a Phase 3, clinical study investigating an anti-myostatin monoclonal antibody (GYM329) in combination with risdiplam, in ambulant pediatric SMA patients aged 2–10 years. RESILIENT (NCT05337553) is a phase 3 clinical trial to evaluate the efficacy and safety of taldefgrobep alfa, another myostatin inhibitor, in both ambulatory and non-ambulatory SMA patients ranging from 4 to 21 years. A proposed classification for therapeutic combinatorial approaches has been recently published [93].

In mice, there is evidence of experimental success from combined SMN-dependent/independent therapy in SMA. A combination treatment with a histone deacetylase (HDAC) inhibitor and an ASO mimicking nusinersen, led to widespread H4 acetylation of the entire *SMN2* locus in type I SMA fibroblasts, showing additive effects on *SMN2* splicing and SMN protein production [94]. Histone deacetylase inhibitors cooperate with a nusinersen-like ASO by reducing the negative feedback on the promotor to upregulate exon 7 inclusion. In the Taiwanese mouse model, combined systemic administration of the nusinersen-like ASO and HDAC inhibitors had strong synergistic effects on SMN production, growth, survival, and neuromuscular function [95]. In another study in the severe mouse model of SMA, an ASO augmenting *SMN2* exon 7 inclusion was used together with AAV-anti-myostatin to block the myostatin pathway [86]. Myostatin inhibition acts synergistically with ASO therapy and improvement of sensory circuits in MNs was observed, but also an increase in body weight, muscle mass and muscle fiber size, and prolonged survival rate. Co-administration of ML372, a protein-stabilizing compound that inhibits SMN ubiquitination without affecting mRNA expression [96] with a splicing-modifying ASO to

incorporate exon 7, led to increased growth, motor function and survival in the SMNΔ7 mouse model [97].

In SMA patients, though sparce, there is also published evidence of combined SMN-dependent therapies. A retrospective report on dual treatment of five children with type I SMA who received nusinersen and OA resulted in overall improvement with no adverse effects, showing that combination therapy is tolerated in these patients [98]. Seven SMA type I patients who received both treatments were assessed for motor function trajectories, ventilation hours and cough assist sessions. The second therapy improved ventilation, but there was no improvement in motor function trajectory compared to single therapy, highlighting the importance of early treatment onset [99]. Another report of four cases of type I SMA initially treated with OA (one of them also with nusinersen) who later received risdiplam, showed increased therapeutic benefits from the combination therapy, with no significant adverse effects [100].
Treatment of cases with 1 and 4-copy individuals have also been reported. One patient with SMA type 0 was treated with both nusinersen and OA and although modest motor improvements since treatment initiation were achieved, with continued motor gain at age 13 months without regression of function, she remained profoundly weak with continued systemic complications from SMA including chronic respiratory failure, dysphagia, congenital heart malformation, digit necrosis, and diffuse macular rash [101]. Another patient with one copy of *SMN2* received early treatment with nusinersen at the age of 13 days and although mild motor improvements 2 months after treatment as well as minimal respiratory improvement were achieved, tracheostomy at 4 months of age was still required with increasing cardiac and autonomic dysfunction, ultimately leading to exitus at 5 months [102].
While combination therapy shows promising results in mice and humans so far, further preclinical and clinical trials are needed to fully discern its effects on patients and to also outline rare adverse events that may occur.

5. Preclinical models of SMA

Preclinical models have been indispensable in unraveling disease mechanisms and evaluating potential therapies across multiple diseases. Specifically, the practice of animal research to understand human biology and disease has evolved significantly, ever since the passage of the Federal Food, Drug, and Cosmetic Act in 1938, which formalized the use of animal models in preclinical validation for the U.S. Food and Drug Administration [103].

5.1 SMA Mouse Models

Genetically engineered mouse models have provided key insights into SMA pathogenesis and therapeutic response. However, despite their utility, current models may also present notable limitations. Unlike their human counterparts, mice only possess a one *SMN* gene and homozygous knockout of *SMN1* in mice leads to massive cell death during early embryonic blastocyst formation resulting in lethality [104]. This suggested that the *SMN* gene in mice has a highly similar biological function to its human counterpart, resulting in the generation of multiple mouse models to replicate the genetic and phenotypic features of SMA, particularly the *SMN1* gene deletion and the presence of human *SMN2* transgenes.

The severe mouse model of SMA (SMN-/-;*SMN2*) was first reported by Monani *et al.* [105] and is usually characterized by a lack of the endogenous mouse *SMN* gene, however they carry two copies of the human *SMN2* transgene [105]. This mouse model has a lifespan of 4 to 6 days and during the first 48 hours of birth they appear to be indistinguishable from their unaffected littermates. Then however rapid deterioration is visible, displayed by decreased movement and heavy breathing [105]. Around postnatal day 3-4 (P3-4), a marked phenotype is displayed with decreased size and body weight, tremors and bell-shaped trunks. At the time of their death their size is decreased on average by 68% and their weight is reduced by half, compared to their healthy littermates [105]. At birth they present a normal amount of MN's, which however significantly drop as they reach their expiration. Similar to humans, increased copy numbers of *SMN2* positively correlate with disease progression in transgenic mouse model, as SMN-/- mice with 8 copies of the human *SMN2* transgene showed no obvious signs of pathology [105]. This model most closely resembles the phenotype displayed by SMA type I patients, recapitulating many key clinical features and therefore is also the most studied mouse model for severe SMA.

The Taiwanese mouse model of SMA (SMN-/-;*SMN2*+/-) is another widely used and well-characterized model first described by Hsieh-Li *et al.* [106]. Five independent transgenic founder lines were generated and cross-bred with SMN+/– mice to create a variety of SMN-/-;*SMN2* mice, that developed progressive MN disease, depending on *SMN2* copy number and were termed SMA-like mice [106]. Riessland *et al.,*

[107] adapted this breeding strategy and created mice with four copies of *SMN2*, allowing for a full lifespan, with almost no visible SMA pathology which most closely resemble findings of mild human SMA [107]. These mice were then crossbred with heterozygous SMN+/- mice lacking the *SMN2* transgene, resulting in litters of 50% control (SMN+/-;*SMN2*+/-) carriers and 50% SMA affected (SMN-/-;*SMN2*+/-)mice [107]. The Taiwanese mouse model has a lifespan of 11 days but develop a severe SMA phenotype displaying reduced body weight, muscle wasting and MN degeneration around P6-7 [106]. This model is frequently used in preclinical studies to evaluate therapeutics that increase SMN protein levels or modify disease progression.

The SMAΔ7 mouse model (SMN-/-;*SMN2*;SMNΔ7) is one of the most commonly used models, as it carries a homozygous deletion of the murine *SMN* gene (SMN−/−) and is rescued by the two human transgenes *SMN2* and SMNΔ7 [28]. SMAΔ7 exhibits a lifespan of about 13–17 days, with pathology onset around P4, characterized by MN loss, muscle weakness and reduced weight gain, which become more pronounced throughout life [28]. Their phenotype resembles a more intermediate severity of SMA.

Another mild to intermediate model is the SMN2B/− mouse model, which exhibits a slower disease progression and longer lifespan than severe or SMNΔ7 models [108], [109]. The SMN2B/− model is a genetically engineered knock-in model where one allele of the mouse *SMN* gene is replaced with the SMN2B allele, containing a mutation in the exon splicing enhancer region of exon 7, altering splicing to mimic the human *SMN2* gene, producing the truncated version of SMN [108]. Unlike other models, the SMN2B/− mouse model solely relies on murine alleles, making it genetically simpler and more stable. Their lifespan is ~25 days and while disease onset occurs around P5-7, progression occurs more slowly, yet gradually [108].

Mouse models have been crucial and very insightful in SMA research, however like any animal model they come with their own limitations. First and foremost, their lifespan is very short. Many models, such as the SMAΔ7 model, exhibit early lethality. In addition, phenotypic severity progresses rapidly, which restricts the ability to study long-term outcomes or chronic therapies [110]. Most importantly though, mice differ significantly from humans in neuromuscular development, immune response and metabolism, significantly limiting translational relevance. It is highly possible that some therapeutic responses seen in mice do not fully predict human outcomes. In addition, as mice do not carry the human *SMN2* gene, mouse models often use human *SMN2* transgenes, which may not recapitulate the full splicing complexity and regulatory landscape of the human genome.

The development of transgenic mouse models has enabled researchers to closely replicate the genetic basis of SMA, producing a molecular phenotype in which SMN protein is expressed in all cells. A range of mouse models has been created that reflect the spectrum of disease severity observed in human SMA patients. These models not only capture the genetic and molecular features of SMA but also accurately reproduce characteristic symptoms and progression of the disease.

5.2 Other Animal Models of SMA

While mice are most often used for studying SMA other models also exist. For instance, *SMN* knockout in *Drosophila, C. elegans* and zebrafish are not embryonically lethal, providing a model in which no functional SMN is present at later developmental stages [111], [112], [113]. In *Drosophila*, SMN knockdown showed progressive loss of mobility and coordination before death, caused by reduced post-synaptic currents and disorganized motor neuron boutons, indicating of NMJ defects [113]. Similarly in zebrafish, knockdown of *SMN1* leads to varying degrees of defects in axon and NMJ development [111], [114]. Zebrafish specifically benefit from their relative ease of genetic manipulation and their optical transparency allowing for real time study the effects of low levels of SMN on motor axon development and growth [114]. In *C. elegans* the orthologue of the human *SMN1* gene, smn-1, had been identified and its knockdown resulted in reduced lifespan and impaired locomotor and pharyngeal activity [112]. *C.elegans* shines as a model due to their rapid lifecycle and ease of maintenance.

Pigs have also been introduced as a model, as their NMJs much more closely resembles human NMJs. By knocking down *SMN1* through means of introducing AAV-9 delivery of short harpin RNAs a new pig model was published [115]. The pig model allowed the characterization of the postnatal role of SMN in motor neurons and the evaluation of clinically relevant biomarkers, such as CMAP and MUNE in response to SMN restoration [115].

5.3 Patient-derived Cellular Models

As previously mentioned, a major challenge in using animal models for SMA is that the *SMN2* gene is specific to humans, limiting how accurately these models can reflect the disease. Recent advancements in *in vitro* disease modeling, particularly using patient-derived cells, offer valuable alternatives.

One of the most basic models are primary fibroblasts. Fibroblasts can be easily obtained from skin biopsies and collected, cultured and analyzed large-scale. They reflect key features of SMA, such as reduced *SMN2* RNA and SMN protein levels, and respond well to *in vitro* treatments [58]. Additionally, primary patient-derived fibroblasts can be used without further modifications, therefore preserving genetic variants that may be modifiers of SMA outcomes [116].

A step further are the use of iPSCs, which allow researchers to generate various cell types from patient fibroblasts. Currently however, many studies still rely on a limited number of cell lines, reducing the potential to capture genetic variability [117]. Interestingly, SMN has also been suggested to play a role in fibroblast differentiation to an iPSC state, which may cause additional variation and differentiation, when trying to mimic SMA [118]. The most direct approach would be a conversion of fibroblasts into neurons, however this will limit the ability to expand cells in large quantities [119]. More complex models, like *in vitro* NMJ systems using co-cultures of iPSC-derived neurons and muscle cells could enable detailed analyses of SMN function and treatment responses [120], [121].

Finally, recent advancements in organoid technologies offer great promise for SMA. The use of organoids in SMA has been limited as of now, due to reliability issues in mimicking organs [122]. Nonetheless, organoids are a promising field of study. Specifically, patient-derived organoid cultures could be used to gain a more in-depth understanding of the consequences of low levels of SMN on organ function, not solely focusing on spinal cord and brain organoids.

These approaches represent a significant step forward in understanding SMA and developing targeted treatments. Although the SMA field has already benefited from numerous valuable disease models, there remain several opportunities for further optimization and advancement.

6. Developmental Aspects of SMA

The neuromuscular system undergoes multiple steps during fetal development (summarized in Table 2).

Gestational Age	Developmental Milestone
7–8 weeks	First interneural connections form between MNs and muscles, leading to early flexion and trunk movement [123]
~12 weeks	Increased sporadic, fetal movement begins due to ongoing axonal outgrowth and neuronal connectivity [124]
~15 weeks	Most NMJs are forming; more specialized and coordinated gross motor movements emerge [124]
~20 weeks	Increased bilateral fetal movements, indicating more advanced neuromuscular coordination [124]
Throughout gestation	Muscles exhibit polyneuronal innervation [125]
Until term	Polyneuronal innervation regresses to mononeuronal innervation, with variability among muscles, such as diaphragm and intercostal muscles regress fastest [126]
Postnatal to early childhood	Maturation completion of the entire motor unit [126]

Table 2: Developmental timepoints and key events in the formation and maturation of the neuromuscular system in the developing fetus

It has previously been shown that SMA pathology in the neuromuscular system already occurs during prenatal development (summarized by Tizzano & Zafeiriou [127]). SMN is ubiquitously expressed in all tissues, yet levels are significantly higher prenatally compared to the postnatal period [128]. Especially MNs require significant amounts of SMN for proper development and maintenance in pre- and early postnatal stages [129], [130]. Using immunoblot studies (mainly available from fetuses at approximately 14 weeks of gestation, predicted to be type I with two *SMN2* copies), it was shown that SMN protein levels are even more diminished, both pre- and postnatally, with the decrease correlating with severity [128]. During the second and third trimester of gestation and the first three months after birth, the amount of SMN protein decreases quite rapidly, specifically in SMA spinal cord samples (4-fold prenatal and 6-fold early postnatal, when compared to age-matched controls) [130].

The magnitude of SMN protein reduction in SMA may be most marked during fetal and neonatal stages. The lack of sufficient SMN levels during development could increase the sensitivity of motor neurons to specific pathological defects, although it has been seen that the most notable reduction of SMN protein levels was actually found in SMA skeletal muscle [128], [131]. In control samples the relative amounts

of SMN in kidney, brain and heart were similar to skeletal muscle, however the decline in postnatal tissues was not as severe [128]. The need for SMN protein to sustain MNs appears to be inversely correlated with age, as older SMA patients showed a slower rate of decline in function and by neurophysiological testing. This is further supported by the observations of disease-modifying therapies (DMTs) which showed a decreased effect, when administered to older children and adults with milder pathology than in infants with more severe disease [124].

In mice and zebrafish SMA was demonstrated to disrupt sensory-motor connectivity [132], [133]. MN development directly influences dorsal root ganglion (DRG) neuron and Schwann cell development [129]. In severe SMA mouse models, embryonic abnormalities include underdeveloped radial growth of motor axons and insufficient Schwann cell wrapping, leading to impaired axonal function at birth and rapid degeneration of unmyelinated axons [129]. Neonatal administration of *SMN2* splicing modulators could enhance axonal radial growth, but prenatal intervention was necessary to fully restore developmental axon growth and maturation in severe SMA mice [129]. Similarly, in zebrafish SMN mutants, Schwann cells failed to properly ensheathe axons and DRG neurons show stunted peripheral axons and failure to divide [132]. Increasing SMN expression in MNs rescued both Schwann and DRG cells, highlighting the cell-autonomous effect secondary to primary MN dysfunction.

Histopathological studies on SMA-affected human fetuses also exhibited changes in nerve and muscle development. Previous reports also showed abnormal nuclei shape, high nucleoplasm density, an increase in deoxyribonucleic acid (DNA) fragmentation and lower choline acetyltransferase (ChAT) expression in SMA motor neurons of fetuses with two *SMN2* copies, indicating impaired MN development [131], [134]. Furthermore, in addition to reduced axon size and varicosities NMJs displayed disorganized acetylcholine receptor (AChR) clusters and an increase in presynaptic vesicles, indicating synaptic defects caused by SMN deficiency [135]. SMA skeletal muscle in the fetus showed a significant reduction in myotube diameter, accompanied by increase of fast myosin heavy chain (MHC) expression at the expense of slow MHC expression, potentially indicating delayed muscular maturation [136], [137]. Collectively, these findings indicate a prenatal onset of SMA pathology of the neuromuscular system, already before MN denervation.

As previously mentioned, in humans more *SMN2* copy numbers, result in more full-length SMN protein production, subsequently leading to a milder SMA phenotype. Some variants in *SMN2* have been identified that further alter SMN production, e.g. c.859G>C which increases SMN production and is associated with a milder course of disease [138]. It is likely that there is a threshold for SMN levels, below which pathology in the SMA fetus emerges and above which the development occurs relatively normal. For example, patients with one *SMN2* copy have been presented with cardiac malformation, arthrogryposis and congenital anomalies that are potentially detectable by ultrasound assessment already during the prenatal period [14]. Individuals with one or two copies usually show pathological changes before or shortly after birth, often developing symptoms within the first three months of life [124]. In contrast, patients with three or more copies show later symptom onset, generally after six months of age.

Furthermore, there is currently no data on prenatal motor neuron loss or abnormal axonal/NMJ development at birth in individuals with three or more copies, allowing for a larger therapeutic window for intervention. While most individuals with four or more copies usually remain asymptomatic for years, some may still experience earlier symptom onset [139].

Regulation of SMN expression is more complex than in species with two *SMN* genes than in rodents or other animals, which only possess a single gene. In humans, *SMN1* is the primary source of full-length SMN mRNA and protein in the spinal cord during development, whereas in other tissues such as muscle and kidney, *SMN1* and *SMN2* contribute more evenly [131]. As *SMN2* cannot compensate sufficiently with full-length SMN, its regulatory mechanisms during fetal development should also be considered for early therapeutic intervention.

Postnatal CNS pathology is most pronounced in the spinal cord, where progressive loss of α-motor neurons in the anterior horn is accompanied by chromatolysis, axonal swelling and a reduction in excitatory synaptic inputs from interneurons [140]. Surrounding glial cells, including astrocytes and microglia, show activation and contribute to neuroinflammation [141], while distal axons and NMJs display immaturity, denervation, and impaired neurotransmission [124].

7. Peripheral Organ Development and their Non-Neuronal Contributions to Pathology

In the last decades many scientific breakthroughs led to the assumption that SMA does not only target MN's but that multiple systems beyond the CNS are affected by SMN deficiency [142]. A still increasing number of reports from multiple SMA mouse models show multisystem involvement. In human SMA cases several non-neuronal organs have been described with disease mechanisms are correspondent to mice, albeit not as severe (summarized in Yeo & Darras, 2020 [143]). This section describes peripheral organs which are not directly associated with SMA, that have previously shown pathology resulting from SMN deficiency and were also analyzed during the doctoral study and later described in the thesis.

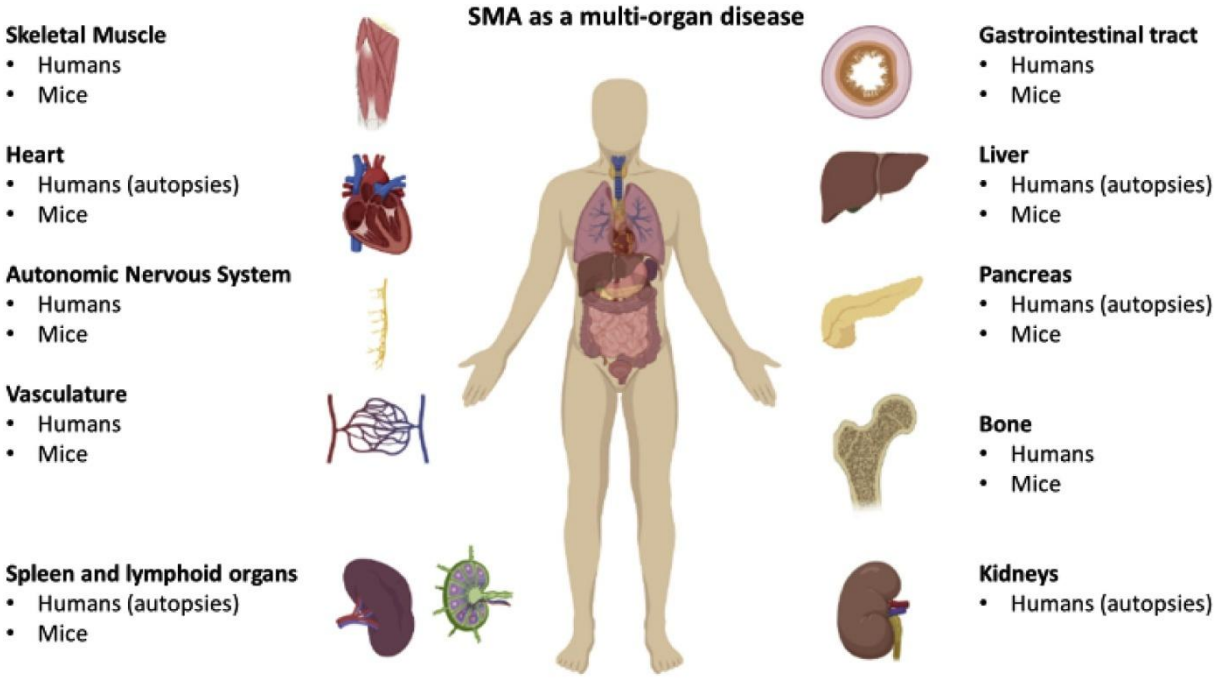


Figure 2: Non-neuronal organs with previously described pathology in SMA. Figure from [143].

7.1. Cardiovascular System – Heart

The first system that develops is the cardiovascular system at the fourth week of conception, due to the embryos growing metabolic demand and the placenta not being able to sustain via diffusion of nutrients [144]. At first, cardiac progenitor cells migrate in the epiblast to the mesoderm and mature into cardiac myoblasts and from the same splanchnic layer so-called "blood islands" undergo vasculogenesis to form vascular structures [144]. As development continues two endocardial tubes form and fuse together to create the primitive heart tube, comprising five main segments: truncus arteriosus, bulbus cordis, primitive ventricle, primitive atrium, and sinus venosus [144]. Around day 23 cardiac loop formation begins by elongation of the heart tube, which takes approximately five days. By the end, trabeculated regions begin to form which serve as primitive ventricles [144]. The sinus venosus and atria then start

to develop as the sinus venosus receives blood the vitelline, umbilical, and cardinal veins and right horn of the sinus venosus incorporates into the right atrium, forming the smooth-walled sinus venarum and the left horn regresses forming the coronary sinus. The pulmonary veins are absorbed into the left atrium, creating its smooth-walled part [144]. Septation occurs in two separate processes: Atrial septation involves the septum primum and septum secundum, forming the foramen ovale and ventricular septation involves a muscular septum and a membranous septum to separate the right and left ventricles with the sinus venosus incorporating into the right atrium, resulting in the formation of the sinoatrial node[144]. From the fifth to the ninth week the atrioventricular and semilunar valves from mesenchymal tissue and primordial valves [144]. The cardiac conduction system forms around the same stage as valve formation, with the sinoatrial taking over as the pacemaker in the fetal heart and atrioventricular node, which receives the electrical impulse from the sinoatrial valve, passing them to the ventricles [145]. By week 10 of fetal development the major components of the heart cardiovascular have formed, which then grow and mature during the remainder of prenatal development.

The heart as the main organ of the cardiovascular system pumps blood and oxygen around the body. As the first functional organ to form, the heart plays a critical role in establishing circulation, supplying oxygen and nutrients, essential for the growth and survival of the developing embryo.

In multiple SMA mouse models previous pathology has already been described, in form of altered gross morphology, dilated ventricles and disorganized cardiomyocytes, already occurring in pre- and early symptomatic stages [146]. SMA type III patients were frequently presented with cardiac rhythm disorders [147]. In addition, a growing number of congenital heart defects have been recognized in patients with severe SMA, including atrial septal defects, dilated right ventricle (RV) and ventricular septal defects (summarized in Palladino *et al.,* [148]). These findings in humans highlight the role of the heart in both the progression and systemic manifestations of SMA.

7.2. Digestive System – Intestine, Liver, Pancreas

The prenatal development of the digestive system involves all three germinal layers, which form different parts of each system and organogenesis occurs from the third to the eight week [149]. The endoderm gives rise to the epithelial lining and glandular structures, the mesoderm contributes to connective tissue and smooth muscle formation and the ectoderm contributes to the enteric nervous system. When the yolk sac incorporates in the embryo around the fourth week, the primitive gut is formed which further develops and divides into fore-,mid- and hindgut [149]. From the foregut, liver and pancreas, along with the esophagus, stomach, duodenum and the gallbladder arise; from the midgut the duodenum, jejunum, ileum, cecum, appendix and parts of the colon; and from the hindgut the rest of the colon along with the anal canal develop [149]. From the sixth until the 11th week the midgut starts to rotate and mature bringing the intestine into its final position in the embryo [149]. Around week nine, villi development begins and by week 12 crypts are starting to form in the intestine [149]. The last part

of the intestine to form is the muscularis mucosae along with additional intestinal villi lining specialized for absorption and by week 24 fetal intestinal function begins [150].

Gastrointestinal defects in patients with SMA have previously been described and include gastroesophageal reflux, constipation and delayed gastric emptying [151]. Significant histopathological abnormalities, with striking reduction of vascular density and increased macrophage infiltration, were detected in the small intestine in the taiwanene SMA mouse model [152]. Severe SMA mouse models have also reported reduced villi length with a blunt, “club-shaped” appearance and severe intramural edema in the lamina propria [153]. In addition, mice with SMN deficiency showed slow intestinal transit and reduced colonic motility [154]. The effects of reduced SMN levels on morphology of the human intestine has yet to be described and given the functional abnormalities in the GI tract of mice, it is likely that the human GI tract might be affected in similar fashion.

Fetal liver development starts with hepatobiliary morphogenesis around the fourth week of gestation [155], when a primordial extension of the foregut, the liver bud, pushes into the mesenchyme where then proliferation of hepatoblasts, with later differentiation into hepatocytes occur [156]. Vitelline veins which penetrate the septum transversum mesenchyme differentiate into intrahepatic components, including hepatic chords and portal sinusoids [157]. The intrahepatic biliary tree begins to form from week five to nine. It is believed that biliary epithelial cells develop from bipotential precursor hepatoblasts toward a biliary fate [158]. Hepatoblasts at the outer boundary of the portal tracts acquire a biliary phenotype, forming the ductal plate and by week 12 the liver begins bile production [159]. Around week 13 maturation of functional hepatocytes and the formation of a biliary network connected to the extrahepatic bile ducts occurs, further developing remodeling to give the classical liver characteristics even until after birth [160].

During fetal development, the liver is the largest organ and the main contributor to hematopoiesis. During adult life it functions mainly as a filtration system removing toxins and metabolites from the body's blood supply. A study by Coovert *et al.,* [161], in which SMN expression of a multitude of organs was measured, showed that the liver is among the organs with the highest expression levels, leading to the hypothesis that it could be affected in SMA patients. In fact, in the Taiwanese mouse model of severe SMA, disorganized sinusoids could be found and no clear sinusoidal spaces between the hepatic plates were visible [162]. Sinusoids overall appeared to be poorly developed with red blood cells (RBC) being accumulated, rather than the liver’s major blood vessels [162]. In addition, hematopoietic elements were still present throughout P5-P9 in the Taiwanese mouse model. In human patients, post-mortem studies on a cohort of 72 SMA cases found an increased susceptibility to developing dyslipidemia and fatty liver (steatosis) [37,5%], compared to the general population with a prevalence of 20% [163]. These findings in both mice and humans highlight the liver in both the progression and systemic manifestations of SMA.

The pancreas also starts to develop at week four, with the outgrowth of the dorsal and ventral pancreatic bud from the endoderm, which fuse together during the seventh week, with the ventral pancreas making

up a significantly larger portion [164]. From week eight to ten the main pancreatic duct is formed as it joins other ductal systems, with the ventral pancreas forming the main part and the dorsal pancreas becoming an "accessory" pancreatic duct [165]. From week ten on until term pancreatic tissue differentiates into the endocrine and exocrine pancreas [165], and by week 12 the pancreas develops insulin-secreting capabilities, though the endocrine pancreas is not fully functional until birth [149].
The pancreas is divided into two parts, the endocrine and exocrine pancreas, with the exocrine pancreas making up 95% of the whole tissue, comprising of acinar cells and the duct system. The endocrine portion of the pancreas is arranged as discrete islets of Langerhans which mainly compose of glucagon producing α-cells and insulin producing β-cells, to regulate blood glucose concentrations [166]. In the SMN2B/− mouse model fasting hyperglycemia, hyperglucagonemia, and glucose resistance were observed, but they also displayed reduced β-cell and increased α-cell populations [167]. Furthermore, in human pancreatic samples of SMA type I patients (p = 6; Age Range 7 – 35 months), a similar abnormal pancreatic islet composition could be observed, with all cases displaying increased glucagon-producing α-cells [167]. These findings suggest that alterations in pancreatic islet composition, particularly the imbalance between α-cells and β-cells might already occur during prenatal development. Given what has been previously shown in SMA mouse models, it is possible that pathology might indeed already occur during development in several organs belonging to the digestive system in humans affected by severe SMA.

7.3. Urinary System – Kidney

The urinary system start to form around the fourth week from the mesoderm as it reorganizes itself to form epithelial buds [168]. From the buds, nephric structures form sequentially, the pronephros which is nonfunctional and regresses by day 25, the mesonephros which is temporarily functional by excreting small amounts of fluid into the amnion between the sixth and tenth week of development, and the mesonephros which continues to differentiate in the permanent kidneys [168]. Interestingly, as opposed to females, in males the mesonephros ducts persist and develop in the testis and ejaculatory duct, becoming part of the reproductive organs [168]. During the sixth week of development, the basic renal architecture begins as branching occurs and collecting ducts are formed. The formation of functional nephrons begins when the ends of developing collecting ducts signal the surrounding cap mesenchyme to form nephric vesicles. These vesicles mature into nephron tubules with distinct regions: a Bowman's capsule, proximal and distal convoluted tubules and the loop of Henle [169]. These formation processes occur until the 32nd week of gestation, forming around 1-3 million collecting tubes [170]. Bladder development also begins in the fourth week with the division of the cloaca by the urogenital septum, leading to the formation of the urogenital sinus, subsequently growing and maturing into the bladder and urethra [168]. Mesonephric ducts and ureteric buds contribute to bladder wall formation and trigone

development, which is eventually lined by endoderm-derived epithelium, completing development [171].

Kidneys are vital organs responsible for filtering blood, removing waste products, toxins, and excess fluids through the production of urine. Additionally, kidneys play a key role in regulating electrolyte levels, such as sodium, potassium, calcium, and maintaining acid-base balance to ensure proper physiological function.

The kidney of the SMA mouse model has been presented with overall smaller kidney size, reduced nephron density, fibrosis and vascular deficits [172]. To add previous reports in a portion of human kidneys in SMA type I patients revealed histopathological abnormalities, including tubular injury and fibrosis, but also glomeruli presenting rare focal mesangial hypercellularity suggesting impaired kidney function and renal tubular dysfunction [173]. Considering that renal pathology has been described in human autopsies of SMA type I patients in addition to pathology in presymptomatic SMA mice, it is likely that onset of pathology may already occur during prenatal development.

7.4. Lymphoid System - Spleen and Thymus

The lymphoid system can be separated into primary lymphoid organs where lymphocytes are produced and mature, and secondary lymphoid organs where lymphocytes interact with antigens to initiate an immune response.

Lymphoid system development begins during the fifth gestational week [174]. The spleen develops from the mesenchyme and at week six forms the lobulated spleen primordium, which coalesces into a single lobulated spleen by week seven [174]. At week 10 the fetal spleen starts to support the liver in hematopoiesis. Although at this stage the splenic system is formed, its development is quite primitive as there is no distinct architectural organization. The parenchyma is predominantly occupied by hematopoietic precursors and almost no lymphocytes are present at this stage [175]. From week 19 to 23 both B- and T- lymphocytes start to condensate around splenic arterioles, with splenic sinuses beginning to form. As the spleen continues to mature, the red and white pulp segregate and develop, with increased numbers of lymphocytes forming until term [176]. After birth splenic hematopoietic function decreases, thus becoming a crucial component of the immune system.

The spleen is the second largest source of lymphatic tissue that performs several important functions in the body such as filtering and cleaning blood by removing damaged RBC's, platelets, and other debris to help in maintaining the quality of the blood circulating in the body [177]. Splenic functions are important for maintain body homeostasis as well as serving a large role in inflammatory immune responses [178]. In the SMA mouse model SMN levels in the spleen were relatively high compared to skeletal muscle and spinal cord [179]. Splenic architecture in SMA mouse models was impaired as overall size was decreased and the red pulp, along with its distinctive macrophages, were reduced [180].

Additionally, immune cells were severely mislocalized, highlighting that SMN is required for the normal development of lymphoid organs [179].

The thymus also starts to develop during the fifth gestational week yet unlike the spleen, derives from the endoderm along other lymphoid organs [181]. Around the seventh week the classic lobular architecture begins to develop, during the eight week the thymocyte precursors start enter the thymus and thymopoiesis begins [164]. From week 10 to 12 formation of early cortical and medullary areas occur and around week 20 the thymus becomes more organized and fully functional [164]. At term, the thymus contains vast amounts of mature T cells and thymopoiesis continues to occur.

The thymus is a primary lymphoid organ, essential in T-cell development and maturation [182]. The thymic B-lymphocytes present a unique group of B-cells, believed to originate from thymic B-lineage committed to be progenitors and they have been thought to play a role in T cell negative selection due to their location in the medulla [183]. Similar to the spleen, thymic architecture in SMA mouse models is also impaired as overall size is decreased, along with a reduction of the thymic cortex and cortical cellularity [179]. Additionally, T- cell development is impaired, as abnormal levels of different maturation stages of T-cells were identified, which may impact immune response [179].

Spleen and thymus are key players in the immune system, contributing to the development and maintenance of immune cells, yet they have not yet been investigated in human SMA patients. Understanding the connections between spleen and thymus development and SMA may further deepen our knowledge on possible immune dysregulation of the disease.

7.5. Respiratory System - Lung

Human lung development occurs in five distinct stages starting with the embryonic stage at week four, when the lung bud arises from the endoderm and from week five to seven, primary bronchi form, which branch into secondary bronchi [164]. The second stage, the pseudoglandular occurs from week 5 to 17 and mainly revolves around continued extensive branching and the formation of terminal bronchioles [164]. At this point, the fetal lung does not resemble the adult lung, as alveoli are still absent. In the canalicular Stage from week 16 – 25, respiratory bronchioles form and vascularization increases [164]. From week 24 until term primitive alveoli form and capillary-alveolar contact increases as the lung expands, eventually becoming the gas-exchange surfaces of the lung [184]. The final stage of lung development is the alveolar Stage occurring from term till 8 years postnatally, when maturation and multiplication of alveoli occurs. Especially in the first 2–3 years most alveolarization occurs [185].

Respiratory impairments are the most frequent non-neurologic complication and the leading cause of mortality in SMA. Type II and nonambulant type III SMA patients present variable severity of chest wall distortion, paradoxical breathing, and impaired airway clearance and cough, compounded by bulbar muscle weakness [186]. The severe form of SMA and lung however was not yet described. Generally, the lung has not been extensively described in either human patients or SMA mouse models in detail.

While the lung of the SMA mouse model has previously been described with variable degrees of emphysema with ruptured alveolar septa and enlarged alveolar spaces [153], no histomorphometrical analysis in SMA patients was carried out. Most reports focus on lung function of SMA patients with milder forms, thus highlighting the novelty in this study, especially considering developmental stages of severe SMA in human patients.

As stated before, an increasing number of reports from peripheral organ pathology in mouse models of SMA, and to lesser extend in human patients have emerged, such as heart [148], [187], kidney [172], [173], spleen [179], [180], thymus [179], pancreas [167], [188], liver [162] and intestine [152]. Findings from SMA mouse models clearly show a multisystem involvement, and to a lesser extent in human patients, yet disease mechanisms are correspondent (summarized in Yeo and Darras, 2020 [143]).

Given the previously described peripheral organ pathology in SMA mouse models and SMA patients beyond the neuromuscular system, we aim to uncover possible pathology that might occur during development of several peripheral organs in humans affected by severe SMA and to compare their findings.

II. HYPOTHESIS AND OBJECTIVES

1. Hypothesis

This thesis focuses on a critical, unresolved issue with significant clinical relevance, especially amid the emergence of novel, mostly CNS-targeted therapies: the pathology affecting peripheral organs outside the CNS. There currently is no cure for SMA. However, the unique genetic situation in SMA patients allowed for the successful development of a number of competing therapeutic approaches (previously introduced in Introduction Section 4), making SMA a model disease for the evaluation of innovative therapeutic concepts of the future. Those treatments however focus on motoneurons as the major cellular therapeutic target and while they represent major advances, they may also give rise to unforeseen clinical challenges. Furthermore, given that SMA therapies are among the most expensive currently available, identifying the appropriate indications and optimal use of such treatments will be vital to ensuring sustainable healthcare.

Although becoming increasingly important, peripheral organ pathology in SMA is still an understudied topic and a comprehensive and joint approach is urgently needed in the light of emerging treated patient populations.

Given that pathological changes have previously been identified in a variety of clinically relevant organs in both the SMA mouse model and human SMA patients, this thesis hypothesizes that severe SMA leads to further pathological defects in non-neuronal organs. In addition, SMN expression levels are significantly higher prenatally and motor neuron degeneration onset already occurs prenatally, leading to the proposition that peripheral organ development should start before birth.

2. Objectives

Based on the proposed hypothesis, this thesis aims to create a baseline of peripheral organ defects in SMA predicted to develop SMA compared to control fetuses.

The detailed objectives of this thesis are as follows:

- Description of the histopathology of SMA during human development in different extra-neuronal tissues. Evaluation by histomorphometric analysis in liver, pancreas, intestine, heart, kidney, lung, spleen and thymus as well as other staining methods adjusted to each organ respectively

- A comparison with age-matched control samples, including a description of the normal developmental histology in these organs

- Comparison of human findings with the features reported in SMA mouse model pathology

III. MATERIALS AND METHODS

Sample procurement

SMA cases for a post-mortem study were identified at the Vall d'Hebron University Hospital Pathology Department. Four of them corresponded to legal pregnancy interruptions (see Table 3). In all cases, macroscopic study was conducted according to stablished protocols, the specimens were fixed in 10% buffered formalin for 1-2 weeks. All SMA samples were genetically confirmed by bi-allelic absence of the *SMN1* gene and the presence of 1 or 2 copies of *SMN2*, as previously described (Table 3) [19]. All cases have had previous family history of severe SMA. The study was approved by the Clinical Investigation Ethical Committee of the Vall d'Hebron University Hospital, Barcelona, Spain PR(AMI)458/2021.

A control group of 8 cases matched by age, or gestational age was gathered. The post-mortem procedure in the control group was the same as in the SMA group.

Developmental Stage	n: control	n: SMA	gestational age of non-SMA	gestational age of "SMA"	Predicted SMA type	Number of *SMN2* copies
Prenatal	2	2	13 weeks	13 weeks	SMA 1	2
	2	1	18 weeks	18 weeks	SMA 1	2
	0	1	21 weeks	21 weeks	SMA 0	1
Perinatal	0	1	36 weeks	36 weeks	SMA 0	1
	2	1	1 month	1 month	SMA 0	1
Postnatal	2	1	8 months	8 months	SMA 1	2

Table 3: Overview of control and SMA samples of this study including SMA genotype (n= sample size; based on family history in prenatal cases) Cases were grouped into 3 types (prenatal, perinatal and postnatal) according to age. For better analysis and comparisons, the case of 1 month was grouped into the perinatal group.

Immunohistochemistry

Immunohistochemistry (IHC) was performed with the Benchmark Ultra staining module, using the ultraView Universal DAB kit (Ref: 760-500) from Ventana Medical Systems, which includes the necessary reagents to perform IHC. For deparaffinization EZ prepTM solution (10x) (Ref: 950-102) was used. Antigenic retrieval was performed with the solution (Cell Conditioning 1 pH 8) (Ref: 950-124). Blocking with 3% hydrogen peroxide solution and washing with Reaction Buffer (10X) (Ref: 950-300) each step was performed automatically.

Antibodies for Immunohistochemistry Staining

CD68 (PG-M1) Monoclonal mouse (Ref:PDM065) was prediluted and incubated for 36 minutes. CD31 (JC/70A) Monoclonal mouse (Ref: 760-4378) was prediluted and incubated for 40 minutes. CD34 (QBEND/10) Monoclonal mouse (Ref: MA1-10202) was prediluted and incubated for 40 minutes. Glycophorin (GA-R2) Monoclonal mouse (Ref:760-4257) was prediluted and incubated for 28 minutes.

CD117/C-KIT(EP10) Rabbit monoclonal (Ref:08763909001) was prediluted and incubated for 36 minutes. CD3 (2GV6) Rabbit Monoclonal (Ref: 790-4341) and CD20 (L26) Mouse Monoclonal (Ref: 760-2531) were prediluted and incubated for 40 minutes.

Primary antibodies were prediluted and incubated (See Table 4). Antibodies were localized by the secondary antibody (HRP Multimer; which contained a cocktail of HRP-labelled antibodies (goat anti-IgG/IgM mouse and rabbit antibodies. Then the resulting complex was visualized using DAB Chromogen; (tetrahydrochloride of 0.2% 3, 3'-diaminobenzidine) and finally counterstained with Hematoxylin (Ref: 760-2021) and Bluing Reagent (Ref: 760-2037). Once the automated process is finished, the preparations are washed, dehydrated and assembled for further usage.

Immunofluorescence

Pancreas, spleen and thymus sections were first heated overnight in a 60°C oven and then deparaffinized in xylene (2 × 10 minutes), fixed in 100% ethanol (1 × 10 minutes), rehydrated in 95% and 75% ethanol (5 minutes each), and washed for 5 minutes in 1xTBS pH7.6 (5 minutes). Sections were then placed in unmasking buffer (citric acid monohydrate pH 6.0) and put in a microwave at full power until boiling. Sections were removed from microwave and rested for 5 minutes. The process was repeated at 20-30% of full power until bubbles started to appear (4< times completing 20 minutes). Sections were allowed to warm for another 20 minutes and then washed 3x5 minutes in 1xTBS pH7.6.

Sections were then incubated for 1 hour at room temperature (RT) in blocking buffer (5% BSA - 1xTBS) inside a humid chamber. Samples were then quickly washed for 30 seconds in 1xTBS. This was followed by an overnight incubation at 4°C with the primary antibodies diluted in 2,5% BSA – 1xTBS. Subsequently, sections were washed 1× with water and 1x in PBS – Tween. Afterwards sections were incubated 1 hour at RT in a dark chamber with the secondary antibodies diluted in 2,5% BSA – 1xTBS and then washed 1× with water and 1x in PBS – Tween. ProLong™ Gold Antifade Mountant with DNA Stain DAPI (Invitrogen™) was added, followed by the slides being mounted.

Antibodies for Immunofluorescent Staining

The primary antibodies for pancreas used were as follows: mouse monoclonal glucagon antibody (1:2000; Ref: G2654; Millipore Sigma) and rabbit recombinant monoclonal insulin antibody (1:200; Ref: EPR17359; Abcam). The secondary antibodies used were as follows: Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (1:1000, Thermo Fisher Scientific), Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (1:1000, Thermo Fisher Scientific).

The primary antibodies for spleen and thymus used were as follows: rabbit recombinant monoclonal HLA-DR antibody (1:100; Ref: EPR3692; Abcam) and rabbit recombinant monoclonal HLAE antibody (1:100; Ref: EPR25300-104; Abcam). The secondary antibodies used were as follows: Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (1:1000, Thermo Fisher

Scientific), Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (1:1000, Thermo Fisher Scientific).

IHC/IF	Ref:	Target	Objective	Incubation Time
Hematoxylin & Eosin	760-2021 760-2037	Cell Nuclei, Extracellular Matrix and Cytoplasm	Visualization of cellular tissue structure	Automated Process
Anti-GYPA antibody[GA-R2 (HIR2)]	760-4257	GYPA protein	Visualization of erythrocytes for quantification	28 minutes
Anti-CD31 antibody[JC/70A]	760-4378	CD31 protein	Visualization of liver sinusoid endothelial cells for observing sinusoid development	40 minutes
Anti-CD34 antibody	760-4378	CD34 protein	Visualization of liver sinusoid endothelial cells for observing sinusoid development	40 minutes
Anti-CD68 antibody[PG-M1]	PDM065	CD68 protein	Visualization of macrophage membranes for quantification	36 minutes
CD117 (EP10)	08763909001	Tyrosine-protein kinase KIT	Visualization of interstitial cells of Cajal	28 minutes
Von Kossa	-	Phosphate Deposits	Quantification of Calcification	1 hour
Anti-Collagen IV antibody (ab6586)	PDM276	Collagen IV	Quantification of Collagenic tissue	36 minutes
Anti-CD3 antibody	2GV6	CD3-T cell receptor	Visualization of T-cell Population	40 minutes
Anti-CD20 antibody	L26	CD20 B-cell molecule	Visualization of B-cell population	40 minutes

Anti-Glucagon[G2654]	EPR17359	Pro-glucagon (Human) in endocrine pancreas	Visualization of glucagon producing α-cells	2 hours
Anti-Insulin[EPR17359]	G2654	Pro-Insulin (Human) in endocrine pancreas	Visualization of insulin producing β-cells	2 hours
Anti-HLA-E antibody	EPR25300-104	MHC class I receptor	Quantification of MHC I expression	2 hours
Anti-HLA-DR antibody	EPR3692	MHC class II receptor	Quantification of MHC II expression	2 hours

Table 4: List of used IHC/IF methods, corresponding target and intended purpose. Grey highlighted antibodies were used for IHC. Yellow highlighted antibodies were used for IF.

Scanning

Whole slide digital images were prepared using PANNORAMIC 250 Flash III scanner (3DHistech) and "SlideScanner software" (3DHistech). Whole slide images were analyzed in CaseViewer software (3DHistech). 10x FOV's of specific sections on different organs were scanned and processed into.gif images. The images were then read and analyzed directly using ImageJ software (FIFJI Build) [189].

Quantification of Immunohistochemistry/ Immunofluorescence

Quantification of different sections using whole-slide images was carried out using tools in ImageJ (FIJI) with its built-in image processing plugins [189]. Results represent data from at least n=10 different sections of each sample. For IHC and IF samples, each section image was converted into 8-bit and colour separation for further analysis.

For IF analysis of the pancreas specifically, at least 100 Langerhans islets per sample were chosen. Criteria for each analyzed islet included the presence of both insulin- and glucagon- expression. Each islet image was converted into 8-bit and colour separation for further analysis.

Statistical analysis

Statistical analysis was carried out on GraphPad PRISM software (GraphPad Software Inc.). Data are presented as mean ± SD. Statistical testing utilized unpaired, two-tailed t-tests.

IV. RESULTS

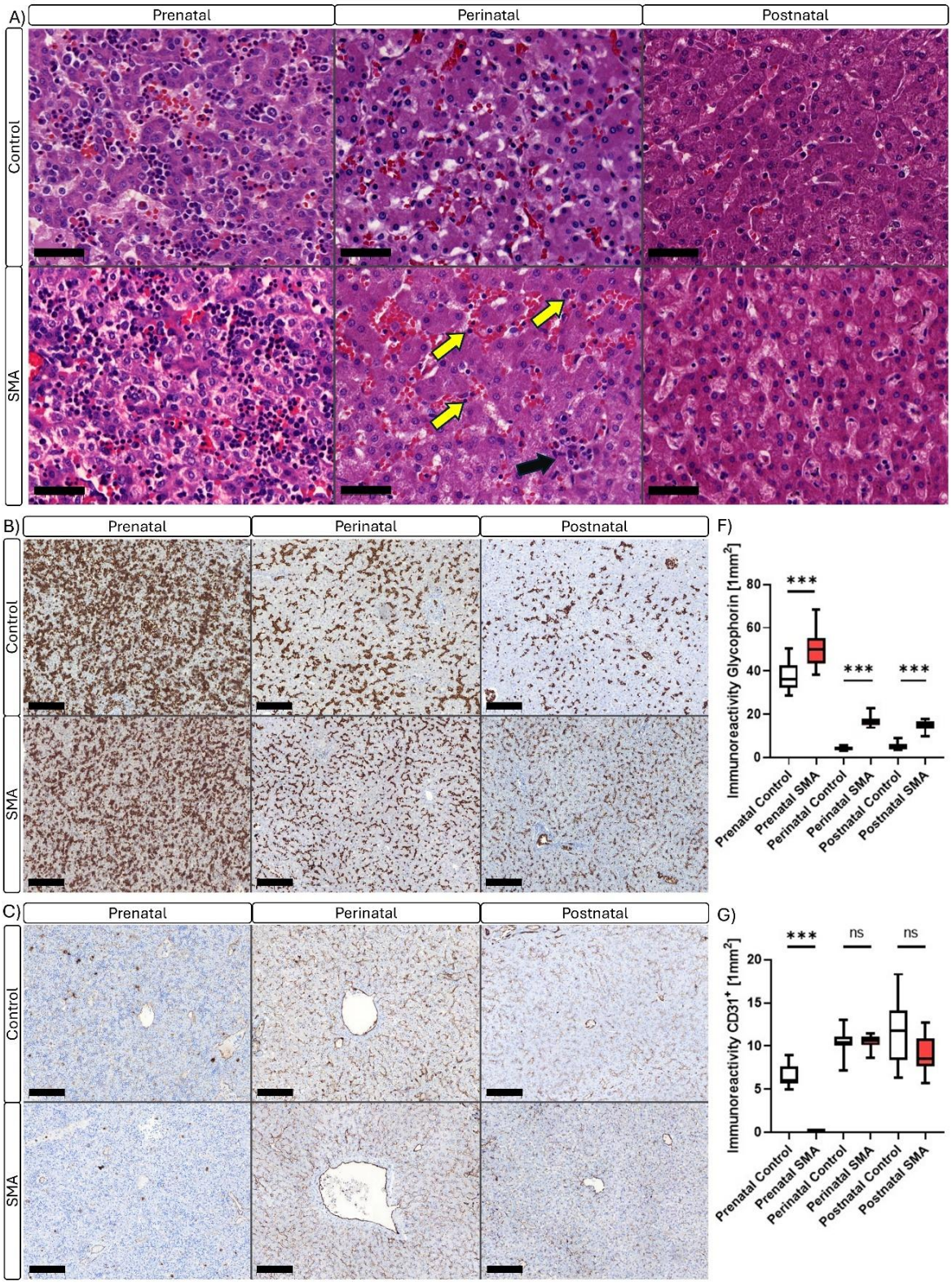
Due to previously described peripheral organ pathology in mouse models of SMA and human patients we aim to see if possible pathology in central organs of the digestive system (liver, pancreas, intestine), the immune system (spleen, thymus) but also heart, kidney and lung already occurs during pre- and perinatal development and if disease mechanisms are correspondent with SMA mouse model pathology.

1. Histomorphometric analysis of the liver

The main components of the liver are the central vein, portal triads, hepatocytes, sinusoidal capillaries and bile ducts. Prenatally the formation of the classic, roughly hexagonic, lobular structure appears similar to their age-matched controls, as the classical structure becomes more visible with maturation (Fig. 3). The sinusoidal spaces show intense hematopoietic activity. Hepatic cords develop the classic structure of one-cell thick hepatocytes, which radiate outward from the central vein of the lobule. The portal triads show no structural differences as bile ducts are emerging at similar gestational ages, hepatic arteries are present with an abundance of red blood cells (RBC) and portal venules appear to be similar in size.

Perinatally, the lobular structure is clearly visible with one-plate thick hepatic cords. RBCs are mainly located around fully formed portal triads, with significantly less presence around the hepatic arteries compared to prenatal stages. In the SMA tissue however, RBCs appear to be more prominent in the sinusoidal spaces, compared to their age-matched controls. Additionally, RBCs appear to be more present in the sinusoidal spaces surrounding the portal veins in SMA. Location and increased presence of RBC's indicate that the sinusoidal spaces could be congested with blood.

Postnatally, no structural differences are visible, and hepatic architecture appears to be similar to perinatal stages. SMA tissue at postnatal stages also show RBCs much more concentrated around portal triads, similar to perinatal and postnatal control tissue.


A)
Prenatal
Perinatal
Postnatal
Control
SMA
B)
Prenatal
Perinatal
Postnatal
Control
SMA
C)
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Control
SMA
F)
Immunoreactivity Glycophorin [1mm²]
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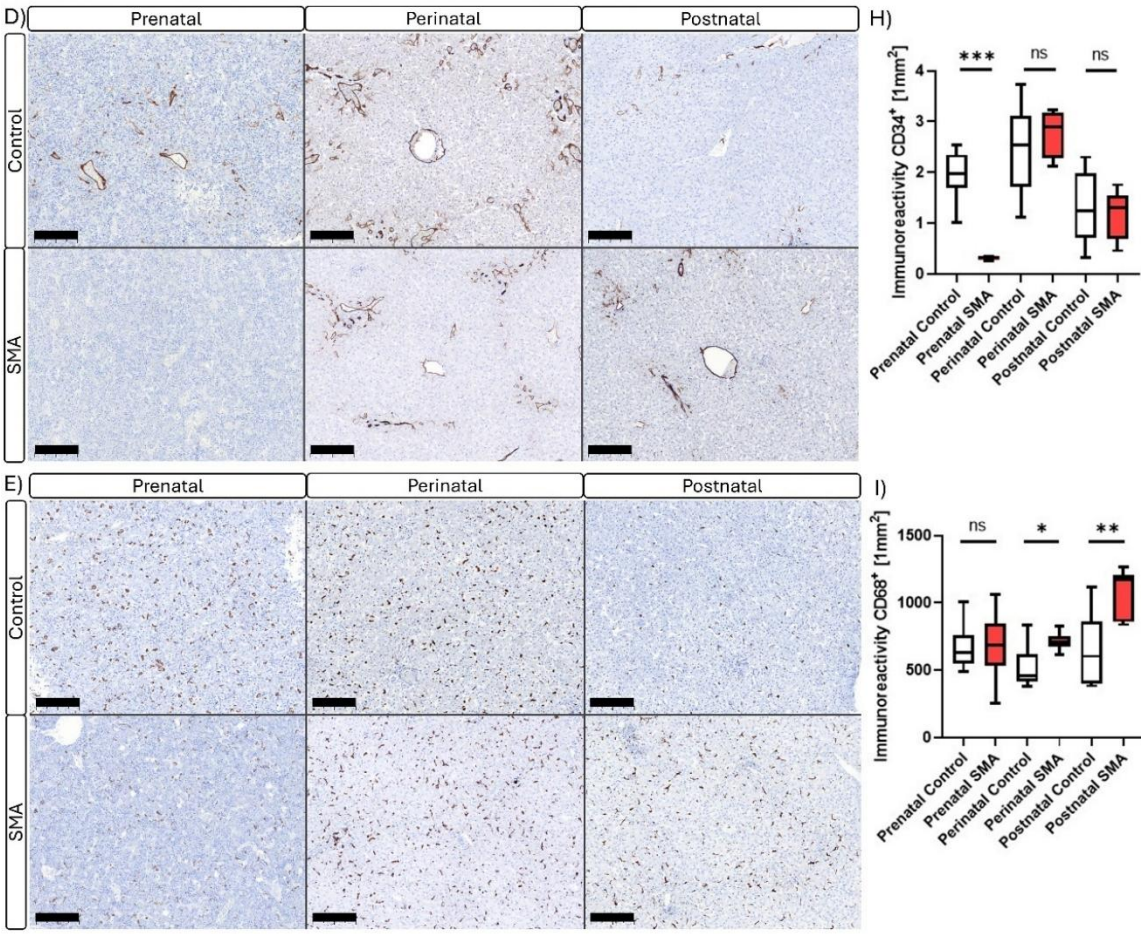


Figure 3: Liver Histology of control and SMA tissue A) Representative images of H&E-stained prenatal, perinatal and postnatal liver tissue sections at higher magnification. Yellow arrowheads indicate nucleated RBCs and black arrowheads indicate proerythroblasts in perinatal SMA samples. [Scale bar 50µm]. Representative images of B) GYPA, C) CD31, D) CD34, E) CD68 stained prenatal, perinatal and postnatal liver tissue sections [Scale bar, 200µm]. Immunoreactive Area of F) GYPA, G) CD31, H) CD34, I) CD68 represented as a Box and Violin Chart. Comparison between control and SMA tissue were significant (control = White; SMA = Red (mean, SD, n = see Table 3,, n^{ns} = P>0.05, n* = P < 0.05, n** = P<0.002, n*** = P < 0.001, unpaired t-test))

Hematopoietic elements remain in the liver throughout development

A closer look at the composition of the RBCs for hematopoietic progenitor cells were taken to gain more insight on hematopoietic elements (Fig. 3B). Progenitor cells can be differentiated in the sinusoids as they still contain their nucleus. In prenatal control and SMA samples progenitor cells are visible throughout the whole tissue, compatible with active hematopoiesis.

Perinatally a multitude of proerythroblasts and nucleated RBCs can be identified in SMA tissue, but not in control tissue, also suggesting prolonged hematopoiesis (Fig. 3B). At the postnatal stage no more erythropoietic elements remain in SMA tissue, appearing similar to controls.

Glycophorin-A expression in SMA liver tissue

To further examine the hematopoietic properties of the analyzed liver tissue, Glycophorin-A (GYPA) antibody staining was carried out to visualize RBCs in tissue (Fig. 3B). To compare and quantify the data from the GYPA stained whole-tissue slides, random sections were analyzed and overall GYPA expression in the sections were measured.

Prenatally, there is a significant increase in GYPA expression of SMA compared to the controls throughout developmental stages. During the perinatal stage overall GYPA expression levels drop significantly in both control and SMA samples compared to prenatal samples and remain consistent throughout postnatal development, yet increased GYPA expression continues to be significantly higher in SMA tissue (Fig. 3B). The continuous increase of GYPA could also be attributed to vascular congestion.

Sinusoid formation throughout liver development

CD31 and CD34 act as markers for liver sinusoid endothelial cells (LSECs) during development with different developmental patterns. Early prenatally a significant decrease in both CD31- and CD34- expression in SMA tissue could be seen, as little to no staining was detected (Fig. 3C,D). During the perinatal stage, LSEC staining of SMA tissue much more resembled their age-matched controls, not only in expression levels, but also in location. This trend continued throughout postnatal development, with no differences in expression profiles and location of both CD31 and CD34.

Investigation of Kupffer cells by CD68 expression

Using CD68 as a marker we investigated Kupffer cells presence in the liver (Fig. 3E). Prenatally no significant difference in the Kupffer cell count could be observed. However, throughout perinatal development the presence of Kupffer cells increased and was significantly higher in SMA compared to age-matched controls. This trend continued throughout postnatal development. Whereas Kupffer cell levels in control tissue remained consistent throughout development, SMA tissue showed a gradual increase of Kupffer cells correlating with development.

2. Histomorphometric analysis of the pancreas

The exocrine pancreas is composed of clusters of cells called acini and the pancreatic ductal system. The endocrine pancreas is composed of islets of Langerhans, which are mainly comprised of α-cells and insulin producing β-cells. Prenatally both control and SMA pancreas depicted the classic acinar structure, with an abundance of connective tissue (Fig. 4). Overall, the histomorphometry of all tissue analyzed corresponded to their age-matched controls.

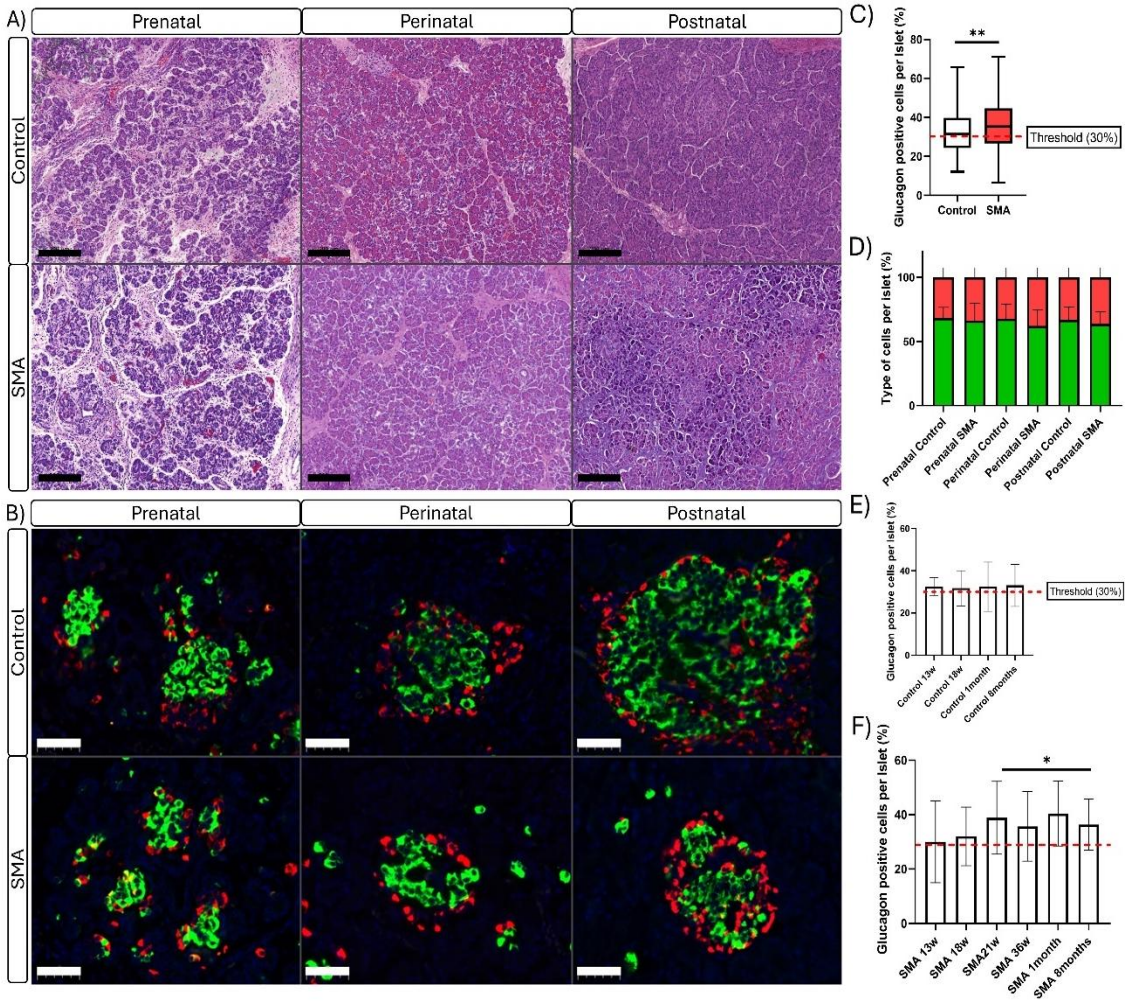


Figure 4: Pancreas histology of control and SMA tissue A) Representative images of H&E-stained prenatal, perinatal and postnatal pancreas tissue sections [Scale Bar, 200µm]. Perinatally and postnatally the SMA tissue does not appear to differ from control samples, as the gross overall structure appears to be highly similar with acini growing in size and connective tissue diminishing. B) Representative pancreatic cross-sections from controls and severe SMA cases co-labelled for glucagon (red; α-cells),insulin (green; β-cells), and DAPI (blue; nuclei). [Scale bar = 50µm]C) Quantification of islet composition represented as a Box and Violin Chart shows a significant increase in glucagon-expressing α-cells ($p<0.002$, unpaired t-test). D) Quantification of islet composition during developmental stages represented as a Box and Violin Chart between control and SMA tissue were significant (Glucagon = red; Insulin = Green; (mean, SD, n = see Table 3, $n^{**} = P<0.002$, $n^{*} = P<0.05$, $n^{ns} = P>0.05$, unpaired t-test) E) Glucagon-positive cells per islet of individual controls. F) Glucagon-positive cells per islet of each severe SMA case individually. Four out of six pancreatic islet compositions revealed a significant increase in glucagon-positive α-cells (mean, SD, n = see Table 3, unpaired t-test)

Islet composition in human developing pancreas

Using immunofluorescence SMA pancreatic samples, co-labelled for glucagon-producing α-cells and insulin-producing β-cells, were compared with age-matched controls (Fig. 4B). Taking all SMA samples together, a significant difference in glucagon-producing α-cells in SMA cases was observed compared to controls (Fig. 4C). Looking at the prenatal stage separately however, α- and β-cells were at relatively normal levels in SMA tissue while during the perinatal and postnatal stage a significant increase in glucagon-producing α-cells, at the expense of insulin producing β-cells was observed (Fig. 4C). Upon further comparing SMA cases individually, we observed that cases with impaired islet composition begin from 21 weeks of gestation and onward in

development (Fig. 4F). Interestingly three of the four cases with abnormal islet composition were from SMA type 0 cases.

3. Histomorphometric analysis of the intestine

The small intestine is separated into duodenum, jejunum, and ileum as major compartment, which vary significantly from one another in length and thickness and cellular structure. In all 3 regions, the intestinal wall is composed of mucosa, submucosa and the muscularis externa. Depending on the part of the intestine, which was removed for analysis the thickness can differ significantly as the 3 layers are highly variable depending on the tissue region. In our analyzed SMA tissue all 3 layers had a similar structural layout as their age-matched controls (Fig. 5A). Additionally, the shape and cellular composition of the villi in the intestine did not appear to significantly change from control tissue and no abnormally-shaped villi were spotted.

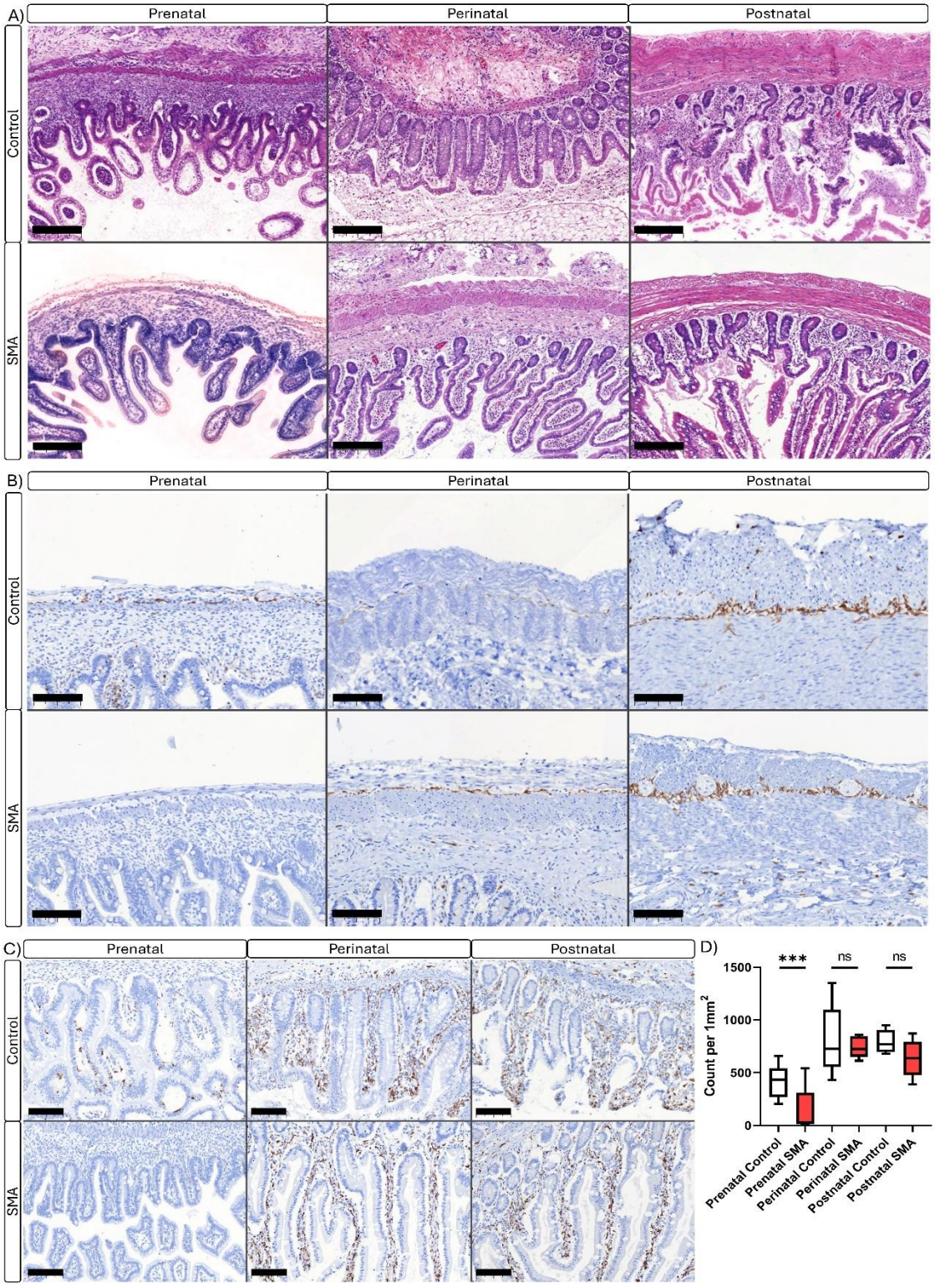


Figure 5: Intestine histology of control and SMA tissue A) Representative images of H&E-stained prenatal, perinatal and postnatal intestine tissue sections [Scale bar, 200µm]No differences in intestinal structure could be observed between control and SMA samples. Representative images of B) CD117 and C) CD163 stained prenatal perinatal and postnatal intestine tissue sections [Scale bar, 100µm]. D) Macrophage Count per mm2 represented as a Box & Violin chart. No significant difference could be observed during all developmental stages. (mean, SD, n = see Table 3, n^{ns} = P>0.05, unpaired t-test; Control = White; SMA =Red)

Investigation of interstitial cells of Cajal (ICC)

C-KIT positive ICC's were stained with CD117. Contrary to 13 week control tissue, ICC's could not be spotted within the whole tissue at 13 weeks prenatally in SMA. However, already at 18 weeks prenatally CD117 staining became visible in the muscularis externa and regions of the mucosa in both controls and SMA. ICC staining intensity increased as development progressed with controls and SMA showing no significant difference (Fig. 5B).

Investigation of intestinal macrophage population

CD68 staining was carried out for quantification of macrophages (Fig. 5C). The population of macrophages in prenatal SMA tissue was significantly reduced compared to their age-matched controls. However, at later prenatal stages of development SMA macrophage population started to normalize, and during peri- and postnatal stages no significant difference in macrophage count could be observed. Furthermore, the location of macrophages in SMA tissue appears to be similar in control and SMA tissue during all developmental stages, with their location being primarily in the lamina propria and the intestinal crypts. No increased infiltration in the lumen of the intestine could be spotted.

4. Histomorphometric analysis of the heart

Due to the region from autopsy samples of the heart, only the myocardium was analyzed, as a full histological structure could not be harvested. Specifically, cardiac muscle cells were analyzed (Fig. 6A). In both control and SMA prenatal tissue myocytes appear to be high in cytoplasm yet already arranged in fibers. Intercalated discs are not as prominent and cellularity appears to be very high. During development myocyte-organization into tubular structures appeared to be much more prominent in perinatal and postnatal stages, and overall cellularity diminished throughout development. As development progressed, myotubes were becoming thicker and more eosinophilic. Both intercalated disks and sarcomeres were clearly visible at term and did not appear to differ in control and SMA tissue. Overall significant structural differences were not visible between control and SMA tissue throughout development.

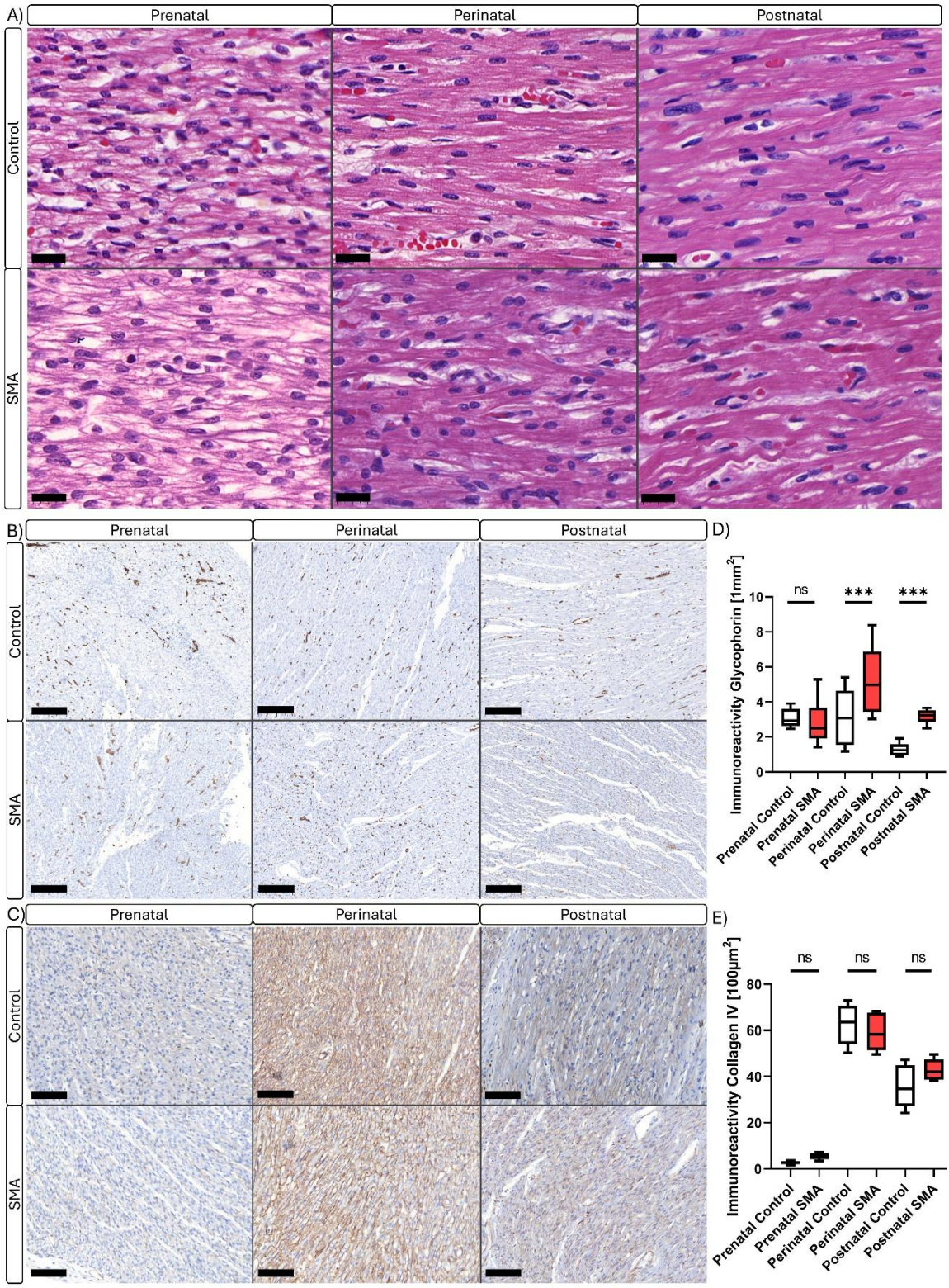


Figure 6: Heart histology of control and SMA tissue A) Representative images of prenatal, perinatal and postnatal heart tissue sections [Scale bar, 20µm]. Representative images of B) GYPA and C) Col4 prenatal, perinatal and postnatal heart tissue sections [Scale bar, 200µm]. Immunoreactive Area of D) GYPA and E) Col4 represented as a Box and Violin Chart. Comparison between control and SMA tissue were significant (control = White; SMA = Red (mean, SD, n^{ns} = P>0.05, n = see Table 3, ***P < 0.001, unpaired t-test))

Glycophorin-A expression in SMA heart tissue

To examine the hematopoietic properties of heart samples, Glycophorin-A (GYPA) antibody staining was carried out to visualize RBCs in the tissue (Fig. 6B). To compare and quantify the data from the GYPA stained whole-tissue slides, random sections were analyzed and overall GYPA expression in the sections were measured. Prenatally, no significant difference in GYPA expression was observed between control and SMA tissue. Yet, during the perinatal stage overall GYPA expression levels in SMA increased significantly, while in control tissue GYPA expression levels remained similar to prenatal tissue. Postnatally, expression levels drop significantly in both control and SMA samples, yet in SMA, GYPA levels remain significantly higher than controls.

Collagen IV expression in SMA heart tissue

To check for the structural framework of the heart, collagen IV (Col4) staining was carried out (Fig. 6C). Prenatally, Col4 expression levels remained relatively low and no significant difference was observed between control and SMA tissue. As development progressed, Col4 levels increased significantly reaching their peak at the perinatal stage, yet control and SMA levels remained similar. Postnatally, expression levels dropped significantly in both control and SMA samples yet no significant difference was observed. It may be possible that expression levels dropped postnatally due to myofibers gradually increasing in thickness, yet area measurements remained the same. This would need to be further confirmed though, to rule out a decrease in Col4.

Heart autopsy reports of SMA cases

Heart defects are becoming more associated with severe SMA and autopsy reports of our analyzed tissue, revealed that specifically SMA with one SMN2 copy (type 0 patients) were presented with heart defects. Cardiac abnormalities included a case with low ventricular septal defect, another case with coarctation of the juxtaductal aorta, and a third case with dilation of the vena cava and permeable foramen ovale respectively. On the other hand, SMA with two SMN2 copies (type I patients) were not described with any gross heart alterations.

5. Histomorphometric analysis of the lung

The fetal lung differs in many ways from the adult lung, as throughout development developing bronchi enlarge, with alveolar ducts increase in size as well. Upon comparing the prenatal lungs to their age-matched controls, structure appears to be highly similar (Fig. 7). The bronchioles do not appear to differ in size and both are lined with the club-like simple cuboidal epithelial cells. Alveolar ducts are also lined with a simple cuboidal epithelium and do not appear to differ in size. In postnatal tissue, the significant increase in alveolar spaces is visible in both control and SMA

tissue and do not appear to differ. Additionally, no prominent ruptures in the alveolar septa were visible. Alveolar space could not be accurately measured as their size is highly variable depending on the tissue.

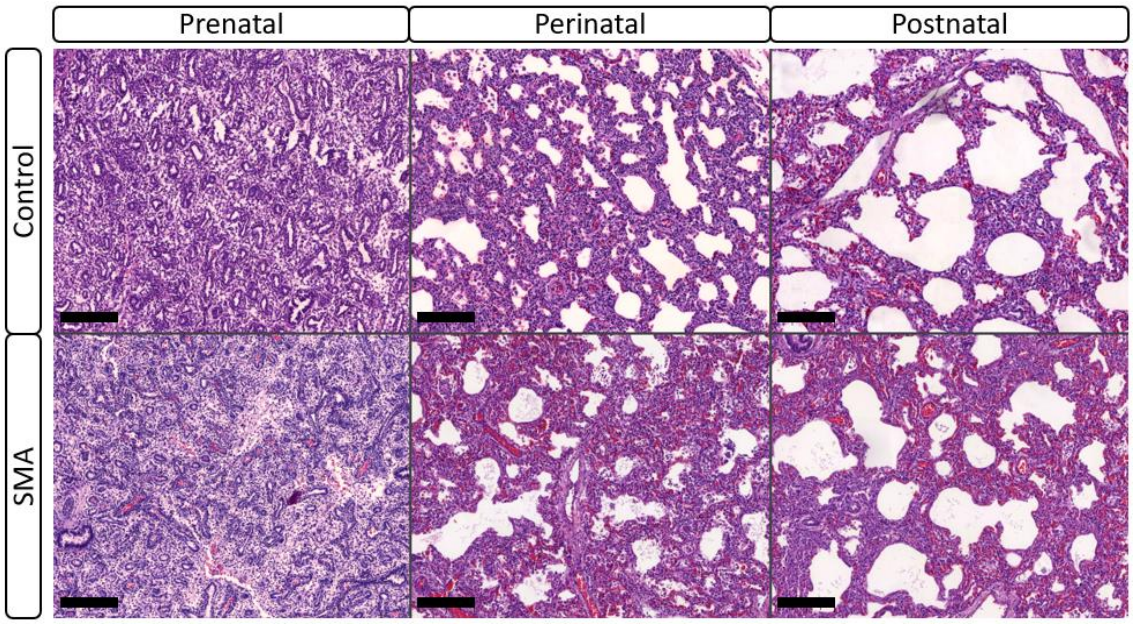


Figure 7: Lung Histology of H&E-stained prenatal and postnatal control and SMA tissue Representative images of H&E-stained prenatal, perinatal and postnatal lung tissue sections from autopsies [Scale bar, 200μm]. No structural differences could be observed between control and SMA samples during development.

6. Histomorphometric analysis of the kidney

The kidney consists of the cortex and medulla, each with specific structures. The cortex contains renal corpuscles (glomeruli and Bowman's capsule) and convoluted tubules, while the medulla houses the loop of Henle, collecting ducts, and renal pyramids.

The glomerular structure did not appear to differ between control and SMA tissue during all developmental stages (Fig. 8A). Nephron arrangement remained highly similar as well and the tubular structure of SMA tissue remained indistinguishable in cellular arrangement to control tissue. Furthermore, no sign of inflammation or fibrosis in medulla or cortex were spotted in SMA tissue in all analyzed tissue. Also, no mesangial hypercellularity could be spotted in control and SMA tissue during all developmental stages. On the other hand, as capsule diameter of the kidney gradually increased throughout development, a significant increase in thickness could be observed, especially in postnatal SMA tissue compared to their age-matched controls.

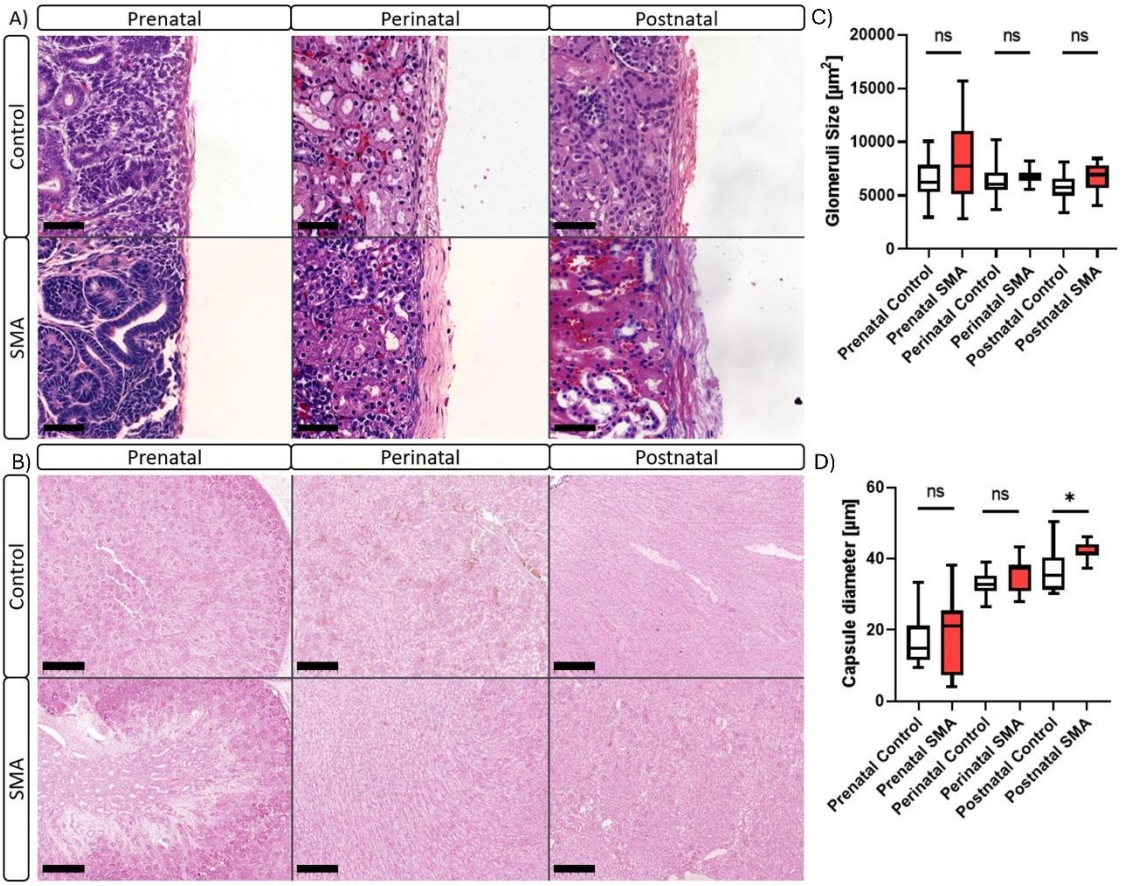


Figure 8: Kidney Histology of H&E-stained control and SMA tissue A) Representative images of H&E-stained prenatal, perinatal and postnatal kidney tissue sections [Scale bar, 50µm]. B) Representative images of Von Kossa stained prenatal, perinatal and postnatal kidney tissue sections. Calcification would be indicated by black spots in the tissue [Scale bar, 500µm]. C) Quantification of glomeruli size represented as a Box and Violin Chart shows no significant difference during all developmental stages (mean, SD, n = see Table 3, n^{ns} = P>0.05, unpaired t-test) D) Quantification of capsule diameter represented as a Box and Violin Chart shows no significant difference during the prenatal and perinatal stages. During the postnatal stage SMA tissue capsule size is significantly increased (mean, SD, n = see Table 3, n* = P<0.05, unpaired t-test).

Von Kossa staining in SMA kidney tissue

Von Kossa staining was carried out to highlight if calcium deposits were present in the kidney. During all developmental stages both control and SMA tissue showed no indication of increased calcification in either medulla or cortex.

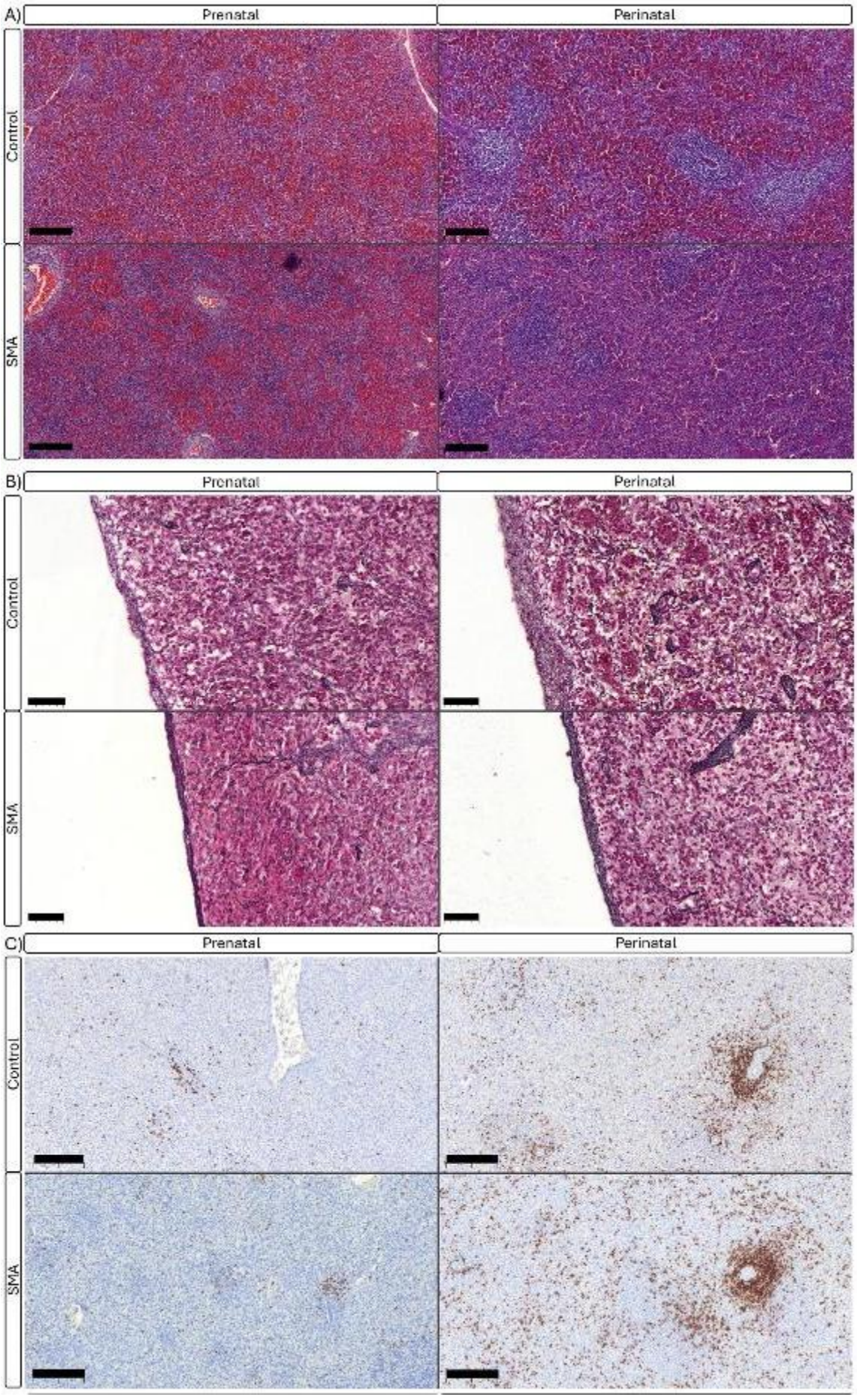
7. Histomorphometric analysis of the spleen

The parenchyma of the spleen is divided into two functionally and morphologically distinct compartments (red pulp and white pulp) separated by a tissue layer called the marginal zone. During the prenatal stage, in both control and SMA tissue no clear distinction of red and white pulp was visible, likely due to formation still occuring (Fig. 9A). As development continued red

and white pulp formation became more evident in control tissue. In the perinatal stage, the control tissue showed a clear red/white pulp distinction and the red pulp indicated a high concentration of RBCs. Perinatal SMA tissue on the other hand did not show as clear of a distinction as their age-matched controls. While the white pulp could be spotted, it was not as defined and RBCs in the red pulp did not appear to be as abundant.

Reticulin Expression in the Spleen

Reticulin fibers make up the framework of the spleen and reticulin staining was carried out to analyze the structural integrity. Reticular fibers were already abundantly present in prenatal tissue, with no significant changes between control and SMA. Furthermore, no significant difference of reticulin arrangement in the red and white pulp of the spleen in SMA samples during all developmental stages could be identified (Fig. 9B). However, upon inspecting the splenic capsule, which mostly consists of reticulin fibers, the density was measured. In the prenatal stage the density did not significantly differ in SMA tissue compared to age-matched controls, however perinatal tissue revealed a significant decrease of ~30% in SMA tissue (Fig. 9E).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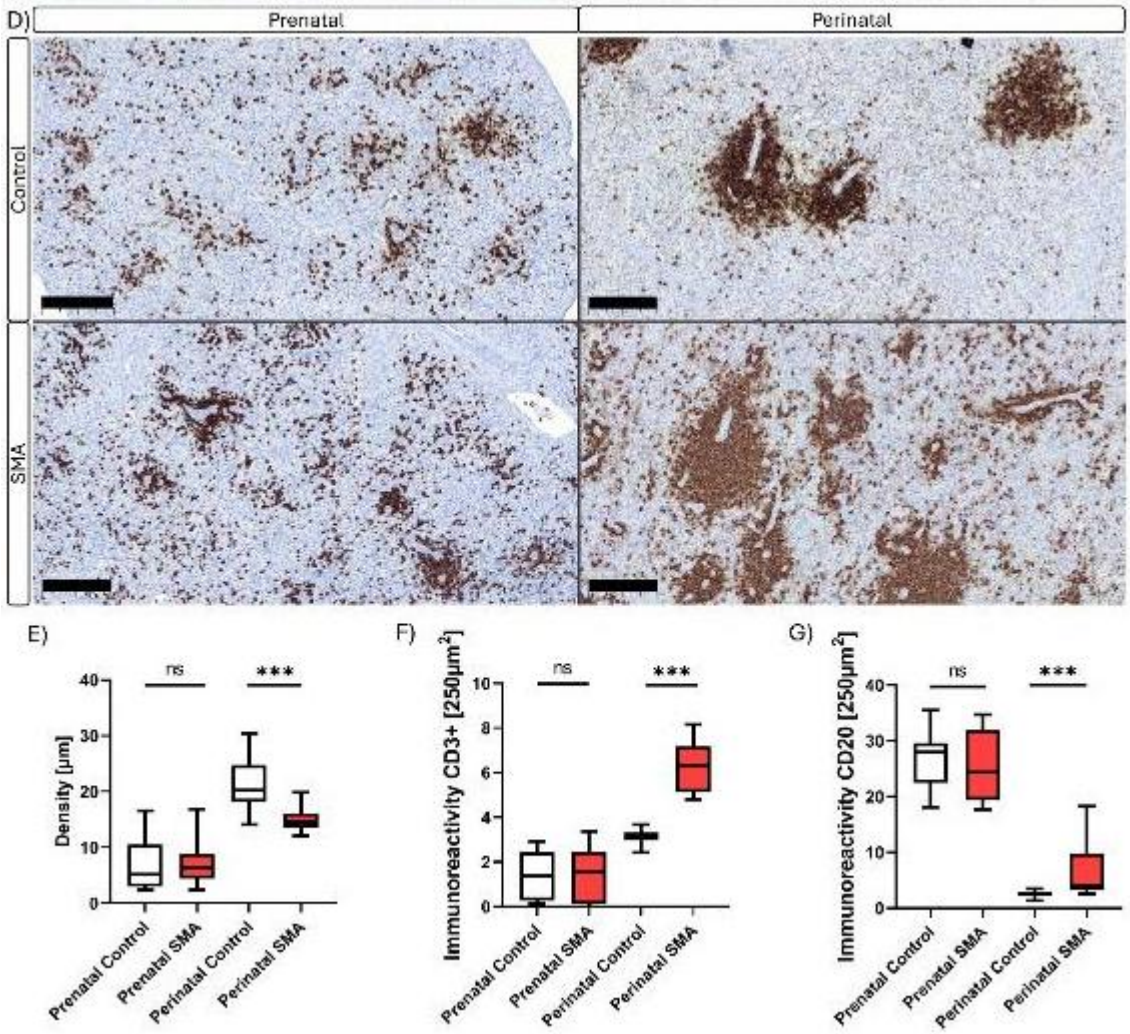
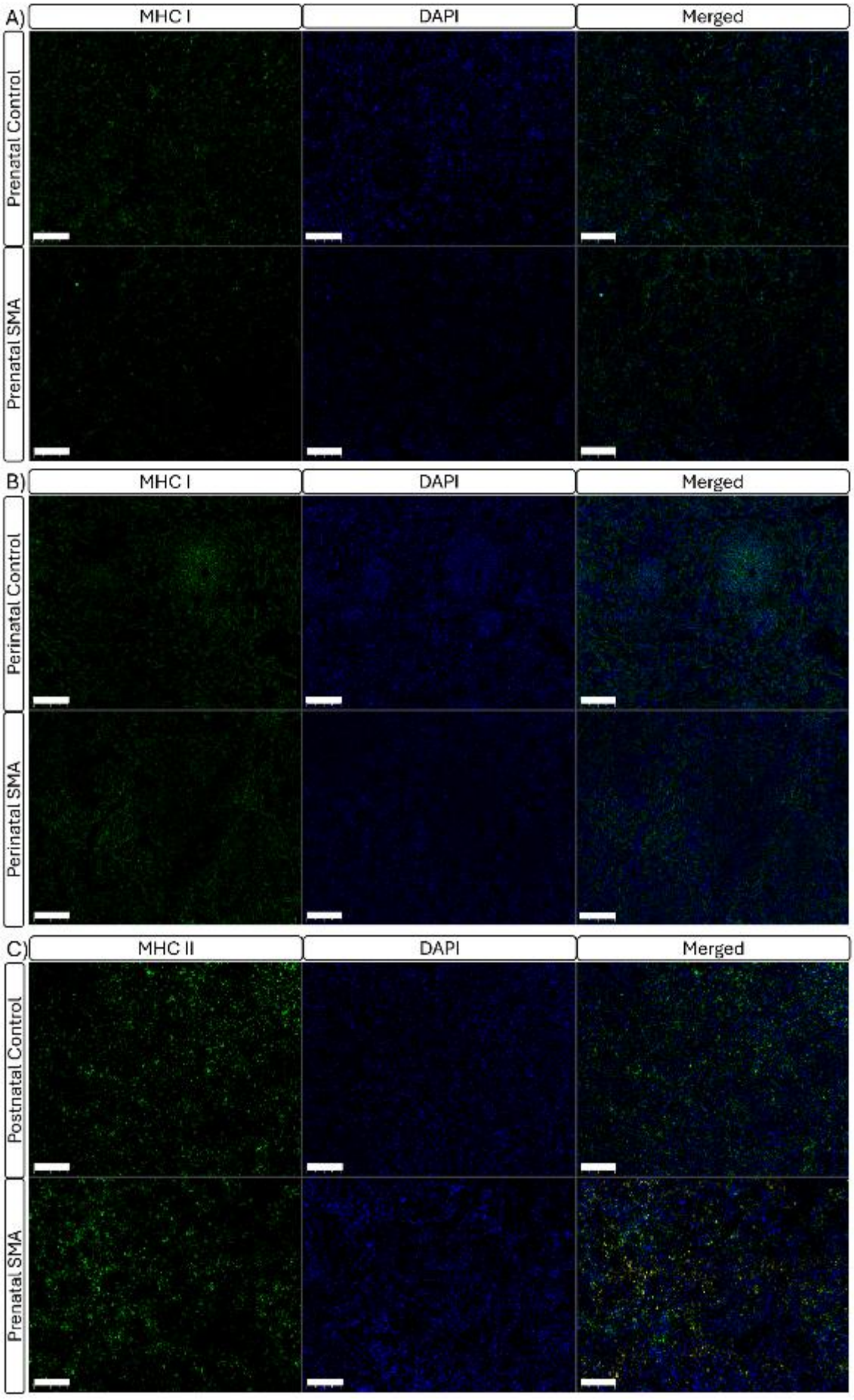


Figure 9: Spleen histology of control and SMA tissue A) Representative images of H&E-stained prenatal and perinatal spleen tissue sections at higher magnification [Scale bar 200µm]. Representative images of B) Reticulin, C) CD3, D) CD20 stained prenatal and perinatal spleen tissue sections [Scale bar A) 200µm, B) 50mm, C),D) [200µm]. E) Splenic capsule density represented as a Box and Violin Chart. Immunoreactive area of F) CD3 G) CD20 in the red pulp represented as a Box and Violin Chart. White pulp clusters were manually removed from the images during statistical analysis. Comparison between control and SMA tissue were significant (control = White; SMA = Red (mean, SD, n = see Table 3, n^{ns} = P>0.05, n^{***} = P < 0.001, unpaired t-test)).

Investigation of splenic immune cells using CD3 and CD20

To better highlight the immune cells of the spleen immunohistochemistry was performed to label T-cells and B-cells (Fig. 9C,D). At 13 weeks prenatally, no immunoreactivity for CD3 or CD20 could be spotted in either control or SMA tissue. Scattered B- and T- lymphocytes start to become visible around 14-15 weeks of gestation. Since no immunoreactivity could be observed, this developmental stage was excluded from the quantitative analysis as only background could be measured. In addition, the white pulp area can vary in size and skewer the measurements of larger areas, therefore the quantitative analysis focused on immune cell presence in the red pulp. In prenatal splenic tissue no significant difference in both CD3 and CD20 immunoreactivity could be observed (Fig. 9F,G).

It is to note though, that CD20 immunoreactivity is significantly higher prenatally than perinatally. At this developmental stage, the spleen is relatively small in overall size and red and white pulp formation is still ongoing, therefore the increased presence of CD20 in the red pulp prenatally could be attributed to the size difference of developmental stages, yet the same area was measured. Perinatally the formation of the periarteriolar lymphoid sheaths was clearly visible, as T-cells arranged around central arterioles with a surrounding B-cell zone. However, compared to perinatal control tissue, the red pulp of perinatal SMA spleens showed a significant increase of ~151% of CD3 immunoreactivity and of ~247% of CD20 immunoreactivity (Fig. 9 F,G).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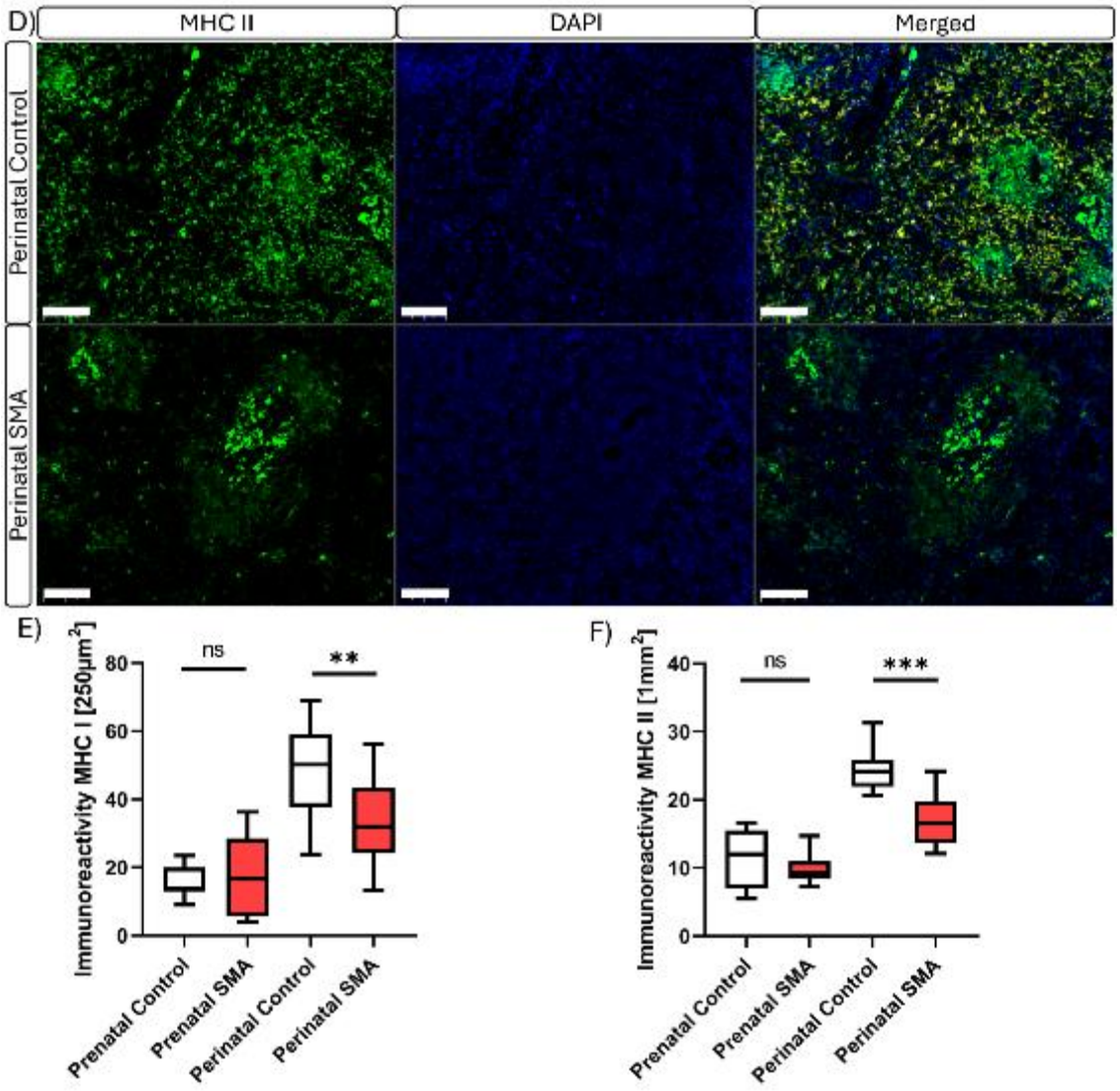


Figure 10: Spleen immunofluorescence of control and SMA tissue Representative splenic cross-sections from controls and severe SMA cases co-labelled for A,B) MHC I (green), C,D) MHC II (green) and DAPI (blue; nuclei) [Scale bar 200µm]. Quantification of E) MHC I and F) MHC II immunoreactivity during prenatal and perinatal development represented as a Box and Violin Chart between control and SMA tissue were significant (MHC I and MHC II = Green; (mean, SD, n = see Table 3, n*** = P<0.001, n** = P<0.002, n^{ns} = P>0.05, unpaired t-test)

Investigation of splenic immune cells using MHC I and MHC II

The further investigate immune cell maturation of the spleen, antibodies targeting MHC I and MHC II were used (Fig. 10 A-D). First, overall MHC II immunoreactivity is significantly higher than MHC I immunoreactivity in both pre-and perinatal spleen tissue.

In prenatal spleen tissue no difference in MHC I and MHC II immunoreactivity could be observed. Both control and SMA tissue showed immunoreactivity spread throughout the whole tissue, which is likely due to the spleen still forming the red and white pulp areas.

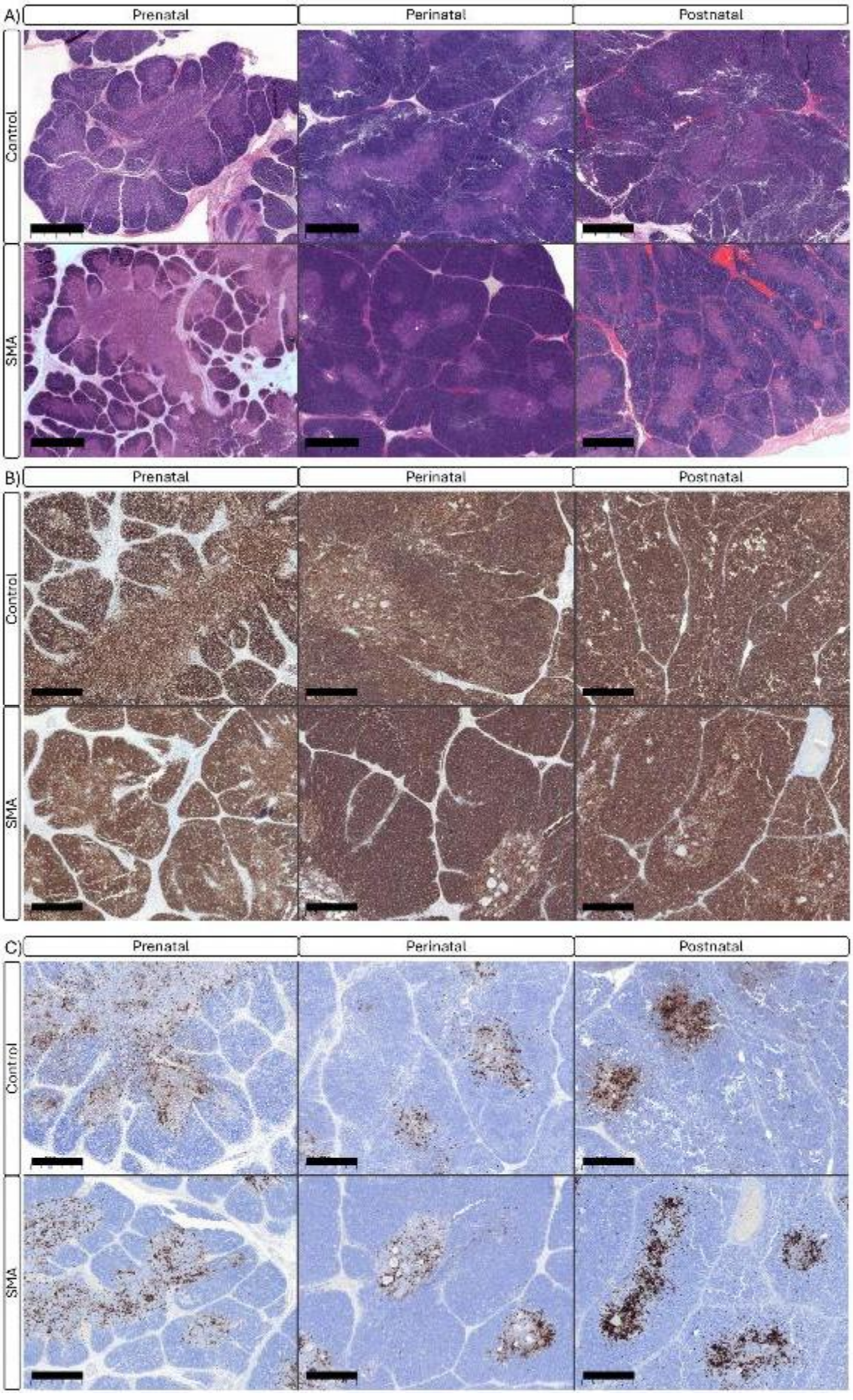
Perinatally MHC I immunoreactivity show strong immunoreactivity around arterioles in control tissue. In SMA tissue on the other hand MHC I immunoreactivity is still evenly present throughout the whole tissue.

Perinatally both control and SMA tissue showed strong MHC II immunoreactivity around arterioles. However, upon comparing with age-matched controls, SMA tissue showed less immunoreactivity in the red pulp. Interestingly both MHC I and MHC II immunoreactivity are significantly decreased in perinatal SMA tissue (~28% for MHCI and ~32% for MHCII respectively) (Fig. 10 E,F) compared to controls.

8. Histomorphometric analysis of the thymus

The thymus is a primary lymphoid organ, divided into cortical and medullary regions within each lobule. To better highlight the structure of the thymus H&E staining was performed (Fig. 11A). During the prenatal stage, both control and SMA tissue depict the classical lobular structure and the cortex and medulla are visible, showing no significant differences. Peri- and postnatally no differences could be seen between SMA and control tissue either. As development progressed, a clear increase in cortex and reduction in medulla is visible compared to the prenatal stage. Comparing the peri- and postnatal stage no visible structural differences could be identified as both control tissue and SMA tissue revealed a highly similar structure.

Hassall’s corpuscles are characteristic of the medulla and although the function of Hassall’s corpuscles has yet to be fully explored it is suggested that Hassall's corpuscles have a critical role in dendritic-cell-mediated secondary positive selection of medium-to-high affinity self-reactive T cells, leading to the generation of immunoregulatory T cells that suppress polyclonal T cell activation [190]. While Hassall’s corpuscles increase in size as development progresses, no structural differences could be seen during all developmental stages of SMA compared to controls.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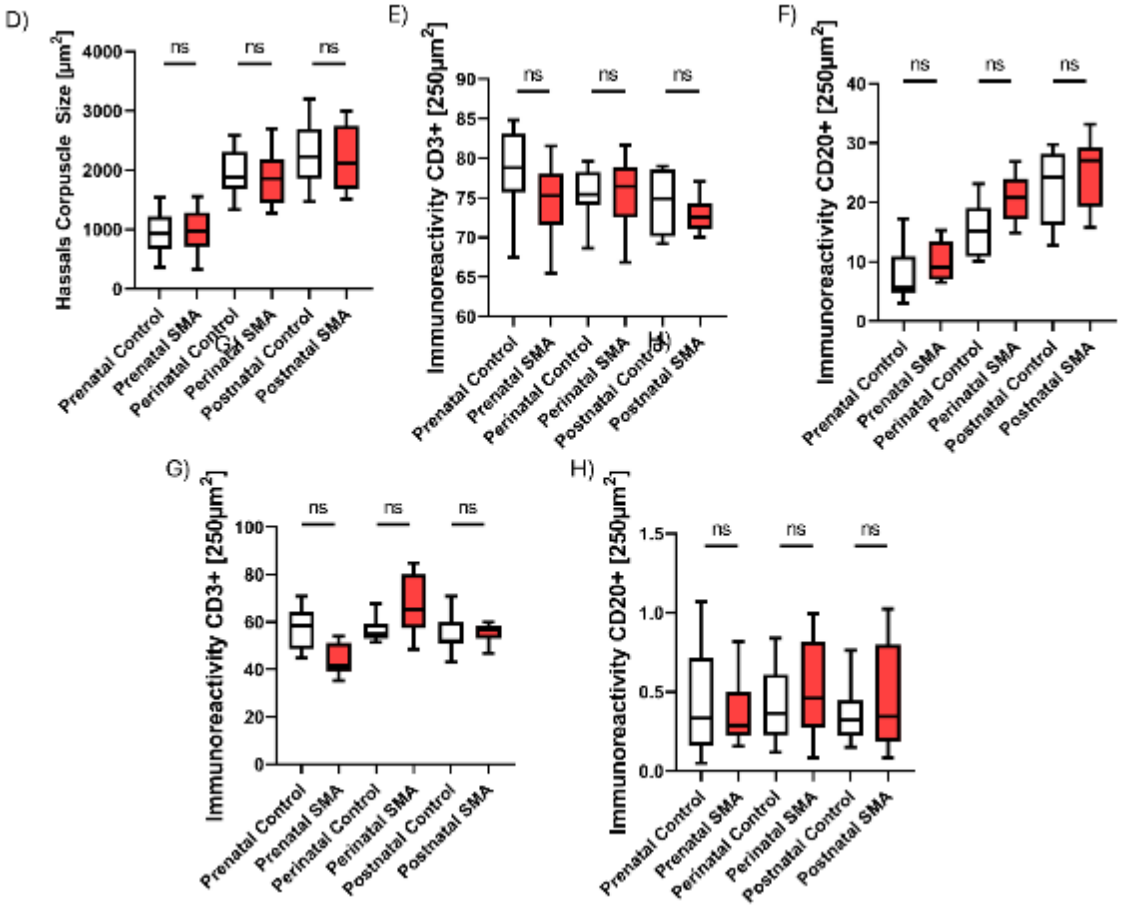


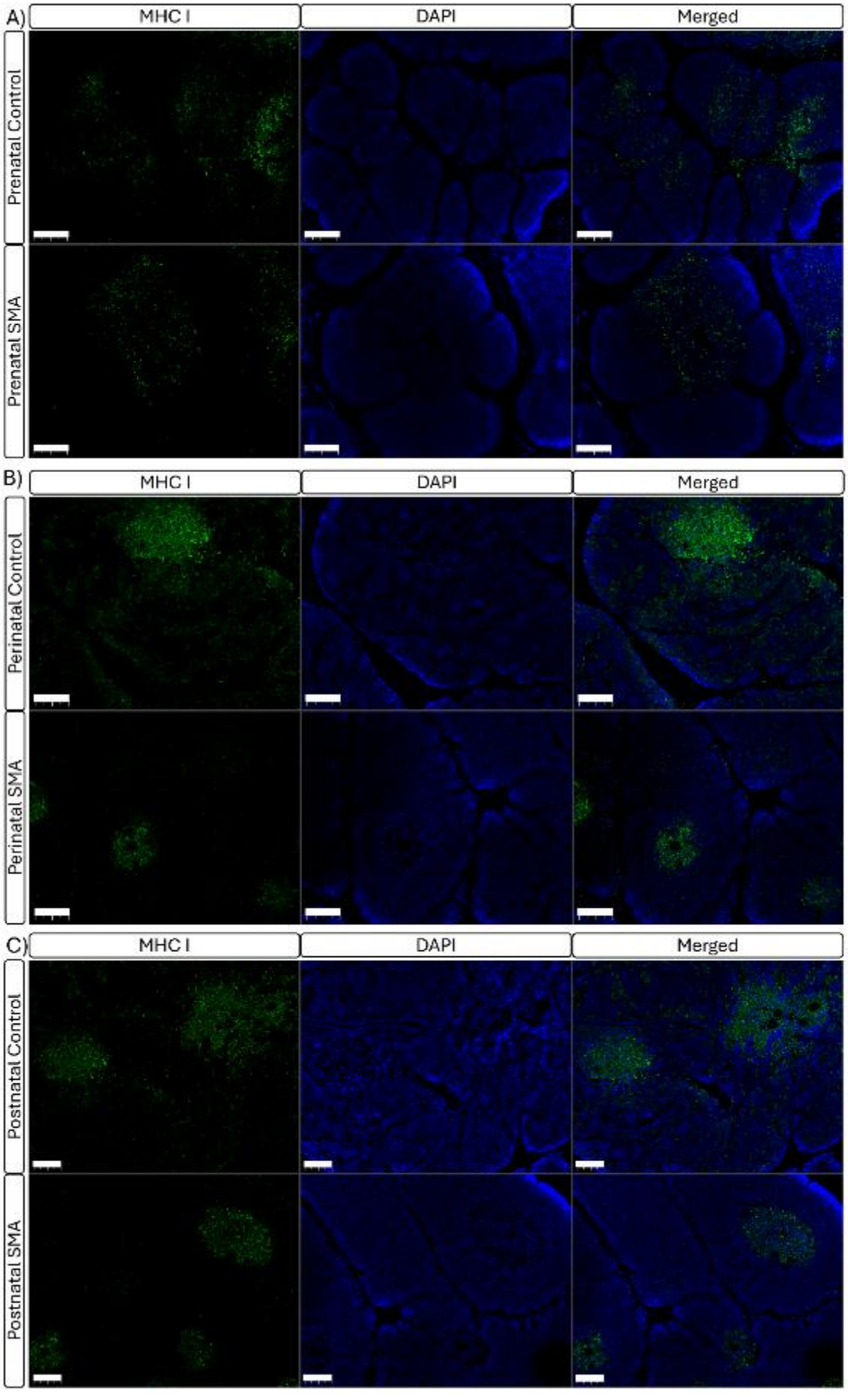
Figure 11: Thymus histology of control and SMA tissue A) Representative images of H&E-stained prenatal and postnatal thymus tissue sections at higher magnification [Scale bar 1000µm]. Representative images of B) CD3 and C) CD20 stained prenatal, perinatal and postnatal thymus tissue sections [Scale bar 500µm]. D) Overall size of Hassall's corpuscles represented as a Box & Whiskers chart. Immunoreactive area of E) CD3 medulla, F) CD3 cortex, G) CD20 medulla and H) CD20 cortex represented as a Box and Violin Chart. Comparison between control and SMA tissue were not significant (control = White; SMA = Red (mean, SD, n = see Table 3, n^{ns} = P>0.05, unpaired t-test)).

Investigation of thymic immune cells using CD3 and CD20

To better highlight the lymphatic elements of the thymus immunohistochemistry was performed to label T-cells and B-cells (Fig. 11 B,C). Similar to the splenic tissue analysis, at 13 weeks no immunoreactivity for CD3 or CD20 could be spotted in either control or SMA tissue. Scattered B- and T- lymphocytes start to become visible around 14-15 weeks of gestation. Since no immunoreactivity could be observed, this developmental stage was excluded from the quantitative analysis and only background could be measured. Regarding the amount of T-lymphocytes in both cortex and medulla, no significant differences could be seen during all developmental stages. CD3 immunoreactivity is very high in both cortex and medulla further increasing with development. While not significant, there appears to be a tendency of increased immunoreactivity of CD20 in peri- and postnatal SMA tissue as development progressed.

Regarding the B-lymphocyte population, no significant differences in CD20 immunoreactivity were observed in both the medulla and cortex during prenatal development. While occasional

scarce immunoreactivity of CD20 could be seen in the cortex peri- and postnatally, the medulla hosts most of the CD20-expressing B-cell population.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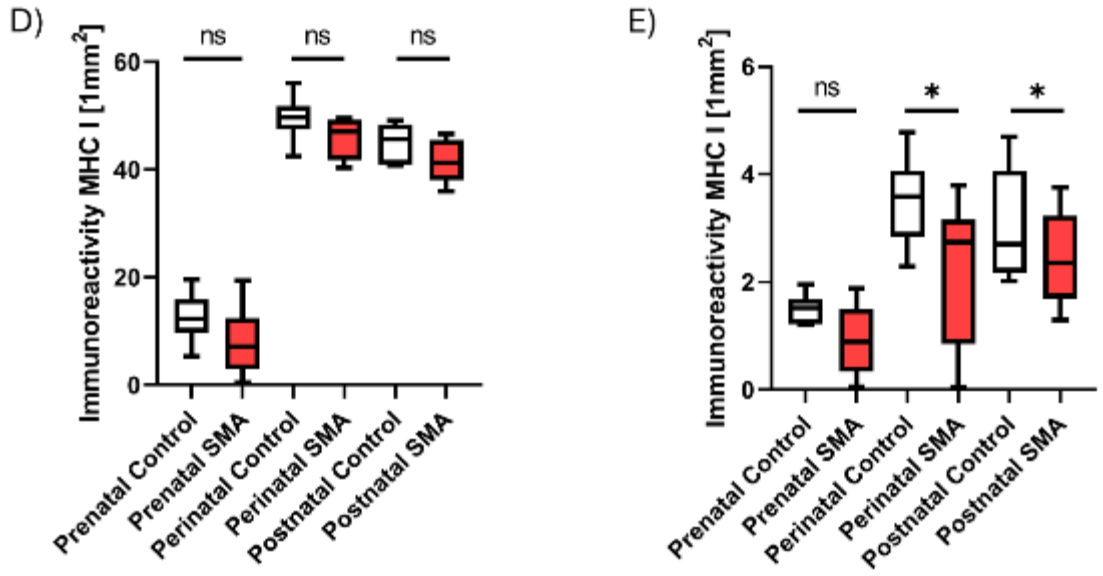
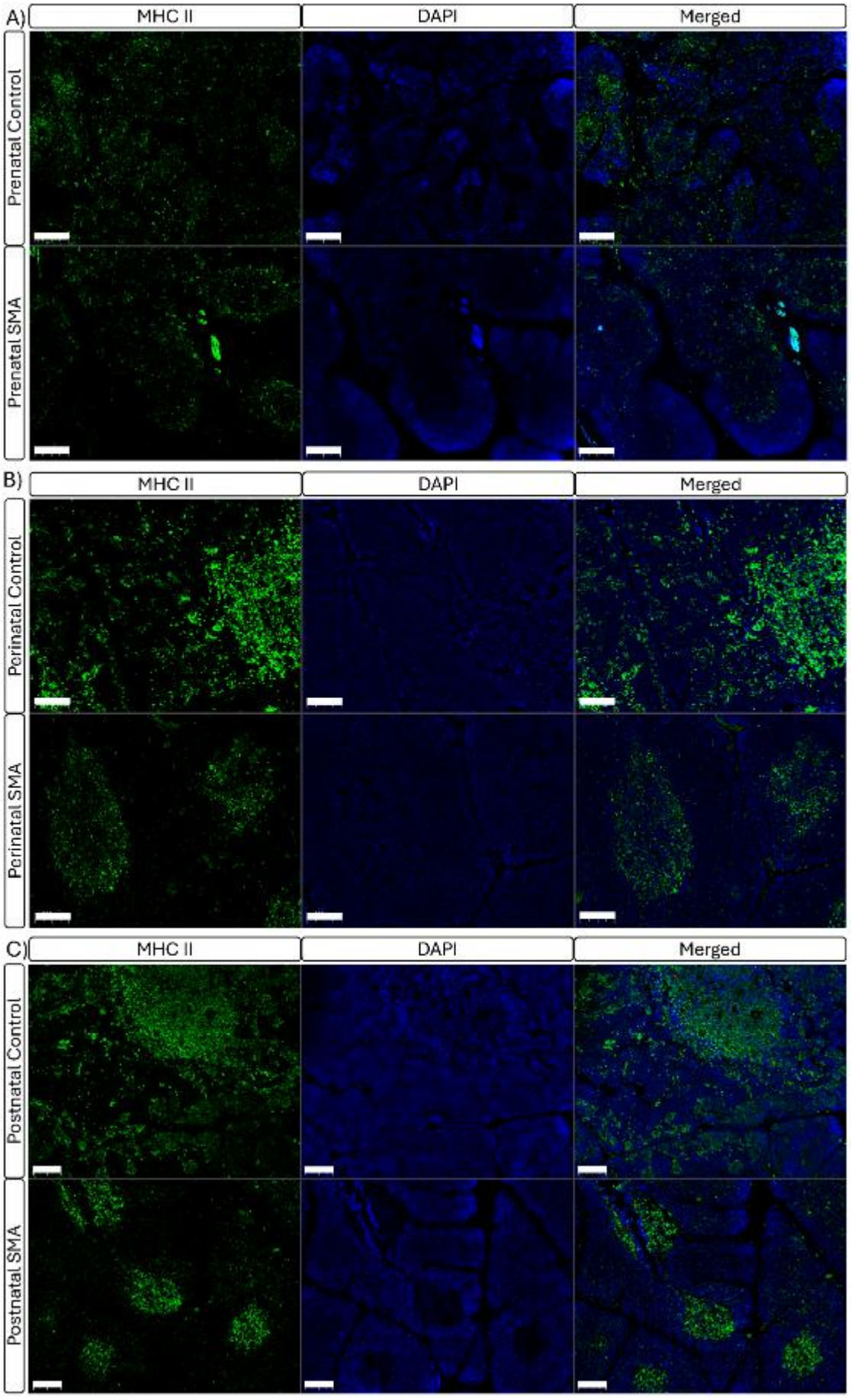


Figure 12: Thymus MHC I immunofluorescence of control and SMA tissue Representative splenic cross-sections from controls and severe SMA cases co-labelled for A) prenatal, B) perinatal and C) postnatal MHC I, (green), and DAPI (blue; nuclei). [Scale bar 200µm]. Quantification of D) medullary MHC I and E) cortical MHC I immunoreactivity during pre-, peri- and postnatal development represented as a Box and Violin Chart between control and SMA tissue were significant (MHC I = Green; DAPI = Blue (mean, SD, n = see Table 3, n* = $P<0.05$, n^{ns} = $P>0.05$, unpaired t-test)).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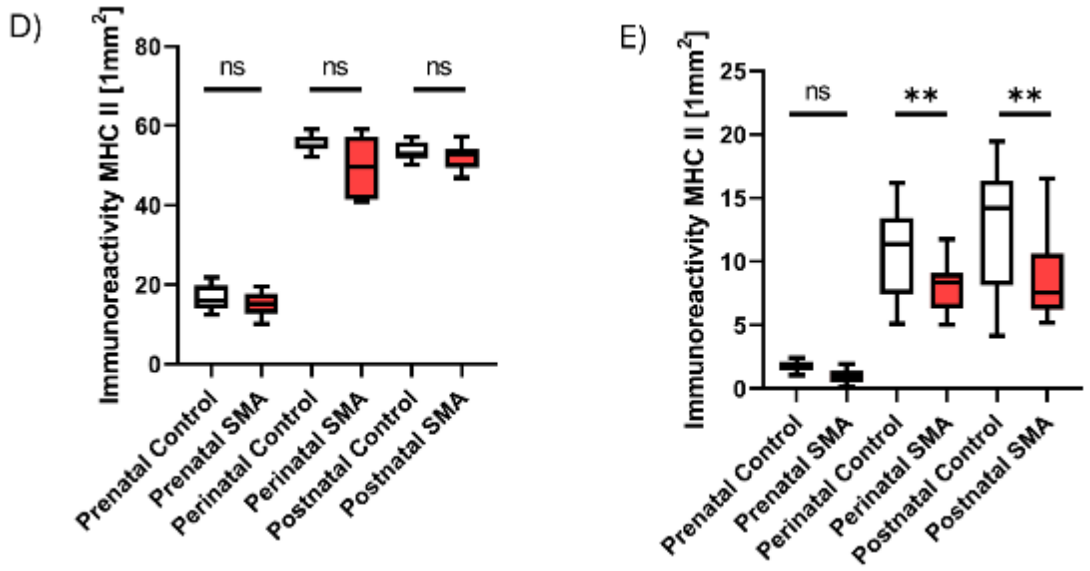


Figure 13: Thymus MHC II immunofluorescence of control and SMA tissue Representative splenic cross-sections from controls and severe SMA cases co-labelled for A) prenatal, B) perinatal and C) postnatal MHC II, (green), and DAPI (blue; nuclei). [Scale bar 200µm]. Quantification of D) medullary MHC II and E) cortical MHC II immunoreactivity during pre-, peri- and postnatal development represented as a Box and Violin Chart between control and SMA tissue were significant (MHC I = Green; DAPI = Blue (mean, SD, n = see Table 3, n** = $P<0.02$, $n^{ns} = P>0.05$, unpaired t-test)).

Investigation of thymic immune cells using MHC I

The further investigate the immune response of the thymus MHC I were used as antibodies (Fig. 12 A-C). In prenatal thymus tissue no difference in MHC I immunoreactivity could be observed. Both control and SMA tissue revealed immunoreactivity mainly in the medulla of the thymus, with isolated signals in the cortex.

Perinatally MHC I immunoreactivity are significantly higher than in prenatal tissue. Furthermore, MHC I immunoreactivity was significantly reduced in the thymic cortex of SMA tissue compared to their age-matched controls (~37% decrease), where an abundance could be spotted. This trend further continued throughout postnatal development (~17% decrease in SMA), however mean values became more adjacent (Fig. 12E).

Investigation of thymic immune cells using MHC II

The further investigate the immune response of the thymus MHC II were used as antibodies (Fig. 13A-C). In prenatal thymus tissue no difference in MHC II immunoreactivity could be observed. Both control and SMA tissue show immunoreactivity mainly in the medulla of the thymus, with isolated signals in the cortex.

Perinatally MHC II immunoreactivity are significantly higher than in prenatal tissue. Furthermore, MHC II immunoreactivity is significantly reduced in the thymic cortex of SMA tissue compared to their age-matched controls (~25% decrease). This trend further continued throughout postnatal development, with MHC II immunoreactivity decreasing further as development progressed (~32% decrease) (Fig. 13E).

V. DISCUSSION

In the past decade, SMA has seen remarkable progress in both diagnosis and treatment. The development of the FDA-approved SMN-targeting therapies nusinersen, OA, and risdiplam have markedly improved prognosis [162] and quality of life in SMA patients by restoring SMN protein expression, especially when administered early. Current research is now shifting toward combinatorial strategies that go beyond SMN restoration, highlighted by multiple clinical trials currently ongoing [333]. This now allows for the focus expanding to non-neurological symptoms of SMA, such as immune, cardiac, and metabolic dysfunctions, particularly in severe or untreated patients.

This study provides a first systemic analysis of human peripheral organ development in severe SMA, revealing subtle yet relevant histopathological alterations in multiple organ systems already occurring during fetal development. Previous studies on this topic have largely relied on SMA mouse models, highlighting the aspect that our investigation offers novel human-specific insights that expand our current understanding of SMA as a multisystem developmental disorder.

This study describes a systematic approach to investigate the human development of histopathology of SMA organs and compares with age-matched controls and with previous results of SMA mouse models and literature.

Liver

Structurally the liver did not appear to be affected by SMA in humans as all major architecture did not exhibit clear differences with their age-matched controls. In the SMA mouse model failure to develop distinctive sinusoidal spaces between hepatic plates and immature morphology were reported [162]. In our human SMA samples most developmental stages had a highly similar developmental pattern of sinusoids compared to their age-matched controls, indicating that sinusoid development may not be affected by SMN deficiency (Fig. 3C,D).

However, we did observe persistent presence of hematopoietic elements perinatally, similar to the SMA mouse model phenotype, where prolonged erythropoiesis were described (Fig. 3B). The bone marrow gradually takes over as the hematopoietic site starting around 4-5 months gestation and by term it is the sole organ responsible for hematopoiesis [334]. Our findings regarding the presence of proerythroblasts and nucleated RBCs in SMA liver sinusoids perinatally, indicate that erythropoiesis still occurs in the liver hinting at developmental delay.

Furthermore, in the SMA mouse model an increase of RBCs was reported with sinusoids being congested, attributed to prolonged erythropoiesis [162]. GYPA is a single-span membrane protein of RBCs [335] and therefore a suitable indicator for RBC count. GYPA expression is indeed higher throughout all developmental stages in sinusoidal spaces of our SMA tissue and in agreement with reports of the SMA mouse model liver [162]. It is to note though, that vascular

congestion might also lead to the increase of RBCs in the liver and the amount of analyzed samples might not be significant enough to fully attribute increased RBCs to prolonged erythropoiesis.

Kupffer cells are the resident macrophages of the liver and play a role in a multitude of liver functions [13]. In the SMA mouse model, systemic inflammation with marked increase in leukocytes and Kupffer cells in the liver was observed [336]. While the Kupffer cell count in our human SMA samples appeared to be similar to control tissue prenatally, during peri- and postnatal stages a significant increase was observed.

Taken together Our results support prior findings in SMA mouse models that suggest persistent hematopoietic activity in the liver. The increased presence of erythropoietic progenitors and elevated GYPA expression in perinatal and postnatal SMA liver tissue suggests a delay or disruption in the typical shift of hematopoiesis from liver to bone marrow. Though we did not observe significant structural abnormalities in sinusoid formation or bile duct development, the increased Kupffer cell density in human SMA tissue further supports the hypothesis of ongoing inflammatory mechanisms in the hepatic microenvironment, which was also observed in the severe mouse model of SMA [336]. The liver's high SMN expression, especially during prenatal development may render it particularly susceptible to SMN deficiency, highlighting the importance of monitoring hepatic function in SMA patients over time.

Pancreas

The histopathological analysis of the pancreas was conducted by first analyzing the structure of the exocrine pancreas, which does not appear to be affected by SMA as all major structures are present in the SMA tissue and did not exhibit significant differences compared to their age-matched controls (Fig. 4). As SMA pancreatic tissue develops in a highly similar fashion to control tissue and to what is described in literature [337], it is likely that reduced levels of SMN do not affect structural development.

It is of interest though, that human Langerhans islets significantly differ from mouse islets as their composition does not display a strict core-mantle segregation of α- and β-cells, as seen in mice [338]. To add, α-cells localize along the mantle and internal vascular channels of human islets. It is to note though that the core of normal human islets is typically composed of more β-cells than α-cells (~70% β-cells) [338]. SMA mice are characterized by fasting hyperglycemia, glucose intolerance, hypersensitivity to insulin, and hyperglucagonemia, further accompanied by abnormal pancreatic islet composition as a marked decrease in core β-cells is visible with a significant increase in the mantle- α-cells [167]. Previously both SMA mice and human patients have been reported with an abnormal increase in α-cells, suggesting a conserved role for the SMN

protein in normal pancreatic development [167]. Indeed, an abnormal islet composition was observed in our tissue from 21 weeks gestation onward (Fig. 4F).

Furthermore, several SMA type I patients have been described with hypoglycemia and several type II and 3 patients reportedly have glucose intolerance and dyslipidemia [167], [339]. Taking together previously described pathology and our results, it is likely that SMN-deficiency might lead to alterations in glucose and lipid metabolism disorders, with onset already during prenatal development in severe SMA patients.

The absence of structural changes in the exocrine pancreas suggests a selective vulnerability of endocrine pancreatic components, possibly due to glucose and lipid metabolism disorders, with are more often seen in SMA patients with milder forms [340], [341]. As has previously been shown metabolic abnormalities are becoming a more important feature of SMA, suggesting a more systemic therapeutic approach to ensure more beneficial outcomes throughout the patient's life.

Intestine

Our analysis of control and SMA tissue during all developmental stages did not reveal any significant differences, as a gradual increase in thickness of the 3 prominent layers of the small intestine in both control tissue and SMA samples could be observed, with no extraordinary features during all developmental stages. To add, the shape of villi in the analyzed human SMA samples did not exhibit a "club-shaped" structure as was previously described in the SMA mouse model [152] (Fig. 5A).

ICC's function as "pacemakers" of the intestine and play a critical role in small-intestinal contractile activity [342]. It has further been shown that myenteric deep muscular plexus ICC's are mediators of enteric motor neurotransmission. A reduction of ICC's could explain previously reported issues of SMA patients in form of constipation and delayed gastric emptying. At 13 weeks gestation myenteric ICC's in control tissue could be observed, but no staining was visible in SMA. As development progressed, at 18 weeks prenatally in both controls and SMA, ICC staining in the myenteric region became more prominent and staining increased within other muscle layers in the small intestine (Fig. 5B). Therefore, the possible initial delay in ICC development diminishes upon term.

In the SMA mouse model significantly increased macrophage populations were observed in the lumen of the intestine [152]. Contrary to the SMA mouse model, in our analyzed SMA cases no significant differences could be observed throughout development. Furthermore, no indication of abnormal location could be observed and most importantly macrophage levels in the lumen were almost non-existent (Fig. 5C), further indicating normal development of the intestine in absence of SMN.

Overall, it is likely that intestinal defects do not form during pre- and postnatal development but warrants investigation at later stages of the disease. It may also be possible that lower GI dysfunction may be attributed to vascular abnormalities, as suggested by Corsello *et al.,* [343], due to the SMA mouse model exhibiting decreased vascular density [152], a fact that should be more extensively investigated in SMA patients.

In summary, the absence of long-term histological differences suggests that gastrointestinal manifestations in SMA may develop more from functional dysregulation or secondary effects than from intrinsic developmental defects.

Heart

Cardiac abnormalities in patients with SMA have been described, ranging from mild to severe forms and mainly include structural defects and arrhythmia [147]. The presentation of structural defects in all SMA type 0 patients described in this study, however not in patients with SMA type I at autopsy, may indicate that developmental pathology of the heart and reduced SMN levels are inversely correlated and suggests a minimal threshold of SMN to ensure proper heart development. Furthermore, considering that SMA type 0 patients were presented at pre- and perinatal stages, it is highly likely that the architecture of the heart is already affected by SMA during prenatal development.

Gross morphological alterations have also previously been described in the SMA mouse model, in addition to disorganization of cardiomyocytes and thinner ventricular walls, with possible presymptomatic onset [146]. However, In our analyzed cases we did not observe any structural differences in the myocardium of SMA hearts compared to their age-matched controls. While gross morphological changes in the human heart affected by SMA are present, architecture of the myocardium remained unaffected.

The surrounding basement membranes of cardiomyocytes provide structural support and its proper development is important for the formation of sarcomeres [344]. Gross pathology in the heart wall of SMA mice and disorganized arrangement of cardiomyocytes have been described as consequence of decreased levels of Col4 [344]. Contrary to the SMA mouse model, in our analyzed SMA cases no significant differences could be observed throughout development.

In addition, in the SMA mouse model an increase of RBCs was reported in the heart, with blood clotting of major arteries [162]. While major arteries of the heart could not be analyzed, GYPA expression remained higher throughout all developmental stages in the myocardium of our SMA samples. Similar to previously described liver pathology (Fig. 3A), the significant increase in RBCs could be attributed to vascular congestion, however In SMA patients' arrhythmias and bradycardias are most predominant in children (summarized in [146]). Therefore, increased presence of RBCs in human SMA tissue might indicate reduced heart function to properly pump blood. This finding resonates with previous observations in SMA mouse models that may help to

explain arrhythmias and conduction abnormalities reported in SMA patients [147]. However, it is important to consider that dysautonomic alterations are observed in type I patients such as increased sweating, altered regulation of vascular tone, irregular skin response to temperature changes. These findings may be correlate by previously described tachycardia and bradycardia in neonates [264].

Lung

The lung has been the least histopathologically described organ in both SMA patients and SMA mouse models. Most analyses revolve around lung expiratory volume, which is mostly affected by muscle strength in the diaphragm and intercostal muscle, yet the architecture of the lung remains to be analyzed in SMA.

While respiratory complications are the most important cause of morbidity and mortality in SMA, they are usually attributed to failure of respiratory muscles [345]. Our histopathological analysis of the lung did not show any significant differences to their age-matched controls. The lung undergoes various changes during fetal development and while those changes are clearly visible upon comparing prenatal and postnatal lung tissue, comparison of control and SMA tissue did not show structural differences. In the Taiwanese mouse model of SMA, postnatally, variable degrees of emphysema with ruptured alveolar septa and enlarged alveolar spaces were reported [153]. In our analyzed human SMA tissue, alveolar spaces were neither enlarged nor were alveolar septa ruptured. It is therefore likely that the lung is not affected during development and/or may not be affected at all by SMA.

These results support the prevailing view that respiratory complications in SMA are primarily neurogenic due to diaphragmatic and intercostal muscle weakness rather than pulmonary in origin. Nevertheless, these findings do not preclude the possibility of secondary lung pathology later in disease progression, especially in cases of chronic hypoventilation [345], [346].

Kidney

Pathology in the kidney in SMA mouse models have extensively been described [172], with additional reports in SMA type I patients [173]. In our SMA tissue gross histomorphometry appeared to be highly similar to their age-matched controls. Unlike the SMA mouse model, where impaired nephrogenesis, glomerular sclerosis, and vascular defects were identified, renal analysis of human SMA tissue revealed no overt abnormalities in glomeruli, tubules, or mesangial cellularity, hinting that renal parenchyma is rather spared during prenatal and early postnatal development. We did however observe a significant increase in renal capsule diameter, similar to the Taiwanese mouse model, which may signal the onset of fibrotic changes, possibly related to

early alterations in extracellular matrix dynamics or compensatory responses, similar to renal abnormalities in SMA mice [172].

Renal calcification has been described in two SMA type I autopsies at ~2 years of age [173]. In addition, a retrospective study on SMA type I children, ranging from 9 to 55 months, showed an increased risk of developing kidney stones with hypercalciuria being the most common risk factor [347]. High calcium levels in urine lead to crystal formation, which can then accumulate in kidney tissue, resulting in nephrocalcinosis, which in turn raises the risk of kidney stones. We could not observe any deposits of calcium in either cortex or medulla in any of our analyzed SMA tissue. We therefore assume that if calcium deposits in higher quantities in SMA kidneys, it likely arises later in disease progression, consistent with previous reports [173], [347].

Spleen

The spleen plays a pivotal role in systemic immune surveillance, hematopoietic regulation, and antigen presentation. While prenatal development of the spleen did not appear to differ in SMA tissue compared to their age-matched controls, in perinatal human SMA splenic tissue a significant reduction in splenic capsule size could be observed (Fig. 9E). The capsule allows the spleen to significantly increase in size when necessary and discharge a large amount of blood to contribute to tissue oxygenation [348]. A decrease in size of the splenic capsule could significantly impact its ability to help with tissue oxygenation, however a similar pathological phenotype has yet to be described in other diseases. The splenic capsule has largely been understudied making this finding a novelty.

Furthermore, the formation of red and white pulp in perinatal SMA tissue was not as clearly distinguished as in control tissue and RBCs in the red pulp did not appear to be as abundant as their age-matched controls (Fig. 9A). Therefore, it might be possible that the formation of red and white pulp during SMA development is impaired. In addition, the increase of B- and T-cells in the red pulp is significant in SMA (Fig. 9C,D). I seems, that during perinatal SMA development germinal centers, while already visible, are still forming in the white pulp, and a handful of both B- and T-cells still remain throughout the splenic tissue, which might also indicate delay in development or lymphocyte mislocalization.

Similar results could be seen in the SMN2B/- mouse model, where a mislocalization of B-cells and T-cells could be seen at P2, which intensified during P5 and P14, where a defective segregation of the white and red pulp in SMN2B/- spleens was suggested [179]. A similar labelling pattern of B- and T-cells could also be seen in the SMNΔ7 mouse model, where the splenic structure was disrupted and the red pulp was significantly reduced [180]. Additionally, quantitative immunohistochemistry revealed a substantial increase in CD3+ T cells and CD20+ B cells in the red pulp of perinatal SMA tissue, indicating failure in immune cell

compartmentalization, which could suggest developmental delay. Major Histocompatibility Complex (MHC) plays a central role in the immune response by presenting antigens to T cells [349]. The two primary classes of MHC molecules, MHC I and MHC II, are involved in different aspects of antigen presentation and immune activation [349]. MHC I complexes are presented on the cell surface, where they are recognized by CD8+ T cells. Upon recognition, the CD8+ T cells become activated and can kill an infected cell. MHC II-peptide complexes are recognized by CD4+ T cells, which subsequently become activated to help coordinate the immune response [349]. MHC I and MHC II expression in prenatal splenic SMA tissue remained similar to controls, however as development continued expression levels significantly dropped in the perinatal spleen. This downregulation may indicate defective antigen-presenting cell maturation or survival in the SMA spleen, potentially weakening the host's adaptive immune responses. Previous studies suggest that low MHC I expression impairs CD8+ T cell cytotoxicity [350] while MHC II deficiency is linked to reduced CD4+ T cell activation and susceptibility to infections [351]. Perinatal onset of pathology suggests that while organogenesis proceeds normally in utero, SMN-dependent immune maturation diverges at term.

Collectively, these results reveal that the developing spleen may indeed be affected by SMN deficiency and should be further observed to prevent immunological problems.

Thymus

In contrast to the spleen, the human thymus did not exhibit gross structural abnormalities in SMA tissue across all developmental stages. (Fig. 11). Lobular organization, cortico-medullary compartmentalization, and the presence and size of Hassall's corpuscles did not significantly differ between SMA and control tissue. This contrasts with findings in SMA mouse models, particularly SMN2B/- [179]. With development progressing, the cortex becomes larger and at term should make up significantly more area than the medulla (2:1 Ratio) [352]. In the SMN2B/- mouse model a clear reduction in cortico-medullary ratio and a decrease in cortical cellularity was described [179]. In our available samples the cortico-medullary ratio of SMA did not significantly differ compared to their age-matched controls and no significant reduction in cortex cellularity could be observed. The absence of these features in human tissue may potentially be related to interspecies differences or may reflect a later onset of pathology in humans not captured in the early postnatal stages. Lymphocytes in the cortex consist almost exclusively of T lymphocytes, which undergo a series of steps and as they mature, they descend through the cortex and into the medulla. Fully differentiated thymocytes exit the medulla via the blood vessels and lymphatic channels [353]. CD3 immunoreactivity is very strong throughout the whole thymus tissue, because both mature and immature T-cells express CD3 (Fig. 11B). Yet, no significant difference in the CD3 expressing T-lymphocyte population between control and SMA tissue could be observed during all stages of development (Fig. 11C). The B-lymphocyte population as well,

showed no significant difference in both medulla and cortex between control and SMA tissue during all developmental stages.

Despite preserved morphology, functional deficits were evident at the molecular level using IF. The thymus did not reveal any significant changes in MHC I and MHC II immunoreactivity during the prenatal stage, indicating that prenatal development of the lymphatic system in human SMA patients occurs normally. In the peri- and postnatal thymus on the other hand, while expression in the medulla remains largely similar, a significant decrease of MHC I and MHC II in the cortex was identified. A decrease of MHC I expression has been associated with a comprised immune function, specifically reduced recognition by antigens and cytotoxicity of CD8+ T cells [350]. A decrease in MHC II expression has previously been associated with reduced antigen-presenting function to naïve CD4+ T cells, subsequently resulting in susceptibility to infections to a variety of microorganisms [351].

In the thymus B cells consistently express higher amounts of MHC-II [354], therefore having a higher absence of MHC II molecules in SMA patients may cause severe T-cell immunodeficiency and the ability to mount immune response [355].

Regarding T-cell maturation specifically in the thymus, immature T-cells express both CD4 and CD8 on their surface, which interact with MHCI or MHCII for lineage commitment, resulting in single or double-negative (DN) selection. DN T-cells play a role in maintaining immune tolerance and suppressing immune responses and increased levels have previously indicated with pediatric auto-immune diseases [356]. While SMA is not an autoimmune disease, some studies suggest potential immune system defects in SMA patients [179], [357]. Reduced MHC expression in the cortex may impair the ability of thymocytes to undergo proper selection, potentially resulting in altered T cell concentrations or an increase in DN T cells, a phenotype, which was previously associated with defective immune tolerance and pediatric autoimmune diseases [356].

Interestingly, the medullary expression of MHC molecules remained relatively preserved in SMA samples, implying that late-stage T cell maturation and negative selection may be less affected. Nonetheless, reduced cortical MHC expression alone may impair central tolerance and functional T cell development, which could underline increased infection susceptibility reported in SMA patients [357].

Collectively, these findings underscore that SMA pathology includes early, possibly intrinsic defects in the lymphoid organs, particularly impacting immune cell development, organization, and function. The altered cellular localization and MHC expression patterns in the spleen and thymus suggest that SMN deficiency disrupts immunological maturation processes, which may lead to systemic immune dysregulation in SMA patients. This is of clinical importance, especially in light of emerging SMA therapies that prolong lifespan and may unmask latent systemic phenotypes not apparent in early-lethal disease states.

Summary of Discussion

Our findings contribute to the concept of SMA as a multisystemic developmental disease, particularly in its severe forms (SMA type 0-I). It is also to note that, even though translational pathology could be seen, the evolution and severity of disease in SMA mouse models is far greater differs in several aspects in comparison with severe human SMA cases.

While some organ systems (pancreas, liver) may show evidence of possible developmental pathology, others showed no particular temporospatial alterations, at least in early life (summarized in Table 5). One must keep in mind though, that our sample size was limited due to the rarity of severe SMA tissue availability as treatment became a more viable option for families with children affected by the disease. As human pathology of a disease does not progress unanimously in each individual, it is possible that some fetuses of SMA might have peripheral structural organ defects. Despite this, our research provides valuable insights into early pathological processes across multiple non-neuronal systems.

Moreover, our results highlight the necessity of longitudinal monitoring of peripheral organs in SMA patients, particularly now as with their increased life expectancy due to the success of SMN-restoring therapies.

Analyzed Organs	Main Findings
Liver	Persistent hematopoietic activity and increased Kupffer cell density suggests developmental delay and possible chronic inflammation
Pancreas	Altered islet composition, increased α-cells and decreased β-cells, indicates early endocrine dysregulation, possibly contributing to glucose intolerance in SMA
Intestine	No major structural abnormalities
Heart	No abnormalities in myocardium structure, however congenital heart defects in type 0 SMA and increased GYPA expression point to vascular deficits
Kidney	No overt renal abnormalities, though increased capsule diameter may suggest early signs of fibrosis
Spleen	Reduced capsule thickness, impaired pulp organization, and abnormal B-/T- cell localization and activation
Thymus	Normal structural appearance but reduced cortical MHC I/II expression suggest impaired T-cell development and maturation
Lung	No major structural abnormalities

Table 5: Main pathological findings of each organ

Limitations of our Study

Although the number of cases can be expanded, the samples of this study are unique and exceedingly difficult to obtain, as SMA treatment progresses. SMA occurs in 1/6000 to 1/10000 births globally, making sample-obtaining for extensive comparison very difficult. Furthermore, due to the recent approval of the disease-modifying therapies, many families facing an SMA case in pregnancy choose continuation to birth to start immediate therapy. Therefore, we consider that these samples obtained and selected carefully from our tissue bank represent a valuable collection to perform this type of study. The availability of a limited number of organs, status of the treatment and fixation of these samples precluded some tissues (i.e. bone) more specific molecular analysis to expand the investigation. We have employed a battery of antibodies to highlight specific aspects of developmental profiles in peripheral organs in which previous pathology has been described in the SMA mouse model and SMA patients. However, other antibodies for IHC could potentially be used, to not limit each experiment in a subset to a single target protein or to analyze different pathways.

VI. CONCLUSIONS

Main Conclusions

In this doctoral thesis we provide results that support SMA as a multisystemic developmental disorder, especially in severe forms (SMA type 0–I). SMN is ubiquitously expressed and necessary for fetal development and organ function. From the here described results, we show that SMN deficiency may causes some structural alterations in various peripheral organs, as several observed changes already begin during fetal development.

Organ-Specific Conclusions

Overall SMN-deficiency results in varying degrees of peripheral organ involvement, with some organs being more susceptible at earlier stages of development such as liver, pancreas, heart, kidney, spleen and thymus.

Other organs such as intestine and lung revealed no overt abnormalities during development.

Though peripheral organ complications have previously been described in SMA patients, they did not translate to our developmental findings indicating later onset in life.

Certain pathological similarities with SMA mouse models were observed, yet our analyzed human SMA cases revealed a milder involvement, highlighting the importance of more rigorous studies in humans to fully discern possible clinical features of SMA.

Final Insight

SMN is ubiquitously expressed and necessary for development of many organs and tissue. The longitudinal monitoring of peripheral organs is necessary, especially now that SMA patients have longer life expectancy as a result of effective therapies. The heterogeneity of human SMA pathology reinforces the importance of personalized and multi-targeted therapeutic strategies in the future.

VII. APPENDIX

Georg Lindner contributed as a co-author to both scripts presented in this section.

For the first manuscript, Dr. Mario Marotta was primarily responsible of the work and the contribution of the candidate was related to data interpretation, figure preparation, and drafting sections of the manuscript, with a special contribution to the "Discussion".

For the second manuscript, Dr. Eduardo Tizzano was primarily responsible of the idea, design, illustrations, tables and content of the review implicating the candidate as a second author to compose the main text, preparing figures and tables and revising bibliography and contribution of all authors.

MANUSCRIPT I: Laser microdissection-based genome-wide expression analysis in atrophied and hypertrophied skeletal muscle fibers of spinal muscular atrophy type I patients

Mario Marotta[a,c],¶, Yaris Sarria[a], Georg Lindner[b], Alejandra Fernández-Martín[c], Jose Luis Peiró[c], Francisco Soldado[c], Francina Munell[a,d], Eduardo Tizzano[b,d], Manuel Roig-Quilis[a,d]

a. Child neurology laboratory. Vall d'Hebron University Hospital Research Institute, Barcelona, Spain
b. Medicine Genetics Group, Vall d'Hebron Research Institute (VHIR), 08035 Barcelona, Spain
c. Bioengineering, Cell Therapy and Surgery in Congenital Malformations Laboratory. Vall d'Hebron Institut de Recerca (VHIR). Universitat Autonoma de Barcelona, Barcelona, Spain
d. Child neurology department. Vall d'Hebron University Hospital, Barcelona, Spain
e. Genetics department. Hospital de la Santa Creu i Sant Pau, Barcelona, Spain

Corresponding Author:
Dr. Mario Marotta, Vall d'Hebron University Hospital Research Institute. Passeig Vall d'Hebron 119-129, 08035, Barcelona, Spain. e-mail: mario.marotta@vhir.org

The layout of the was formatted to fit the style of the doctoral thesis.

Abstract

Spinal muscular atrophy (SMA) is characterized by loss of motor neurons (MN) in the spinal cord, which leads to denervation of and wasting of skeletal muscle. The aim of this study was to investigate the specific global gene expression profiles of human control (considered free of muscle disease), hypertrophied- or atrophied-SMA skeletal muscle fibers. In this work we combined Laser Capture Microdissection (LCM) technology with genome-wide microarray-based technology to determine and compare global gene expression profiles in control, hypertrophied- and atrophied-SMA skeletal muscle fibers populations. We obtained hypertrophied and atrophied skeletal muscle fiber groups in human type-I SMA skeletal muscle biopsies and tissue by LCM for gene expression microarray-based analysis at different stages of the SMA disease evolution. Microarray analysis showed a very small difference in gene expression profiles between control vs SMA-hypertrophied fibers, whereas strong differences in gene expression profiles were observed when comparing control vs SMA-atrophied fibers and atrophied vs hypertrophied fibers. Our results also demonstrate the activation of muscle regeneration mechanisms in atrophied cells, mimicking processes carried out during embryogenesis. LCM-based isolation of specific skeletal muscle fiber types, in conjunction with microarrays-based global gene expression analysis, provides a useful approach to the molecular study of processes occurring during innervation and denervation of human SMA skeletal muscle fibers. Knowledge of the specific mechanisms by which muscle fibers become denervated and atrophied could provide valuable information for developing new therapeutic strategies addressed to the treatment of the human SMA disease.

Introduction

Spinal Muscular Atrophy (SMA) affects more than 1 in 10000 infants and children worldwide and causes degeneration of lower motor neurons in the spinal cord and brainstem, therefore leading to severe muscle weakness. Muscle pathology in severe prenatal SMA samples is characterized in the form of overall smaller myotubes compared to age-matched controls [135] but also by more myotubes expressing fast myosin (fMHC) and less myotubes expressing slow myosin (sMHC) [137]. Furthermore, histological analyses of skeletal muscle exhibit a large presence of small atrophic fiber clusters, of both type I and type II origin, but also a select few normal and hypertrophic fiber clusters [191]. Laser Capture Microdissection (LCM) permits to isolate different muscle groups separately from the same tissue as it allows for rapid and reliable collection of homogeneous cell populations based on morphological characteristics, enabling the study of individual muscle fiber populations.

Until now, there have only been a few published works where LCM has been used to obtain LCM-derived skeletal muscle samples [192], [193], but none of them have focused on the study of the specific isolation of atrophied or hypertrophied muscle fiber populations from fresh human SMA skeletal muscle biopsies and the subsequent genome-wide microarray-based study of their specific genome-wide expression profiles. Being able to study atrophic fibers separately provide an excellent approach to study the precise evolution of SMA as well as the affectation of different muscle fibers for the further development of potential therapies.

SMA Therapeutics

Improvements in our understanding of the underlying defects in SMA remain critical for the development of therapeutic approaches. SMN deficiency has been shown to affect more than just MNs, with cells throughout the CNS as well as non-neuronal cells experiencing pathological changes [136], [137], [194]. SMN-dependent treatments, which aim to boost SMN production, have shown positive results, especially in SMA type I, leading to motor improvements and stability in other types. Nusinersen (Spinraza®) was the first approved treatment, working by enhancing *SMN2* mRNA to increase full-length SMN production. However, it requires intrathecal injections due to its inability to cross the blood-brain barrier and does not target non-CNS tissue. Risdiplam (Evrysdi®) is an oral alternative that also promotes *SMN2* exon 7 inclusion. Another approach, gene replacement therapy using onasemnogene abeparvovec (Zolgensma®), delivers *SMN1* through an adeno-associated virus (AAV9) vector, which can cross the blood-brain barrier and target both the CNS and peripheral tissues. While promising, concerns about the long-term effectiveness and safety of these treatments remain.

Additionally, therapeutics that target SMN-independent pathways are also under investigation such as myostatin (summarized in [195]). Myostatin is a cytokine that inhibits skeletal muscle growth. Overexpression of the endogenous myostatin antagonist follistatin has shown increased skeletal muscle mass in mice, as well as improved muscle strength and motor function [85], [86]. Previous study in the mouse model of severe SMA, an antisense oligonucleotide (ASO) augmenting *SMN2* exon 7 splicing was used together with AAV-anti-myostatin to block the myostatin pathway [86]. Myostatin inhibition acted synergistically with ASO therapy and improvement of sensory circuits in motor neurons was observed, also including increase in body weight, muscle mass and fiber size, and prolonged survival rate. Further clinical trials also use investigational anti-myostatin inhibitors in combination therapy. For example, SAPPHIRE (NCT05156320) is a phase 3 clinical study using apitegromab, a fully human anti-proMyostatin monoclonal antibody (mAb) of the immunoglobulin G4 (IgG4)/lambda isotype that binds to human pro/latent myostatin with high affinity, which will be administered intravenously every 4 months in >2-year-old patients for whom nusinersen treatment was initiated prior to 5 years of age. Further clinical studies using anti-proMyostatin antibodies in combination with currently approved SMA-therapies include TOPAZ (NCT03921528), ONYX (NCT05626855), MANATEE (NCT03032172) and RESILIENT (NCT05337553),

To study the gene expression profiles between atrophied and hypertrophied muscle fibers in SMA and compared to age-matched control muscles, could provide valuable information on innervation and/or denervation patterns of skeletal muscle.

Materials and Methods

Human muscle biopsies and tissue processing

Human skeletal muscle biopsies were surgically obtained by pediatric surgeons with informed consent and approval of the Human Ethics Committees of Vall d'Hebron University Hospital, Barcelona, Spain. Human muscle biopsies were obtained from type-I SMA (Werdnig-Hoffman disease) patients at ages ranging from 4- to 12-months-old and from age-matched controls considered free of muscle disease. Human skeletal muscle samples were excised, embedded in OCT compound (Tissue-Tek, Sakura, Zoeterwoude, Netherlands) and immediately frozen in liquid nitrogen, and the tissue blocks were stored at -80°C until used.

Laser Capture Microdissection and RNA Extraction

The architecture of skeletal muscle and the longitudinal length of skeletal muscle fibers allow the obtaining of large amounts of numerous serial transversal sections and the acquisition of large quantities of microdissected tissue from every selected fiber group. As performed in previous studies [196], serial frozen skeletal muscle transversal sections were cut at 20-µm thick using an HM505E microtome (Microm, Walldorf, Germany) at below -20ºC, mounted on pre-treated with 0.1% (w/v) poly-L-Lysine (Sigma-Aldrich Quimica SA, Madrid, Spain) RNase-free membrane-slides (PALM Microlaser Technologies AG, Munich, Germany) and immediately processed under RNase-free conditions. Skeletal muscle sections were fixed with RNase-free ethanol 70% for 1 min, washed in RNase-free water, stained with Hematoxylin-Eosin (10 seconds hematoxylin and 5 seconds eosin), dehydrated with RNase-free graded ethanol solutions (1 min ethanol 70% twice, 1 min ethanol 95% twice, 1 min ethanol 100% twice) and cleared in xylene (two times, 15 seconds each). After air-drying for 5 min, the slides were stored at -80°C until further use. Mayer's hematoxylin solution was purchased from Sigma (Sigma-Aldrich Quimica SA, Madrid, Spain) and eosin solution was prepared just before use by dissolving 0.2 g eosin Y (Merck, Darmstadt, Germany) in 100 ml RNAse-free 80% ethanol acidified with 0.4 ml glacial acetic acid (Sigma-Aldrich Quimica SA, Madrid, Spain).

LCM was performed under direct microscopic visualization on H&E-stained muscle sections, using a Leica® LMD 6000 microdissection system (Leica microsystems, Germany). To prevent RNA degradation and minimize exposure to room temperature, all the RNase-free membrane-slides containing the desiccated skeletal muscle sections were thawed and processed one by one. The selected groups of fibers were collected from each correlative slide into dry 0.5 ml tube caps coated with silicon (MicroDissect GmbH, Germany) and were stored at -80ºC until all the laser captured-sections belonging to each fiber group were obtained. All the microdissected areas belonging to the same fiber group were collected in the same tube, to immediately begin the RNA extraction protocol. Total RNA was extracted by the spin-column-

based method RNeasy Micro Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Before Microarrays analysis, the quality and quantity of the LCM-derived RNA were assessed using the RNA pico assay® LabChips and the Agilent 2100 Bioanalyzer (Agilent Technologies, South Queensferry, Scotland). The RIN (RNA Integrity Number) values determined for all the RNA samples ranged from 6.1 to 7.3, indicating a good RNA quality, according to the grading of the Agilent 2100 RIN Software.

Microarray hybridization

Microarrays were carried out using the Affymetrix microarray platform and the Genechip Human Exon 1.0 ST Array. This array analyzes both gene expression and alternative splicing patterns on a whole-genome scale on a single array with probes covering virtually every known exon on the target genome thus permitting expression summarization at the exon level.

Starting material was 5 ng of total RNA from LCM-microdissected tissue. Quality of isolated RNA was first measured by Bioanalyzer Assay (Agilent Technologies). Briefly, RNA was amplified for target preparation with the Ovation Pico WTA system (NuGEN Technologies Inc.). WT Ovation Exon Module (NuGEN Technologies Inc.) was used to generate the sense strand cDNA target ready for fragmentation and labelling with NuGEN Encore Biotin Module (NuGEN Technologies Inc.) according to the manufacturer’s instructions. Sense ssDNA fragmented and labelled was hybridized to the arrays. Chips were processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000 (Affymetrix).

Microarray data analysis

Images were processed with Expression ConsoleTM Software (Affymetrix). All samples demonstrated characteristics of high-quality cRNA and were subjected to subsequent analysis. Raw expression values obtained directly from.CEL files were preprocessed using the RMA method [197], a three-step process which integrates background correction, normalization and summarization of probe values. These normalized values were the basis for all the analysis.

Prior to any analysis, data were submitted to non-specific filtering to remove low signal genes (those genes whose mean signal in each group did not exceed a minimum threshold) and low variability genes (those genes whose standard deviation between all samples did not exceed a minimum threshold). CEL files were imported into the Affy package (36) in Bioconductor (http://www.bioconductor.org/) and pre-processed using the RMA (robust multi-array analysis) algorithm with default parameters (16). Genes were filtered according to the following criteria: Signal ≥log (100), Mean AbsFC (absolute fold-change) ≥1.5. Genes complying with these criteria were then processed using the package limma (a linear model for microarray analysis by Gordon Smyth, Natalie Thorne and James Wettenhall at The Walter and Eliza Hall Institute

of Medical Research) and fdr (false discovery rate) was used as the method for multi test correction (Benjamini and Hochberg's step-up method).

The selection of differentially expressed genes between conditions was based on a linear model analysis with empirical Bayes moderation of variance estimates following the methodology developed by Smyth, 2004.. This method extends traditional linear model analysis using empirical Bayes methods to combine information from the whole array and every individual gene to obtain improved error estimates which are very useful in microarray data analysis where sample sizes are often small, which can lead to erratic error estimates and, in consequence, to untrustworthy p-values. The analysis yields standard test statistics, such as fold changes and (moderated)-t or p–values, which can be used to rank genes from most to least differentially expressed. In order to deal with the multiple testing issues derived from the fact that many tests (one per gene) are simultaneously performed, p-values were adjusted to obtain strong control over false discovery rate using the Benjamini and Hochberg method [198].

Genes selected as being differentially expressed were clustered to look for common patterns of expression. Hierarchical clustering with euclidean distance was used to form the groups, and heatmaps (color coded graphs with samples in columns and genes in rows) were used to visualize them. Multidimensional scaling was applied on distance matrices used to group samples to produce graphical representations that helped to discover groups in samples.

The analysis of Biological Significance was based on an Over-Representation Analysis aiming to determine genes that were found to be differentially expressed, appeared to be concentrated or particularly absent of some Gene Ontology Categories, could be related to the biological processes involved in the analysis.

All statistical analyses were performed using the free statistical language R and libraries developed for microarray data analysis by the Bioconductor Project (www.bioconductor.org). The main methods and tools used are described in the monograph by Gentleman *et al.,* 2005 [199]. A complete data set of our microarray analysis has been submitted to the EMBL-EBI ArrayExpress repository.

Immunofluorescence analysis

For immunofluorescence analysis, frozen muscle transversal sections were cut at below -20ºC using an HM505E microtome (Microm) and mounted on 0.1% (w/v) poly-L-Lysine pre-treated slides. Muscle sections were fixed in cold (-20ºC) acetone for 5 min, rinsed twice with PBS and blocked with 10% goat serum in PBS for 20 minutes. Primary antibodies against human Myogenin (M-225, Santa Cruz Biotechnology), DMHC (NCL-DMHC, Novocastra) and collagen-I (ab138492, Abcam), were diluted in PBS with 1% BSA and were incubated in a humid chamber for 16 h at 4ºC. Next, muscle sections were washed three times with PBS and incubated with Alexa Fluor® 568 anti-mouse (A-11004, Invitrogen) or Alexa Fluor® 488 anti-

rabbit (A-11008, Invitrogen) secondary antibodies diluted in PBS with 1% BSA in a dark humid chamber for 45 minutes at room temperature. Finally, slides were washed three times with PBS and mounted using a coverslip and Vectashield® mounting medium with DAPI (Vector Laboratories) and coverslip edges were sealed with clear nail polish.

Measurement of muscle fiber diameter

For cross-sectional area (CSA) determination, muscle fibers were counted and measured in collagen-I immunofluorescence microphotographs. The area of muscle fibers were calculated in the images selected within every muscle sample by using a BX-61 microscope (Olympus) equipped with a DP72 camera (Olympus) and CellSens Digital Imaging software (version 1.9). CSA of all muscle fibers was measured using Image-J software (version 1.46; National Institutes of Health) based on a ratio of calibrated pixels to actual size (μm).

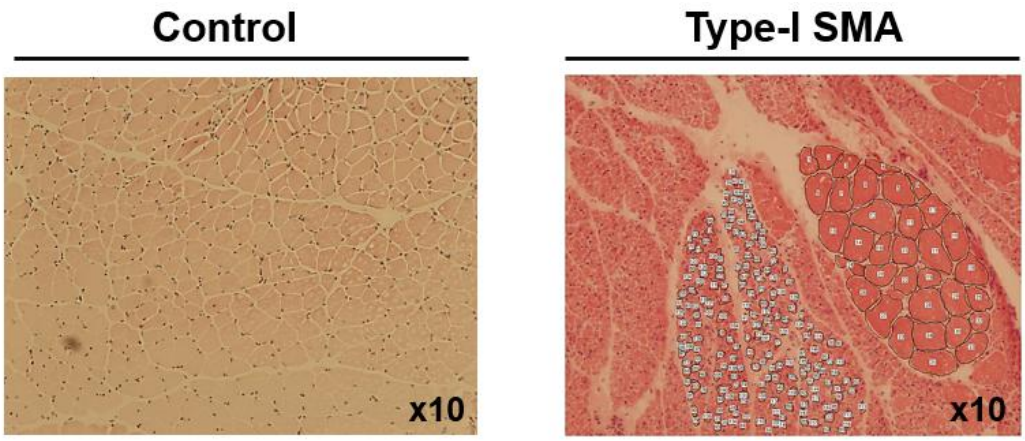
Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 9.1.1 (GraphPad Software). The Shapiro-Wilk test was used to evaluate normality of the data. As a result, a nonparametric Kruskal-Wallis test was performed to assess statistical significance in myofiber CSA and the post hoc Dunn’s test was performed for multiple comparisons between the different groups (Hypertrophic, Atrophic, and control myofibers). Differences were considered significant with an adjusted p-value less than 0.05.

Results

LCM of different fiber group types from control vs SMA skeletal muscles

Based on H&E stains, we identified different fiber group types in human control vs SMA skeletal muscles (Fig. 14). By means of LCM, we separately isolated tissue from 3 types of skeletal muscle fiber groups collected from control or type I SMA human skeletal muscle biopsies: 1) control muscle fibers from skeletal muscle samples free of muscle disease, and 2) hypertrophied-SMA muscle fibers or 3) atrophied-SMA muscle fibers from type I SMA human skeletal muscle samples. To confirm hypertrophy or atrophy of SMA muscle fiber groups compared to control fibers size, the average cross-sectional areas (CSA) of fiber groups were measured by using the Image J software (NIH). Statistically significant differences in myofiber' CSA were found in the different muscle fiber populations. The largest CSA values were found in hypertrophied-SMA fibers (3981.24 ± 1417.13 µm2) with respect to control (1019.89 ± 137.63 µm2) or atrophied-SMA fibers (225.84 ± 61.50 µm2). The adjusted p-values for multiple comparisons between healthy (n=127), hypertrophic (n=44) or atrophic (n=168) fiber populations were: hypertrophic vs. atrophic fibers ($p<0.0001$), hypertrophic vs. control fibers ($p=0.0002$) and atrophic vs. control ($p<0.0001$).



Fiber type	description	Average fiber cross sectional area (%)
C	Control muscle fibers	100
H	Hypertrophied type-I SMA fibers	390
A	Atrophied type-I SMA fibers	22

Figure 14: Determination of fiber areas for Laser Capture Microdissection (LCM): Area for LCM was chosen based on distinct architecture of atrophied and hypertrophied skeletal muscle fibers. The average (%) of cross-sectional areas of each fiber type were calculated respecting to control group.

As LCM was performed in previous studies carried out in mouse skeletal muscle samples [196], the protocol was optimized with the purpose of obtaining large quantities of microdissected tissue from a reasonably small number of human skeletal muscle sections. The specific areas of the different skeletal muscle fiber groups (especially atrophied and hypertrophied fibers in SMA muscle biopsies) were first localized and identified under optical microscopy in conventionally processed H&E tissue sections mounted with glass cover slips. Next, the same fiber groups were localized in LCM membrane-slides containing 20 µm-thickness transversal skeletal muscle sections after H-E staining and air-dried. All fiber group areas of interest were localized and microdissected from several consecutive skeletal muscle sections and ultimately collected in the same tube to proceed with RNA purification (Fig. 15).

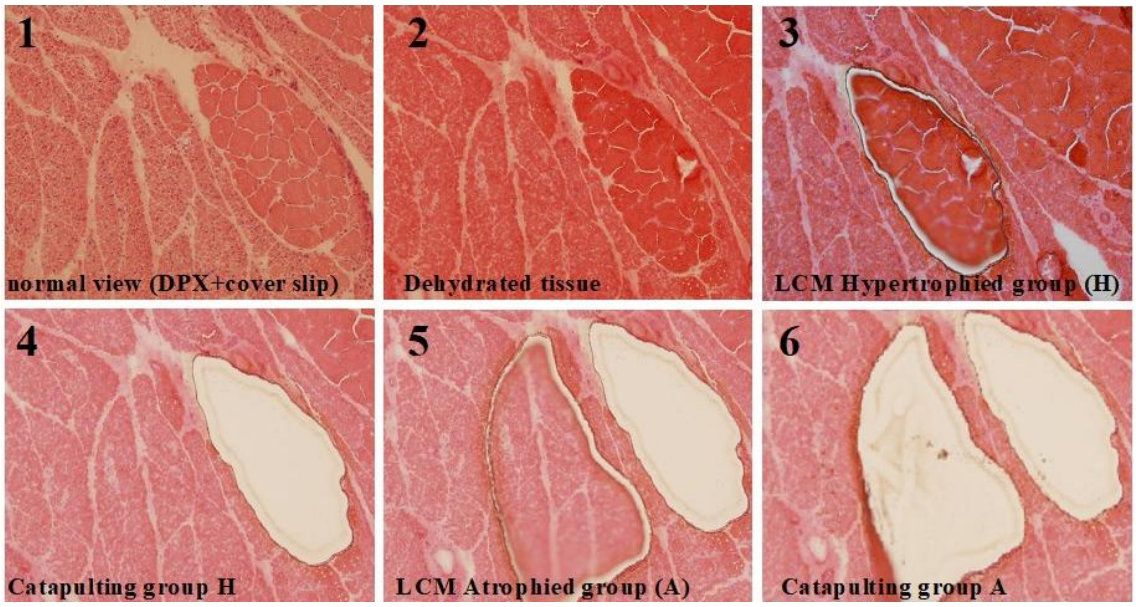


Figure 15: LCM procedure for hypertrophied and non-hypertrophied myofibers from a type I SMA patient muscle biopsy. The different areas were obtained by LCM procedure from H&E stained sections of a type I SMA patient muscle biopsy. The representative LCM processes for two different zones are shown in the figure: Hypertrophied (H) and non-hypertrophied myofibers 1. Normal visualization of the tissue (tissue sections mounted with glass cover slips by placing a drop of DPX onto a glass slide). 2. Desiccated tissue is shown before LCM. 3. Microdissection of Hypertrophied group of fibers. 4. Catapulting of dissected Hypertrophied group. 5. Microdissection of Atrophied group of fibers. 6. Catapulting of dissected Atrophied group. Images show enlarged (100x) photographs for all panels.

Fiber type	description	Yield[a] (ng total RNA/ mm^2 microdissected area[b])
C	Control muscle fibers	0.46 ± 0.20
H	Hypertrophied type-I SMA fibers	1.03 ± 0.44
A	Atrophied type-I SMA fibers	1.47 ± 0.19

Table 6: Yield of LCM-obtained RNA per area of each fiber type. Data are presented as mean ±SD.

Respecting RNA quantities obtained from the different types of fibers, we found that the yield of LCM-obtained RNA was dependent on every specific fiber type group (Table 6). Interestingly, the maximum yield of RNA/total microdissected area was obtained from the fiber groups composed of SMA-atrophic myofibers (with a maximum yield of 1.47±0.19 ng RNA/mm2 of the microdissected area), being more than 3-fold higher than the RNA production in control myofiber-group in the basal state (0.46±0.20 ng RNA/mm2 of the microdissected area). The high yield of mRNA in atrophic fibers could be attributed to upregulation of specific transcription factors and signaling pathways involved in protein degradation and synthesis. Increased mRNA expression might be required to restore their levels [200]. On the other hand, SMA-hypertrophic myofibers showed a more than 2-fold (1.03±0.44 ng RNA/mm2 of the microdissected area) increase in RNA production compared to control myofibers (but lower than SMA-atrophied ones), probably due to the augment in size of these fibers in an attempt to compensate for the lack of muscle strength in SMA skeletal muscles, increasing muscle protein requirements and a higher rate of transcriptional activity in hypertrophied fibers. RNA obtained from the different muscle fiber groups showed good quality (RIN ranging from 6.1 to 7.3) and was suitable for subsequent microarray-based gene expression analysis.

Microarray-based gene expression profiles in control vs type-I SMA skeletal muscle fiber groups.

In order to determine differences in gene expression between LCM-obtained skeletal muscle fiber types, three different comparisons of global gene expression profiles were performed: 1) control vs SMA-hypertrophied, 2) control vs SMA-atrophied and, 3) SMA-atrophied vs SMA-hypertrophied skeletal muscle fibers.

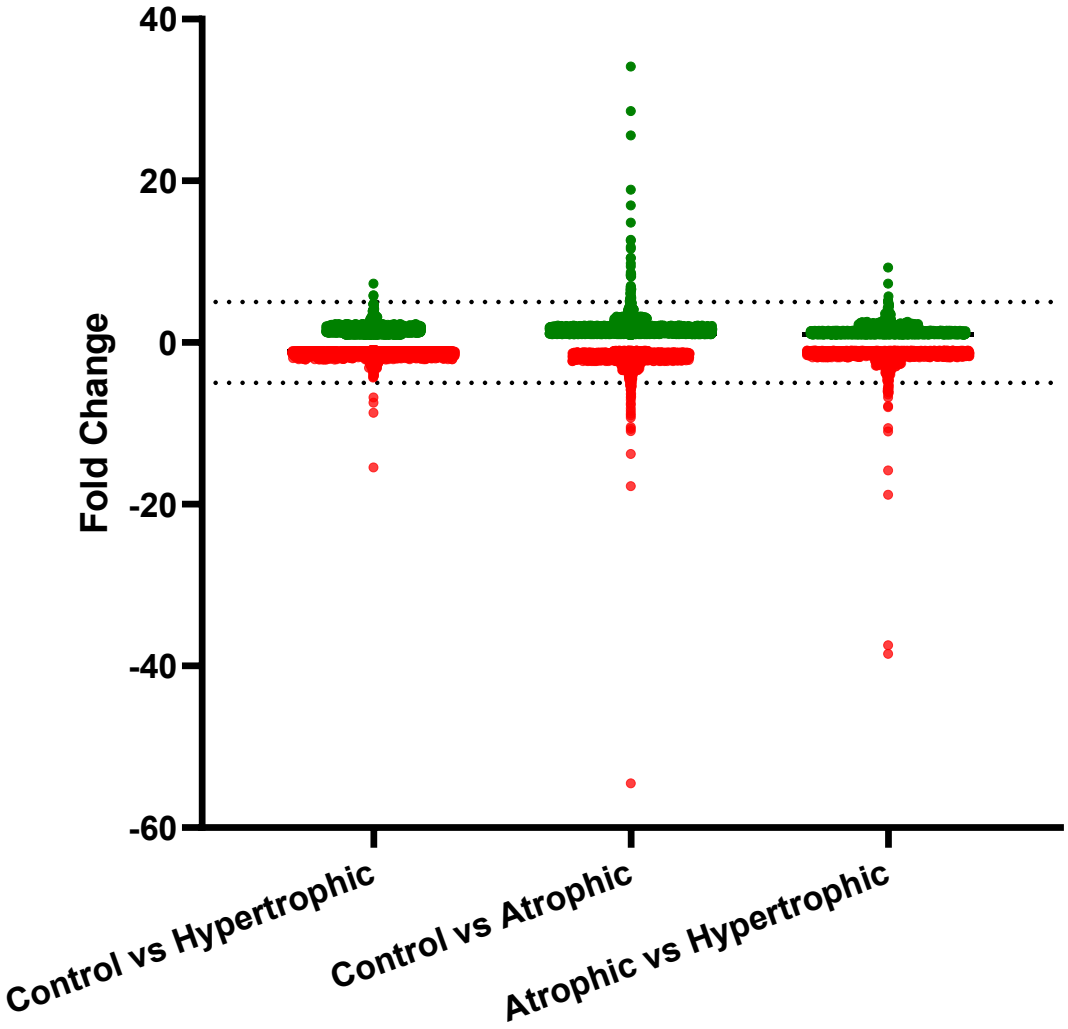


Figure 16: All differentially expressed genes from gene expression analysis between fiber types: Dotted line represents a threshold of p>2 and p<-2 for up- and down-regulated genes. Green-coded genes are upregulated and red-coded genes are downregulated.

Gene expression differences between control vs SMA-hypertrophied fibers

Microarray-based analysis demonstrated a very small difference in gene expression profiles between control vs SMA-hypertrophied fibers (Table 7). Interestingly, only 6 genes genes/ESTs (2 of them being up-regulated and 4 down-regulated) showed statistically (≥1.5 fold, $p<0.05$) significant expression variations. Five of these genes, named MYH8, RSPO3, ATP2A1, CHRNA1 and MYH2 are well-known genes that appeared to be differentially expressed between control vs hypertrophied fibers. MYH8 showed the strongest variation, being down-regulated more than 15-fold, whereas the other myosin MYH2 showed opposite variation, being up-regulated 4-fold in control fibers when compared to hypertrophied ones. MYH8 gene encodes for a perinatal myosin which is predominantly expressed in fetal skeletal muscle whereas MYH2 encodes for an adult muscle myosin isoform, which indicates a fetal-like phenotype in myosin isoform expression of hypertrophic myofibers compared to control ones.

Interestingly, SMA-hypertrophied fibers showed decreased expression levels of CHRNA1 gene, which encodes the alpha-1 subunit of the acetylcholine receptor, suggesting the possibility of signaling impairment of neurotransmission in SMA-hypertrophied compared to control muscles.

Ingenuity pathways analysis (IPA, Qiagen) of filtered (≥1.5-fold and $p<0.05$) gene expression data revealed 3 differentially expressed genes between control vs SMA-hypertrophied fibers, named ATP2A1, CHRNA1 and MYH2, belonging to the Skeletal and Muscular System Development and Function, Tissue morphology and Nervous System Development and Function of the Top Bio Function categories generated by IPA pathways analysis.

Symbol	Entrez Gene Name	ID	P-Value	Fold Change
ATP2A1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	3655060	4.325E-05	4.550
MYH2	myosin, heavy chain 2, skeletal muscle, adult	3745351	8.450E-05	4.069
LOC92973	LOC92973 gene	3168245	3.268E-05	-4.131
CHRNA1	cholinergic receptor, nicotinic, alpha 1 (muscle)	2587937	3.192E-05	-4.254
RSPO3	R-spondin 3 homolog (Xenopus laevis)	2924851	4.782E-05	-7.424
MYH8	myosin, heavy chain 8, skeletal muscle, perinatal	3745161	3.593E-05	-15.443

Table 7: Gene expression differences between LCM-obtained control vs SMA-hypertrophied fiber groups. Human control vs type I SMA-hypertrophied fiber groups tissue were isolated by LCM and the RNA obtained was used for microarrays analysis by using the human exon array 1.0 ST Affymetrix genechips. Fold change ($p<0.05$) indicates the up-or down-regulated genes/ESTs in control fibers respecting SMA-hypertrophied ones.

Gene expression differences between control vs SMA-atrophied fibers

Microarray analysis showed the strongest differences in gene expression profiles when we compared control vs SMA-atrophied fibers (Table 8). 395 Genes/ESTs were differentially expressed (≥1.5 fold, $p<0.05$) between control vs atrophied myofibers, with 226 of them being up-regulated and 169 being down-regulated. The most important gene expression changes (more than 20-fold of variation) were observed in the down-regulation of MYH8, and the up-regulation of IDI2, MYL3 and MYH7 genes in control fibers compared to atrophied areas.

Our microarray-based data demonstrated significant expression changes of several myosin isoforms, the most important changes being the strong up-regulation of MYH8 perinatal myosin which showed the strongest variation, and MYH1 myosin (54.53- and 10.43-fold, respectively) in SMA-atrophied fibers when compared to control muscle, as well as the up-regulation of the light chain MYL3 and MYH7 cardiac myosin isoforms (28.65- and 25.64-fold, respectively). Interestingly, atrophied fibers also showed a strong down-regulation of MYOZ2 (11.58-fold), indicating a decrease in the expression of genes involved in the maintenance of muscle mass. Furthermore, the genes FST (a natural myostatin antagonist) and MUSK (crucially involved in development and maintenance of neuromuscular junctions) also showed a strong down-regulation in atrophied fibers (8.47- and 8,77-fold, respectively). IDI2 gene, which, recently suggested to play a significant role in the pathogenesis of sporadic amyotrophic lateral sclerosis, showed the strongest down-regulation in atrophied fibers (34.14 fold). Moreover, two membrane receptors, CHRNA1 and Foll, were found up-regulated in atrophied compared to control fibers (13.78- and 8.77-fold, respectively). Mutations in both genes have been associated with congenital myasthenic syndromes (CMS), which are characterized by varying degrees of muscle weakness due to impaired neuromuscular transmission [201]. It is also interesting to see that Pax3 was upregulated (3.91-fold) in atrophic fibers as it regulates the myogenic differentiation and plays a major role during early skeletal muscle formation in the embryo [202].

IPA analysis of filtered (≥1.5-fold and $p<0.05$) gene expression data revealed that 5 of the genes differentially expressed between control vs SMA-atrophied fibers, named CDKN1A, EGF, ERBB3, MFAP5 and VEGFA, belonged to NOTCH1 signaling pathway, indicating a potential role of this important pathway in the evolution of denervation-induced skeletal muscle atrophy.

Symbol	Entrez Gene Name	ID	P-Value	Fold Change
IDI2	isopentenyl-diphosphate delta isomerase 2	3273578	8.602E-10	34.14
MYL3	myosin, light chain 3, alkali; ventricular, skeletal, slow	2672442	6.053E-09	28.65
MYH7	myosin, heavy chain 7, cardiac muscle, beta	3557504	2.579E-08	25.64
MYOZ2	myozenin 2	2741206	7.197E-07	11.58
MFAP5	microfibrillar associated protein 5	3443226	2.321E-04	6.52
VEGFA	vascular endothelial growth factor A	2908179	2.531E-03	3.11

EGF	epidermal growth factor (beta-urogastrone)	2739308	3.486E-03	-2.97
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2905169	4.663E-04	-3.83
PAX3	paired box 3	2600881	1.237E-04	-3.90
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	3417249	1.224E-06	-6.78
FST	follistatin	2809399	1.22E-06	-8.47
MUSK	muscle, skeletal, receptor tyrosine kinase	3184710	1.551E-06	-8.77
CHRNA1x	cholinergic receptor, nicotinic, alpha 1 (muscle)	2587937	2.705E-08	-13.78
MYH8	myosin, heavy chain 8, skeletal muscle, perinatal	3745161	4.743E-07	-54.53

Table 8: Gene expression differences between LCM-obtained control vs SMA-atrophied fibers. Human control tissue or type I SMA-atrophied fiber groups were isolated by LCM and the RNA obtained was used for microarrays analysis by using the human exon array 1.0 ST Affymetrix genechips. Fold change ($p<0.05$) indicates the most up- or down-regulated genes/ESTs in control fibers respecting SMA-atrophied fibers.

Gene expression differences between atrophied and hypertrophied type-I SMA skeletal muscle fiber populations

The comparison between atrophied and hypertrophied type-I SMA fibers showed important differences in gene expression profiles (Table 9). 139 genes/ESTs were differentially expressed (≥1.5fold, $p<0.05$), respectively), that showed a tendency of down-regulation of gene expression profiles in atrophied fibers, since the down-regulated genes were almost twice as many as the up-regulated ones (49 up- and 90 down-regulated genes/ESTs). The genes that showed the strongest gene expression variations were mainly down-regulated, such as MYL3, MYH7, MYOZ2, OCC-1, MYH6 and TYRP1 which showed more than a 10-fold decline of gene expression in atrophied fibers compared to hypertrophied fiber groups.

The comparison performed between atrophied and hypertrophied fibers (Table 9) demonstrated a strong down-regulation in atrophied fibers of the light chain myosin MYL3 (38.51-fold), and the two cardiac muscle myosins MYH7 and MYH6 (37.45- and 10.99-fold, respectively) in SMA-atrophied fibers. Significant down-regulation of cardiac hypertrophy-related MYOZ2 gene (18.81-fold), as well as two genes related to protection against neuronal death in neurodegenerative stress conditions (UBE2D3 (6,76-fold) and seladin-1 (5,89-fold) were also

detected in atrophied fiber areas which could indicate an activation of some mechanisms in an attempt to prevent neuronal death and subsequent skeletal muscle denervation-induced atrophy.

Symbol	Entrez Gene Name	ID	P-Value	Fold Change
DHCR24	24-dehydrocholesterol reductase	2413907	7.008E-08	-5.98
UBE2D3	ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	3896293	8.200E-09	-6.76
ASB12	ankyrin repeat and SOCS box-containing 12	2413907	5.164E-10	-7.85
PPP1R1A	protein phosphatase 1, regulatory (inhibitor) subunit 1A	3896293	3.805E-10	-7.98
TYRP1	tyrosinase-related protein 1	3162486	1.118E-12	-10.59
MYH6	myosin, heavy chain 6, cardiac muscle, alpha	3557430	4.908E-13	-10.99
OCC-1	overexpressed in colon carcinoma-1	3429857	8.666E-17	-15.79
MYOZ2	myozenin 2	2741206	8.833E-19	-18.81
MYH7	myosin, heavy chain 7, cardiac muscle, beta	3557504	8.715E-28	-37.45
MYL3	myosin, light chain 3, alkali; ventricular, skeletal, slow	2672442	3.416E-28	-38.51

Table 9: Gene expression differences between LCM-obtained SMA-atrophied vs hypertrophied fiber groups. Human type I SMA-atrophied and hypertrophied fiber groups tissue were isolated by LCM and the RNA obtained was used for microarrays analysis by using the human exon array 1.0 ST Affymetrix genechips. Fold change ($p<0.05$) indicates the most up- or down-regulated genes/ESTs in SMA-atrophied fibers respecting to hypertrophied ones.

Corroboration of Microarray data by immunofluorescence analysis of muscle regeneration-related protein expression

We carried out immunofluorescence analysis with the purpose of corroborating protein expression level results obtained by microarray analysis, which demonstrated a raise in muscle regeneration-related genes, such as myogenin and fetal/embryonic myosin isoforms (Fig. 17). Immunofluorescence analysis demonstrated the presence of both myogenin and developmental myosin heavy chain (dMHC) exclusively in SMA-atrophied fiber areas, indicating an activation of the muscle regeneration processes as a response of muscle atrophy in human skeletal muscle fibers. These data demonstrate that atrophied, but not hypertrophied, muscle fibers start to recapitulate muscle regeneration mechanisms, probably due to signals indicating muscle tissue

loss in areas experiencing atrophy by denervation and as a response mechanism to compensate and strengthen the atrophied muscle fibers. Supporting previous data obtained by other authors, further indicate muscle regeneration marker expression in atrophied skeletal muscles [203], [204].

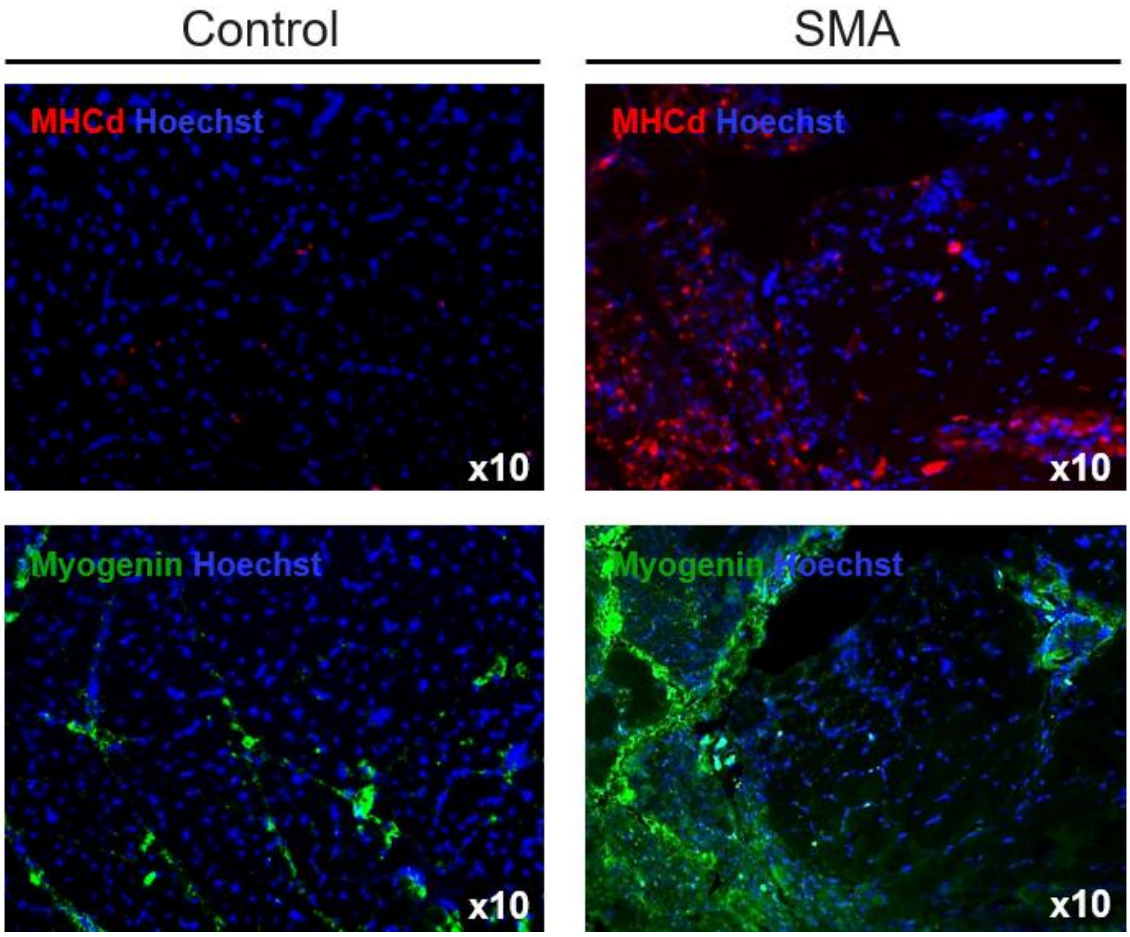


Figure 17: Immunofluorescence expression of fetal myosin and myogenin in SMA. Representative cross-sections of control vs SMA muscle biopsies. Tissue we co-labelled for DMHC (red, DMHC), Myogenin (green, MYOG) and Hoechst (blue, nuclei); x10 Zoom.

Discussion

Deeper knowledge of the molecular bases that cause SMA and the events that occur in the innervation of muscle fibers by MNs may help to develop new therapeutic strategies for this disease. Utilizing LCM, we have obtained RNA samples from specific areas of muscle tissue from SMA patients which allowed us to study global gene expression in three types of muscle fibers. LCM technology allows for rapid and reliable collection of homogeneous cell populations from tissue sections under direct microscopic visualization, but also efficient isolation of specific areas of tissue without contamination from surrounding tissue components, leaving the intracellular structure and molecules intact [155], [205]. Several research fields, such as cancer [206], [207], [208] and neurodegenerative diseases [209], [210], have applied this technology in their studies. LCM-obtained RNA is suitable for performing gene expression analysis by RT-PCR, but also microarray gene expression analysis after RNA amplification. This method was previously used to study DNA mitochondrial deletions in rat [211], [212], human [213] and monkey [214] skeletal muscle samples. Furthermore, angiogenesis in skeletal muscle was studied by isolation of capillaries by LCM [215] and LCM has also been used in human polymyositis skeletal muscle biopsy specimens for the specific isolation of muscle-invading T-cells [216].

For the first time, in this investigation we have applied the LCM technology to study specific gene expression profiles in diverse type-I SMA human skeletal muscle fiber populations.

We used samples from infants with SMA type I during the onset of the pathology, from which we obtained valuable information about the molecular processes that take place in the development of the disease. Using LCM, we were able to separately study gene expression profiles of three different populations of skeletal muscle fibers, thus generating three different comparisons of microarrays-based gene expression profiles. Microarray analysis showed a very small difference in gene expression profiles when comparing control vs SMA-hypertrophied fibers (only 6 genes genes/ESTs showed statistically significant expression variations), whereas strong differences in gene expression profiles were observed, upon comparing control vs SMA-atrophied fibers 124 Genes/ESTs differentially expressed (≥1.5-fold) or SMA-atrophied and SMA-hypertrophied fibers (86 Genes/ESTs differentially expressed ($p<0.01$)).

These data suggest that hypertrophied fibers in SMA skeletal muscles may remain innervated, at least during the onset of the disease, due to their high similarity of gene expression profiles compared to control fibers. On the other hand, atrophied fibers show strong differences compared to control and hypertrophied fibers, and we especially detected an important increase of muscle regeneration markers, such as myogenin and dMHC expression levels, which points

to a possible activation of muscle regeneration mechanisms in response to the atrophy caused by the disease.

The results obtained by microarray-based global gene expression analysis between the three different fiber group type populations demonstrate high similarities between control and SMA-hypertrophied fibers and show the important differences in gene expression profiles of healthy and hypertrophic fibers with respect to SMA-atrophied fibers. Several myosins showed important changes in their expression levels and, in particular, increased levels of several perinatal and embryonic myosin isoforms were detected, whereas significant decrease in adult myosins were found in SMA fibers when compared to controls. This trend was more pronounced in atrophied fibers than in hypertrophied ones, since only two myosins varied in hypertrophied fibers, while almost ten different heavy or light chain myosins were differentially expressed in atrophied fibers, when compared to controls. Interestingly, the gene encoding for the MYOZ2 protein, which is related to cardiac hypertrophy, was found to be strongly up-regulated (18.81-fold) in hypertrophied fibers when compared to atrophied fiber group. Moreover, atrophied fibers also showed a strong decline in gene expression for UBE2D3 and DHCR24, which show to be related to protection against neuronal death in neurodegenerative stress conditions [217]. In contrast, atrophied fibers showed an increase in expression of several genes related to skeletal muscle development and regeneration, such as myoferlin and myogenin. Myoferlin participates in the process of myoblasts fusion in muscle regeneration, and myogenin participates in muscle fiber differentiation process [218]. Therefore, our data indicate that atrophied, but not hypertrophied, fibers are activating several genes involved in skeletal muscle development and regeneration.

In addition, very few differences between control vs type-I SMA hypertrophied muscle fibers were identified, indicating that hypertrophied fibers observed in SMA skeletal muscle biopsies could still be innervated by surrounding living MNs. Our data suggest that muscle fibers could first be innervated in SMA muscles, prior to denervation, and subsequently a lack of SMN leads to motor neuron degeneration followed by a muscular atrophied state. Fibers that remain innervated would become hypertrophied as a compensatory effect to the lack of contractile function of atrophied fibers and therefore explaining why different sized fibers are observed in SMA muscles during the first stages of the disease. By the time, skeletal muscle fibers progressively lose innervation and atrophy begins, and a myogenic regeneration response could be activated in response to atrophy, probably by recapitulating the muscle fiber formation and differentiation processes that occurs during skeletal muscle formation in embryogenesis [219]. Myogenin is essential in myoblast fusion and myofiber formation and dMHC is a well-known marker for muscle development [220], [221], since it is expressed in the first period of young myofiber formation until its replacement by their mature isoforms (fast or slow MHCs). Our immunofluorescence analysis further demonstrated their up-regulation at the protein level (Fig.

17), since both myogenin and dMHC were strongly detected in SMA-atrophied muscle fiber groups, whereas no expression was found in hypertrophied fiber groups. These data demonstrate that atrophied muscle fibers start to recapitulate muscle regeneration mechanisms probably due to signals indicating muscle tissue loss in areas experiencing atrophy by denervation. Similar mechanisms have been reported in mouse embryogenesis during limb skeletal muscle formation [219], where muscle precursor cells are activated to proliferate, migrate from the somite to the limb buds and differentiate to the myogenic lineage to form skeletal muscles. The myogenic program starts when muscle precursor cells begin to express both Myf-5 and MyoD, inducing their determination to muscle lineage, being followed by myogenin expression which activates the differentiation program and induces myoblasts fusion and muscle fiber formation [219]. Our results agree with the activation of mechanisms observed in the limb buds during embryogenesis when skeletal muscles start their formation, suggesting that skeletal muscle fibers that experience denervation-induced atrophy in adult SMA skeletal muscles, could activate the muscle regeneration program which recapitulates the events that take place during embryonic limb skeletal muscle formation.

The presence of dMHC, a commonly used marker for myofiber formation and muscle development [220], [221] and in post-injury regenerating myofibers [222], was also analyzed by immunofluorescence in human skeletal muscle biopsies. A strong dMHC expression was detected exclusively in atrophied fiber areas which demonstrate activation of muscle regeneration processes, in response to the atrophic state of human skeletal muscle fibers in patients with type I SMA. As demonstrated in previous studies [196], dMHC is promptly and highly expressed after 12-24 hours post-injury in mouse skeletal muscle and its expression is detected in areas of regenerating myofibers until it is replaced by the regenerated mature MHC isoforms. Interestingly, atrophied fibers also showed a down-regulation of MYOF gene, which encodes for Myoferlin, a member of the ferlin family. Myoferlin is a type II membrane protein that is structurally similar to dysferlin, and that seems to play a role in calcium-mediated membrane fusion events [223], suggesting that it may be involved in membrane regeneration and repair. It is worth noting that mutations in dysferlin, a protein associated with the plasma membrane, can cause muscle weakness that affects both proximal and distal muscles [224], leading to the suggestion that myoferlin could play a possible role in the muscle weakness and atrophy observed in SMA patients.

The increased expression of myogenic genes in atrophied muscle fibers corroborated by immunofluorescence analysis in type-I SMA human skeletal muscle samples demonstrates that both myogenin and dMHC proteins were exclusively detected in atrophied fiber groups, but not in control vs hypertrophied fibers. This contrast might indicate that skeletal muscle fibers experiencing atrophy probably activate the myogenic program in an attempt to increase muscle mass and strength that is lost after denervation and therefore demonstrate an increased

myogenic regeneration activity in SMA-atrophied fibers. Perhaps the cause could be due to the fact that neuronal signaling activates signaling pathways that inhibit and paralyze the mechanisms of muscle formation and regeneration and when the motor neuron is lost and that signal is no longer active, that inhibition activates those compensatory muscle regeneration mechanisms.

It is also noteworthy that MSTN was not differentially expressed in control vs atrophied, control vs hypertrophied or atrophied vs hypertrophied myofibers (-1,23 fold, +1,3 fold; 1-fold) respectively. Myostatin inhibition has previously been shown to increase muscle mass, with increased amounts and size of myofibers in mice [195] and to lesser extent in humans [225]). Interestingly follistatin, a myostatin-inhibiting protein was strongly downregulated in atrophic fibers (8.74 fold). Increased follistatin levels in SMA mouse models led to increased mass in skeletal muscle, gross motor function improvement and mean lifespan extension, but SMN levels remained unchanged [226]. A case-control study of SMA type I-III patients revealed that reduction of myostatin serum levels among SMA patients correlated with disease-severity, yet follistatin serum levels revealed no significant changes with their age-matched control individuals [227]. The effect of follistatin increase in humans has not extensively been studied, yet it is possible that follistatin may act in a similar SMN-independent manner as in mice [226], [227] and prenatally it might be important in promoting muscle growth and in the induction of myogenesis.

Agrin induces the phosphorylation and activation of MuSK, which ultimately leads to AChR clustering and formation of NMJ's [228]. SMA mice exhibited a reduction in agrin expression levels in skeletal muscle and loss of agrin lead to defects in synapse maintenance and contribute to the overall muscle denervation [229]. Interestingly, an active splice variant of agrin significantly improved motor performance, extended lifespan and positively affected muscle morphology [229]. In our analyzed human skeletal fibers however, AGRN was neither up- nor downregulated, indicating that agrin expression is not directly affected by SMN deficiency.

However, MuSK was also downregulated in atrophied fibers compared to control fibers. MuSK plays a crucial role in the NMJ formation and maintenance between MNs and skeletal muscle [230]. Previous studies have shown that a MuSK agonist effectively enhances NMJ function and muscle morphology in the delta7 SMA mouse model [231]. In human myofibers MuSK likely also plays a crucial role in NMJ maintenance and subsequently preventing denervation already during development in SMA patients.

In summary, we can conclude that SMA atrophied muscle fibers could be activating muscle regeneration mechanisms and recapitulating skeletal muscle formation processes that take place during embryogenesis, in response to denervation caused by the loss of the MN derived from the SMA pathology. Likewise, fibers that remain innervated in these patients experience a

process of hypertrophy to try to compensate for the loss of functionality of denervated and atrophied fibers. This study of fiber populations, isolated from muscles with SMA, provides an excellent approach for the molecular study of the processes that occur during the denervation of human SMA skeletal muscle fibers. The knowledge of the specific response mechanisms of muscle fibers to denervation could provide valuable information for a deep understanding of the pathology and for the development of new therapeutic strategies aimed at the treatment of human SMA disease. Continued research is needed, to clarify optimal protocol parameters and to further understand mechanisms of efficacy.

The first manuscript in the appendix in collaboration with Dr. Mario Marotta from VHIR and Leitat, provides a novel, cell-specific molecular dissection of prenatal SMA type I skeletal muscle fiber pathology, using LCM and genome-wide microarray expression profiling. By independently analyzing atrophied and hypertrophied muscle fibers from SMA patients, this highlights these two divergent phenotypic states and focuses on the muscle's intrinsic response to MN loss and denervation. Importantly, the data obtained underscores the active engagement of regenerative programs in SMA-affected skeletal muscle.

The main finding of this study is the severe contrast in gene expression profiles between SMA-atrophied and SMA-hypertrophied fibers. While hypertrophied fibers display minimal transcriptomic deviation from age-matched controls suggesting a retained degree of innervation and metabolic function, atrophied fibers exhibit extensive transcriptional reprogramming. The over 395 differentially expressed genes between control and atrophied fibers highlight a muscle-intrinsic response to denervation, encompassing both degenerative and regenerative processes.

This transcriptomic divergence supports the hypothesis that SMA pathogenesis in muscle is not uniform but instead fiber-type specific and temporally regulated [250]. Hypertrophied fibers likely represent a subpopulation that remains innervated during early disease stages, compensating for lost contractile function in adjacent denervated fibers through anabolic growth.

Many upregulated genes in atrophied fibers are associated with embryonic myogenesis and regeneration, including MYH8, MYH1, MYL3, MYH7, Myogenin, and dMHC were identified. This indicates that atrophied fibers actively engage in regeneration, perhaps as a compensatory mechanism in response to denervation. This hypothesis is further supported by the expression of Myogenin and dMHC proteins soley in atrophied areas, demonstrated by immunofluorescence (Fig.17).

These findings align with prior studies in both SMA and dystrophic muscle models, where denervation and injury trigger myogenic programs that recapitulate developmental myogenesis [203], [204]. The activation of transcription factors such as PAX3 and Myogenin, and the presence of fetal myosin isoforms, further reinforce the idea that SMA muscle attempts to regenerate via embryonic-like pathways.

This study also reveals downregulation of key genes involved in NMJ maintenance in atrophied fibers, including MUSK and FST, with CHRNA1. These findings suggest a disruption in NMJ signaling, consistent with denervation-induced muscle atrophy. The downregulation of MUSK, a gene essential for NMJ formation and maintenance, is of particular interest as this reinforces the potential for therapeutically targeting NMJ stabilization pathways in SMA patients, considering that upregulation of MuSK agonists have shown efficacy in mouse models [231].

Current SMA therapies primarily aim to restore SMN levels. The differential gene expression in SMA muscle fibers further indicates the importance of SMN-independent pathology in muscle degeneration. Downregulation of DHCR24, UBE2D3, and MYOZ2 in atrophied fibers underscores a broader involvement of oxidative stress response, proteostasis, and cytoskeletal integrity in SMA pathology.

Of particular interest is the finding that a myostatin antagonist FST is significantly downregulated in atrophied fibers. Given the therapeutic potential of myostatin inhibition in SMA mouse models and ongoing clinical trials using anti-myostatin therapies, the reduction in endogenous FST expression in atrophic SMA fibers may make these tissues particularly responsive to such interventions. The inconsistency between mouse and human data here, for example unchanged MSTN expression highlights the importance of human-specific studies in guiding translational research.

The induction of embryonic myosin isoforms and key myogenic regulators in SMA-atrophied fibers suggests partial reactivation. This aligns with assumption that muscle repair processes mimic embryogenesis, as observed in limb muscle formation [219]. The re-expression of the perinatal isoform MYH8, along with other immature myosins and PAX3, indicates that the atrophied muscle environment may strive for regeneration, albeit likely insufficient to fully restore function due to continued MN loss and systemic SMN deficiency.

This study highlights the complexity of postnatal skeletal muscle pathology in type I SMA, demonstrating that hypertrophied fibers maintain near-normal gene expression profiles, whereas atrophied fibers undergo profound transcriptomic remodeling. These findings suggest that early denervation triggers a fetal-like regenerative response in atrophied fibers that is not seen in hypertrophied ones, pointing toward a compensatory interplay between innervated and denervated muscle regions. The insights gained from this fiber-type specific analysis enhance our understanding of SMA pathogenesis beyond the motor neuron and provide a compelling rationale for combining SMN-dependent therapies with interventions that promote muscle regeneration and NMJ maintenance.

MANUSCRIPT II - REVIEW: In utero therapy for Spinal Muscular Atrophy: closer to clinical translation

Eduardo F. Tizzano1,2,&#, Georg Lindner1, Ellie Chilcott3, Richard S. Finkel4&, Rafael J. Yáñez-Muñoz5&

1. Medicine Genetics Group, Vall d'Hebron Research Institute (VHIR), 08035 Barcelona, Spain.
2. Department of Clinical and Molecular Genetics, Hospital Vall d'Hebron, 08035 Barcelona, Spain
3. UCL Institute for Women's Health, 86-96 Chenies Mews, London WC1E 6HX, UK.
4. Center for Experimental Neurotherapeutics, Department of Paediatric Medicine, St. Jude Children's Research Hospital, Memphis, TN, USA
5. AGCTlab.org, Centre of Gene and Cell Therapy, School of Life Sciences & the Environment, Royal Holloway University London, Egham, TW20 0EX Surrey, UK.

&Senior Authors

Corresponding author:

#Eduardo F. Tizzano, MD, PhD, Medicine Genetics, Vall d'Hebron Institut Recerca (VHIR)

Adress: 08035 Barcelona, Spain. Telephone: +34 93274600 ext. 6945.

E-mail: eduardo.tizzano@vhir.org

Present Adress: Neuromuscular Pathology Unit, Fundacion Hospital San Joan de Deu, 08950 Esplugues de Llobregat, Barcelona, Spain.

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Abstract

5q-Spinal muscular atrophy (SMA) has been a trailblazer in the development of advanced therapies for inherited diseases. SMA is an autosomal recessive disorder affecting mainly motor neurons in the anterior horn of the spinal cord and brainstem motor nuclei but currently considered a systemic disease. Advances in understanding of the genetics of SMA led to the development of disease modifying therapies, either transferring a healthy version of *SMN1*, the causative gene absent or altered in SMA, or modulating *SMN2*, a highly homologous but less functional version of *SMN1*, present in all patients. After successful clinical trials, these approaches have resulted in three marketed therapies. Severe SMA, "type I", is the most common type and is considered both a developmental arrest and neurodegenerative disorder. As pathology starts during fetal life in type I patients, a cure is unlikely even when treatment is started shortly after birth in the pre- or mildly symptomatic state. In utero fetal therapy offers the opportunity to mitigate further or possibly prevent manifestations of the disease. This review discusses clinical and developmental aspects of SMA, the advanced therapies approved (gene therapy, antisense oligonucleotide and small molecule compounds), and the rationale, options and challenges, including ethical and safety issues, to initiate in utero therapy. Looking beyond sporadic case reports of prenatal intervention, clinical trials of in utero SMA therapy can be envisaged and should be carefully designed and evaluated to move closer to clinical translation.

Keywords

Spinal muscular atrophy, in-utero therapy, pre-symptomatic, fetus, gene therapy, human development, ethical issues, SMN protein

SMA overview

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder mainly characterized by degeneration and loss of function of alpha motor neurons (MN) in the anterior horn of the spinal cord and brain stem motor nuclei. SMA is the second most common autosomal recessive inherited disorder with a global incidence of approximately 1/11000 [232]. In addition, untreated SMA is the leading cause of death from monogenic disease in infancy. The carrier frequency lies between 1/32 – 1/72 globally [1]. Symptoms in SMA patients derive from an insufficient amount of SMN protein caused by loss-of-function mutations within or deletions of the Survival motor neuron *SMN1* gene. SMN is encoded by two highly similar genes, *SMN1* and *SMN2*, located on *chromosome 5*. A single nucleotide variant (C → T at position c.840) in exon 7 results in the preferential exclusion of exon 7 in ~90% of *SMN2* transcripts, which generates a truncated and unstable protein known as SMNΔ7 [233]. Approximately 10% of functional full-length SMN, identical to that produced by *SMN1*, is produced from each *SMN2* copy, and this can partially compensate for *SMN1* loss and rescue what would otherwise be a lethal disorder. *SMN2* copy number correlates inversely with disease severity [19].

The SMN protein is considered multifunctional and impacts various aspects of RNA metabolism. It is highly associated with the ribonucleoprotein (RNP) complex, regulating biogenesis of small nuclear RNPs. Besides multiple interactions reported [234], SMN has also a role in axonal transport [235], [236], RNA trafficking as well as maintenance of neuromuscular junctions (NMJs) [194]. Despite thorough research, it is not yet well understood why MN's are the most sensitive cells to decreased amounts of SMN.

SMA classification is based on age of onset of symptoms and the highest degree of motor function achieved [237]. The disease can be classified as types 0-IV, with type 0 being the most severe form. It should be noted that regardless of classification, manifestations and symptoms can vary in patients and the distinctions among the types are not absolute. However, the scheme remains relevant in the genetic era and provides useful clinical and prognostic information.

Type 0 SMA is the most severe and rarest of SMA forms [14]. It is associated with prenatal onset of symptoms such as decreased fetal movements. Patients suffer from arthrogryposis, as well as profound hypotonia, bulbar weakness (poor suck and swallow) and respiratory compromise at or soon after birth. Congenital heart defects and skin necrosis are also features of many type 0 cases, indicating systemic pathology from SMN deficiency. Their life expectancy is the lowest of all SMA types, typically with less than one month survival [14]. Type I SMA ("Werdnig-Hoffmann Disease") is the most common form with more than 50% of SMA cases. Disease onset is before six months of age and by definition these individuals fail

to gain independent sitting due to severe muscle weakness. Median survival in these children is less than two years of age, usually due to respiratory muscle dysfunction and respiratory failure [238], [239], [240]. The type II SMA phenotype (intermediate or "Dubowitz" form) presents usually between six and eighteen months of age with developmental arrest after sitting has been achieved, followed by a plateau then a decline in motor skills [241]. While being able to sit upright and sometimes even stand using leg braces, affected children fail to achieve independent walking and are wheelchair-bound. They usually suffer from kyphoscoliosis and if untreated, also from restrictive lung disease. Their cough and airway secretion clearing are progressively compromised. Even though the majority may survive into adulthood, they may require highly supportive management as gastrointestinal and respiratory complications increase [242], [243]. Type III SMA ("Kugelberg-Welander" form) patients are able to walk independently during their early life. They show profound symptom heterogeneity and patients are often misdiagnosed with myopathy or muscular dystrophy. The distribution pattern of muscle weakness is similar to type I and II SMA – with proximal>distal, lower>upper limb predominance - however disease progression is much slower. As muscle weakness progressively increases, particularly of leg muscles, the use of a wheelchair may be required [18]. Type IV SMA is the mildest form of SMA, with symptoms usually arising around the third decade of life. The comorbidities resemble type III SMA, mostly with slowly progressive weakness of lower extremities. People affected have an average lifespan and retain the ability to walk throughout adulthood [18].

It is possible to correlate *SMN2* copy number with clinical characteristics of SMA, whereby copy number increases with decreasing disease severity. This copy-number variation is due to the plastic nature of this region of the genome. Patients with type 0 disease typically have only one *SMN2* copy, most type I cases show two *SMN2* copies, type II generally have three *SMN2* copies whilst long-term walkers usually have four copies [19]. While severity-based classification has clinical advantages, it is not always sufficient to provide prognostic information. However, this inverse correlation is not absolute [19]. Determination of *SMN2* copy number is widely implemented to study SMA patients, provide some level of prognosis, and is frequently used by payers to determine eligibility for SMN-directed therapy. Not all *SMN2* copies are identical, however, and the actual structures and genomic sequences of *SMN2* copies are usually not considered for routine study [244]. In this regard, the detection of positive variants such as c.859G>C and c.835 -44A>G as well as the effect of possible *SMN2-SMN1* hybrids may help to better determine the phenotype [25], [245]. Exploration of the structure and quality of *SMN2* copies and equivalence in different patients may provide clues to the diversity in genotype-phenotype correlation. Combining clinical features and *SMN2* copy number allows for a more precise prognosis. For example, infants with SMA type I and two

copies of *SMN2* have less heterogeneity and follow a more predictable decline in survival (median of 10.5 months) than the overall group of type I patients [239].

Developmental aspects of SMA: a rationale for why and when to treat the disease

Several studies have shown that SMA pathology in the neuromuscular system begins during prenatal development. In the typically developing fetus the neuromuscular system develops from the first interneural connections between motor neurons (MNs) and muscles, formed around 7-8 weeks of gestation and leading to flexion and trunk movement [123]. As neuronal connections are formed during development through axonal outgrowth, increased fetal movement can be seen even from 12 weeks. At around 15-weeks gestation most neuromuscular junctions have been formed, with more specialized gross movement. At around 20-weeks gestation the fetus shows increased bilateral movement [124]. During fetal development, polyneuronal innervation of the muscle is present [125]. Until term, regression from polyneuronal into mononeuronal innervation in muscles occurs, which however differs among various muscles with the diaphragm and intercostal muscles having the fastest regression [126]. Maturation of the motor unit (motor neuron – axon – neuromuscular junction – muscle) continues postnatally and is completed in early childhood.

SMN is ubiquitously produced in all tissues and throughout fetal development and post-natal life. The spatial and temporal requirements for this protein, however, differ throughout development. Motor neurons have the greatest need for SMN protein for proper development [246] and maintenance during fetal and early post-natal life [247], less so in older children and adults. Immunoblot studies for SMN protein production have been performed on skeletal muscle, heart, kidney and brain tissues from postnatal and fetal controls and compared to fetal samples with SMA (predicted to be type I with two *SMN2* copies; mainly at approximately 14 weeks of gestation). It was shown that SMN protein levels were reduced in the SMA samples compared to control tissues, both pre- and postnatally, with the decrease correlating with severity [128].

During the second and third trimester of gestation and the first 3 months after birth, the amount of SMN protein does not increase in SMA spinal cord samples, contrasting with the increase of SMN seen in age-matched controls, remaining 4-fold lower prenatally and 6-fold lower early postnatally [247]. It is likely that these periods of marked reduction in production of SMN protein in SMA during fetal and neonatal stages occur when developing motor neurons are most vulnerable and reliant upon this critical protein. The lack of sufficient SMN during early development appears to be the primary driver of MN dysfunction and death, although it has been observed that the most notable reduction of SMN protein levels happens in SMA skeletal muscle [128], [248]. In control samples the relative amounts of SMN in kidney, brain and heart were similar to skeletal muscle, however the drop in postnatal tissues was not as severe, with

kidney showing the smallest reduction [128]. Older patients with SMA appear to have a lesser need for SMN protein to sustain MNs, as evidenced by a much slower rate of decline in function and by neurophysiological testing. This is also supported by the observations that DMTs appear to have a lesser effect in older children and adults with milder impairment than in infants with more severe disease [124]. Biomarkers of disease activity, for example neurofilament levels in blood, are much higher in newborns (even if pre-symptomatic) and infants than in older individuals with more indolent disease [62], [63], [249].

SMA also affects sensory-motor connectivity in mice and zebrafish models [132], [133]. MN development has a direct influence on the development of dorsal root ganglion (DRG) and Schwann cells [246]. In the severe SMA mouse model, impaired radial growth of motor axons and Schwann cell ensheathment were identified during embryogenesis, hindered neonatal motor axon function and caused fast degeneration of unsheathed axons [246]. Neonatal treatment with *SMN2* splice modifiers increased radial growth of myelinated axons, however in utero treatment was required to restore axonal growth and associated maturation, preventing subsequent neonatal axon degeneration, and enhancing motor axon function [246]. In zebrafish SMN mutants, Schwann cells do not wrap axons tightly and had expanded nodes of Ranvier. DRG neurons showed abnormally short peripheral axons and failure to divide [132]. Increased SMN production in MNs rescued both cell types, highlighting the cell-autonomous effect of SMN, secondary to the developmental MN defects. Thus, these observations in animal models may be relevant for human pre/early postnatal MN network development – both cell-autonomous and non-cell autonomous.

Multiple lines of study have investigated nerve and muscle development and pathological changes in tissues from fetuses with SMA. Abnormal nuclei shape, high nucleoplasm density, an increase in DNA fragmentation and lower choline acetyltransferase (ChAT) levels have been reported in SMA motor neurons from fetuses with two *SMN2* copies, indicating abnormal apoptosis and impaired motor neuron development [134], [248]. Furthermore, axon size was reduced and varicosities could be detected. In neuromuscular junctions, acetylcholine receptor (AChR) disaggregation and increase in presynaptic vesicles could be seen, indicating synaptic defects in SMA development [194]. In skeletal muscle, myotube diameter size was significantly decreased and slow myosin heavy chain (MHC) levels were decreased, while fast MHC production increased, reflecting a possible delay in muscle maturation [137], [194], [250]. In addition, the early studies of muscle histopathology in SMA type I infants identified fetal-like muscle fibers. The combination of all these neuropathologies suggests an element of developmental arrest even before denervation atrophy changes are evident.34 These findings,

summarized in Pérez-García et al. [124] & Tizzano and Zafeiriou [127], point to prenatal effects of SMA on the whole neuromuscular system.

Numerous studies have shown that in humans, the higher the *SMN2* copy number, the more full-length SMN protein is produced and, in general, the milder the associated SMA phenotype. Some variants in *SMN2* have been identified that further alter SMN production, e.g. c.859G>C which increases SMN production and is associated with a milder course of disease [138]. Thus, *SMN2* copy number may serve as a more general prognostic biomarker for disease severity that reflects pathological implications of SMN deficiency (Fig. 18), in addition to motor function impairment. Importantly, there could be a threshold for SMN levels below which the pathology in the SMA fetus emerges and above which the disease process is held in check. Individuals with one copy have cardiac malformation and arthrogryposis, congenital anomalies that are potentially detectable by ultrasound assessment during the prenatal period [14]. Fetuses with one and two *SMN2* copies present with early neuropathological findings [124] with early symptom onset within the first three months of life [239]. On the other hand, fetuses with three and four copies are predicted to produce SMN above the threshold to initiate fetal pathology and instead disease manifests solely in the postnatal period, usually after six months of life in most patients with three copies. There is no evidence currently that demonstrates prenatal motor neuron loss or abnormal axonal/NMJ development at birth in individuals with 3 or more copies of *SMN2*. Indeed, in contrast with fetuses with two *SMN2* copies, fetuses with three *SMN2* copies do not show a detectable alteration in the developmental pattern suggesting a wider therapeutic window to intervene [194]. However, these observations should be taken carefully until more data on neurofilaments, CMAP and *SMN2* variants are available from SMA neonates with three *SMN2* copies. In general, patients with four copies may remain without symptoms for years, but some cases may also present earlier [139].

The regulation of SMN production in different tissues and organs is more complex in humans and species with two *SMN* genes than in rodents and other sub-primate species that have a single copy of the *SMN* gene. In humans, it has been demonstrated that most of the complete messenger RNA and protein in the spinal cord during development originate from *SMN1*, whereas in other tissues such as kidney and muscle the proportion is more balanced between both genes [248] SMN production in the spinal cord is dramatically reduced upon loss of *SMN1*, and *SMN2* is not able to provide a sufficient compensatory source of full-length protein due to the biased expression towards *SMN1* in this tissue. Thus, regulatory mechanisms of *SMN2* expression (including transcript processing) during fetal development should be considered if an early therapeutic intervention such as gene addition therapy is to be administered.

Defining pre-symptomatic SMA

SMA can be identified pre-symptomatically by using prenatal or newborn-specific genetic testing. Most of the cases diagnosed show *SMN1* bi-allelic absence. Rare variants in the *SMN1* gene have been described in SMA patients with compound heterozygous mutations [251], [252]. Non-invasive prenatal screening methods have emerged that use haplotyping of couples at risk [253], [254]. Parents identified as carriers can choose to undergo *in vitro* fertilization and pre-implantation genetic diagnosis, or intrauterine insemination with sperm of a donor who is not an SMA carrier to avoid the risk of SMA [255]. In pregnancies from couples with previous family history the fetus can be screened by chorionic villus sampling (CVS) at 10 to 14 weeks or amniocentesis at 16 to 20 weeks to determine whether SMA is likely. Pregnancy interruption was usually the outcome after diagnosis of SMA, however with the current effective therapies there might be a shift in decision-making towards pregnancy continuation [256]. A confirmed diagnosis of SMA in prenatal screening could create further patient demand for treatment, even prenatally (see below).

Genetic, electrophysiological, and biochemical tests can serve as prognostic biomarkers that predict the future phenotype of groups of individuals with SMA upon diagnosis, both symptomatic and pre-symptomatic [59], [257]. Genetic tests include *SMN1* deletion (for diagnosis) and *SMN2* copy number, which can also be performed in utero from DNA derived from chrorionic villi or an amniocentesis specimen, but in isolation is not fully predictive of the phenotype in an individual patient. For example, two copies of *SMN2* is approximately 79% predictive of a type I phenotype [19]. But, as mentioned, uncommon variants in the *SMN2* gene, such as the c.859G>C and c.835-44A>G predict a milder phenotype. Electrophysiological tests include measurement of the compound motor action potential, motor unit number estimate, and electrical impedance myography, which can serve as predictive, prognostic and pharmacodynamic biomarkers in postnatal individuals, but cannot be performed in utero [257]. Biochemical prognostic and pharmacodynamic biomarkers include SMN protein level (blood or CSF), neurofilament levels (blood or CSF) and creatine kinase levels (blood), and could be tested on a fetal blood sample [258]. In utero sampling of umbilical cord blood could also test for fetal drug level and for safety monitoring, but involves a small but not negligible risk [258] and would need careful medical and ethical consideration in a protocol for prenatal therapy.

In newborn children with an asymptomatic phenotype it is important to assess their disease characteristics if an absence of *SMN1* has been detected by screening. Traits associated with the typical SMA phenotype should be considered, e.g. manifestations in the neuromuscular system (such as hypotonia, decreased movement, reduced/absent tendon reflexes, feeding difficulty) and respiratory system (hypoxemia, hypercapnia, or abnormal breathing pattern). Even without such clinical findings, electrophysiological defects (reduced motor unit number

estimation (MUNE) or compound muscle action potential (CMAP)) [127] or a fluid biomarker (such as an elevated neurofilament level) [254] could indicate loss or non-functional motor neurons undergoing active but rather silent denervation. Collateral sprouting and reinnervation have been identified in symptomatic patients with SMA and may be present during fetal development [259], [260], [261]. We propose that this mechanism, in addition to polyneuronal innervation, could compensate for motor neuron loss or dysfunctional neurons during this clinically silent period. As newborn screening for SMA is implemented more widely, data collection from structured patient registries will allow a thorough assessment of asymptomatic SMA and its baseline characteristics [262], [263]. Eventually, by combining clinical and biomarker assessments, it may be possible to predict whether a neonate is truly asymptomatic or pre-symptomatic and without evidence of silent burden of disease (see below).

There is growing evidence that some babies identified shortly after birth with genetically confirmed SMA and two copies of *SMN2* may not be completely asymptomatic in the first weeks of life. Indeed, early areflexia may be present as well as hypoxemia or hypercapnia, before further signs such as hypotonia, diaphragmatic breathing and tongue fasciculations appear [240], [264]. Results of a pilot trial of newborn screening in Italy show that ~40% of such newborns may already have manifestations at first assessment, before treatment is initiated [265]. Indeed, prodromal symptoms should be investigated and properly documented in all neonates with genetically confirmed SMA. It is possible that biomarkers such as CMAP/MUNE, neurofilament proteins [257], [266] and *SMN2* variants [245] may inform on clinically inapparent disease progression when a neonate with SMA is detected by newborn screening. This information may be very important to initiate early treatment of newborns in SMA. Finkel and Benatar [267] proposed a classification of infants with SMA identified by newborn screening, recognizing an initial clinically silent stage followed by a pauci-symptomatic phase with a later phenoconversion in which the patient becomes truly symptomatic (summarized and further elaborated in Fig. 19).

Brief Overview of SMA therapeutics

Improvements in our understanding of the underlying defects in SMA remain critical for the development of therapeutic approaches. It is now appreciated that SMA is more than a disease of motor neurons. The model proposed by Mentis [133] includes sensory neurons and interneurons of the motor unit circuit. This is consistent with the observation that individuals with SMA type 0 have impaired sensory nerves and proprioceptive sensory neurons in the dorsal root ganglia (DRG) and may be vulnerable to overexpression of SMN [268]. Considerable evidence has emerged that SMN deficiency can create pathological changes and inflammation in neurons, but also glial cells throughout the CNS, as well as peripheral organs

and non-neuronal cells [134], [140], [194], [250] Non-cell autonomous pathology is an increasingly studied topic, the clinical relevance of which is still not fully understood [269]. Therapies for SMA, which can be categorized into SMN-dependent and SMN-independent, vary in their proficiency to target different tissues. The absence or alteration of *SMN1* can be compensated to some extent by SMN protein produced from existing *SMN2* copies, which therefore provides a target for therapeutic approaches. SMN-dependent therapeutic approaches have demonstrated efficacy in several types of SMA, particularly in type I SMA patients, with impressive motor achievements. In other SMA types the outcome of treatment is often stability and non-progression of motor dysfunction, which is very much appreciated by those affected [270]. Other promising treatment approaches, focused on non-SMN-targets in tissues outside the CNS, are currently in clinical trials.

The first marketed therapeutic for SMA was nusinersen (Spinraza®), an antisense oligonucleotide (ASO) which specifically inhibits the ISS-N1 motif in intron 7 of *SMN2* mRNA to promote exon 7 inclusion and hence increased production of full-length SMN protein. Preclinical studies indicated that nusinersen had to be injected very early in neonatal development for maximal efficacy. Nusinersen is unable to penetrate the blood-brain barrier (BBB) and therefore periodic intrathecal injections need to be performed. Such treatment does not address non-CNS aspects of SMA pathology, as leakage of the ASO outside the CNS is very scarce. Promotion of exon 7 inclusion in *SMN2* transcripts can also be achieved with an oral compound, risdiplam (Evrysdi®), recently approved for marketing. Risdiplam has the potential benefit of systemic delivery of drug to non-neuronal organs that may benefit from an increase in SMN protein production, e.g. muscle.

An alternative to *SMN2* upregulation is *SMN1* gene replacement, implemented in the clinic by using onasemnogene abeparvovec (OA; Zolgensma®), an adeno-associated virus serotype 9 (AAV9) vector driven by the ubiquitous and stable CBA promotor, in a single intravenous infusion. AAV9 is able to cross the blood-brain barrier during early development, allowing transgene delivery to both peripheral tissues and the CNS, where it shows an avidity for neurons. Given that SMN is ubiquitously produced, this is expected to provide additional benefit. Concerns about long-term efficacy, safety, durability of effect, and toxicity are still present [22], [271].

There currently is only limited data on treatment of premature babies. A case report on a 30 week-old infant (2 *SMN2* copies), who was treated with Nusinersen as a bridge-therapy until appropriate use of OA, demonstrates feasibility of premature therapy-delivery [272]. A german study also reported successful Risdiplam implementation as a bridge before OA therapy in premature infants [273]. Another case of 30 week-old twins (1 *SMN2* copy) reported favorable

outcome of OA delivery enabled by premature delivery [274]. It is worth to note that in the last case, the single *SMN2* copy had a positive modifier c. 835-44A>G, that should have influenced the phenotype.

Combination therapy has been explored in clinical trials (Table 10) and clinical practice and it is anticipated that there will be further scope for it as more information becomes available [143]. Possible ways in which this could be attempted are outlined in Fig. 20. A proposed classification for different therapeutic combinatorial approaches has been recently published, including switch therapies, bridge therapy, added on or combined [275].

There is published evidence of combined SMN-dependent therapies in SMA. A retrospective report on dual treatment of five children with type I SMA who received nusinersen and onasemnogene abeparvovec-xioi resulted in overall improvement with no adverse effects, showing that combination therapy was tolerated in these patients [98]. Seven SMA type I patients who received both treatments (mostly nusinersen before onasemnogene abeparvovec-xioi) were assessed for motor function trajectories, ventilation hours and cough assist sessions. The second therapy improved ventilation, but there was no improvement in motor function trajectory compared to single therapy, pointing to the importance of early treatment [99]. A report of four cases of type I SMA initially treated with onasemnogene abeparvovec (one of them also with nusinersen) who later received risdiplam treatment showed increased therapeutic benefits from the combination therapy, with no significant adverse effects [100]. The multinational RESTORE registry captures 15-year real-world data on patients treated with the three DMTs, focusing mainly on OA [276]. The majority of these patients are treated with a splicing modifier prior to receiving OA, or the former is added on after OA administration. Informative safety and efficacy data are expected from this registry. The SMArtCARE registry in Germany, Austria and Switzerland will serve a similar function [263].

While the vast majority of DMTs are indicated in patients with 2 or 3 *SMN2* copies, treatments of 1 and 4-copy individuals have also been reported, although they are often excluded by payer coverage policies [101], [102]. One patient with SMA type 0 was treated with both nusinersen and onasemnogene abeparvovec and although modest motor improvements were achieved, with continued motor gain at age 13 months without regression of function, she remained profoundly weak with continued systemic complications from SMA including chronic respiratory failure, dysphagia, congenital heart malformation, digit necrosis, and diffuse macular rash [101]. Another patient with one copy of *SMN2* received early treatment with nusinersen at the age of 13 days and although mild motor improvement 2 months after treatment as well as minimal respiratory enhancement were achieved, tracheostomy at the age of 4 months

was still required with increasing cardiac and autonomic dysfunction, ultimately leading to exitus at 5 months [102].

Given that present SMN-dependent therapies are not able to provide a full cure, therapeutics that target SMN-independent pathways are also under investigation. While neuroprotective agents have shown no clear and convincing results in clinical trials [84]. it has been demonstrated in animal models that MNs in SMA show loss of excitatory input from primary sensory afferents and intermediate neurons, pointing to a neural circuit dysfunction [133]. A possible therapy is under investigation with the implant of epidural electrodes targeting sensory axons over the lumbosacral spinal cord (NCT05430113) [277]. On inhibitors have become promising. Myostatin is a hormone that inhibits skeletal muscle growth. Serum myostatin levels are reduced in patients with SMA and may serve as an informative biomarker for progression of disease and response to an intervention [227]. Three clinical trials are currently in progress to examine the possible added benefit from drugs that inhibits myostatin (Table 10).

General perspective of in utero (gene) therapy

Advancements in maternal fetal medicine and gene sequencing have greatly improved early detection and accurate diagnosis of genetic disorders during pregnancy, paving the way for a new therapeutic field, in utero molecular and cellular therapeutics. There are currently several approaches to in utero therapy with the potential to treat single gene mutations, such as blood transfusion, enzyme replacement therapy (ERT), and stem cell transplantation, offering the opportunity to intervene before irreversible disease onset. For anatomical conditions detected prenatally, fetal surgery can have real impact to improve outcomes [278]: open spina bifida disorders and congenital diaphragmatic hernia have been treated successfully [279].

Anecdotal evidence of treatment during pregnancy with biologics for maternal atopic diseases seems encouraging in terms of lack of detrimental effects on maternal and fetal outcomes [280]. On-going clinical trials of fetal treatment with protein therapy (enzyme replacement for lysosomal diseases, recombinant ectodysplasin protein receptor–binding domain for X-linked hypohidrotic ectodermal dysplasia) and cell therapy (mesenchymal stem cells for osteogenesis imperfecta (BoostB4), maternal bone marrow hematopoietic stem cells for alpha thalassaemia major) will be highly relevant for clinical translation of any in utero therapy for SMA [281], [282]. The PEARL clinical trial (NCT04532047) aims to treat lysosomal storage diseases which already have FDA-approved postnatal treatments, with in utero intervention. Eight diseases are included in this trial, including Mucopolysaccharidosis types 1, 2, 4a, 6, and 7, as well as Infantile-onset Pompe disease, Neuronopathic Gaucher disease and Lysosomal Acid Lipase deficiency.

Recently, in utero ERT for a fetus with Pompe disease has been reported. Six umbilical cord injections at 2-week intervals were administered between 24 weeks 5 days gestation through to 36 weeks 5 days gestation. The benefit of prenatal therapy was reportedly superior in this patient in comparison with treatment after birth in their siblings. The child did not develop cardiac hypertrophy, had normal creatine kinase levels, and no evidence of glycogen buildup in the placenta, suggesting that fetal pathophysiology had been prevented by the prenatal therapeutic approach [283].

Successful in utero protein treatment for X-linked hipohydrotic dysplasia has been reported for six patients [284], [285]. A recombinant form of ectodysplasin A1 (EDA1), Fc-EDA, that includes the receptor-binding domain, was administered at gestational week 26 and later by injection in the amniotic fluid. Treated children demonstrate sweat gland development and pilocarpine-inducible sweating and have not had hyperthermic episodes during the summer nor any significant eye, nose, throat, or respiratory issues. They also have more permanent teeth than untreated siblings, even though oligodontia is not fully corrected. The follow-up has reached six years for the first children treated and shows possible dose-dependency. These results are in stark contrast with postnatal treatment with the same protein, which did not lead to patient improvements. To confirm and extend these prenatal treatment data, the Phase 2 clinical trial EDELIFE has been initiated (NCT04980638) [286]

Furthermore, progress in gene therapy has been steady. Currently over 700 active gene therapy investigational drug applications have been reported (https://www.nhlbi.nih.gov/health-topics/genetic-therapies). While postnatal applications of gene therapy are well developed, in utero gene therapy (IUGT), the delivery of relevant genes to the developing fetus to correct genetic defects is yet to be applied clinically [287]. Prevention of disease onset, immune tolerance to the transgene product due to an immature immune system, increased vector biodistribution due to small body size and the possibility of transducing stem cells leading to permanent genetic correction are all potential beneficial features of this treatment modality [288], [289]. However, proposals for the clinical application of in utero therapy in humans must be firmly rooted on established experience of therapeutic delivery to relevant animal models, including traditional drugs, gene therapies and cell therapies. Animal work should extend to large animals, with gestational age when administered, size, physiology, immune responses and delivery challenges more akin to humans. Risks to the developing fetus and the mother must be thoroughly assessed, as well as germ-line transmission. Both gene therapy and genome editing should be considered as therapeutic options. An initial path towards clinical application has been recently outlined [282].

IUGT has been explored in a variety of animal models, including mice, guinea pigs, sheep, pigs and macaques. Diseases under study have included both inherited and idiotypic disorders: X-Linked hypohidrotic ectodermal dysplasia [290], hereditary tyrosinemia type I [290], [291], thalassaemia [292], [293], haemophilia [294], [295], [296], Gaucher disease [297], Angelman syndrome [298] and fetal growth restriction [299], [300], [301].

As an example, neuropathic Gaucher disease (nGD) is characterized by a mutation in the GBA gene, which leads to glucocerebrosidase enzyme deficiency. The lethal type II form affects newborn children, beginning with prenatal pathology and symptoms starting to develop around three to six months of age, with no currently available therapy. A study in nGD mice using AAV9 vector to deliver the GBA gene, resulted in increased expression of neuronal glucocerebrosidase which eliminated neurodegeneration and led to a high decrease in neuroinflammation and improved survival rate of mice [302]. Importantly, prenatal administration was more effective at improving the phenotype than neonatal delivery. In search for less invasive ways of treating genetic disorders before birth, scientists working in mice with Angelman syndrome, have found that delivering ASO to the fetal brain through amniotic fluid is as effective as delivering it via cerebrospinal fluid [297].

Currently, one of the biggest limitations of postnatal gene therapy is the natural immune response of the patient to the viral vector and/or the transgene product. A significant benefit of IUGT is the tolerogenic immune system of the fetus, but pre-existing maternal antibodies crossing the placenta may limit IUGT. However, this can be avoided by either conducting maternal antibody screening or by carrying out IUGT at the beginning of the second trimester of pregnancy, before significant maternal antibody transfer [303]. This could for instance be of benefit to avoid anti-AAV antibodies, which have been detected in newborns [304]. Hemophilia B can be complicated by the presence of antibodies to human factor IX (hF.IX). A study in which IUGT was carried out using intramuscular injection of AAV1-hF.IX vector in hemophilia B fetal mice and subsequent postnatal challenge, showed preserved expression of hF.IX with absence of hF.IX antibodies. In comparison, mice treated with AAV postnatally and receiving the challenge developed hF.IX antibodies, therefore showing the advantage of preempting the immune response [305]. In a more recent study IUGT was used to achieve immune tolerance to foreign protein using AAV-GFP vectors in fetal sheep [306]. The treated sheep developed postnatal immune tolerance to GFP, however such tolerance did not extend to AAV vector capsid proteins, with serotype-specific neutralizing antibodies being detected. Durable hepatic transgene expression was also serotype-dependent. Another study also used AAV9-GFP vector delivery in fetal sheep, whereby widespread distribution of vector genomes was present in all

tissues harvested at term. Transduction in maternal ewes and also germline transduction were observed [307].

IUGT may also enhance the chance of transducing stem and progenitor cells as their presence is increased in a multitude of developing fetal organs. To maximize efficiency, vector serotype and vector delivery route should be extensively explored. In a study on BALB/c mice fetuses at 14-15 days gestation, lentiviral vectors with different pseudotypes were used. The comparison of intramuscular and intrahepatic injection with the various vectors showed different patterns of expression of the marker gene.112 Intrahepatic injection mostly led to marker gene expression in liver and heart, while most organs were negative, however distinct positive cells could be seen in the lung and muscles near the injection site. Intramuscular injection led to primary expression in the targeted muscle groups and the heart, with isolated cells being visible in the liver [308].

While fetal gene therapy offers opportunities for actual prevention of genetic disorders, there are potential risks involved. First, gene therapy might disrupt normal fetal development as prenatal production of a particular transgenic protein might have unknown side effects, even if postnatal therapeutic function has been established [309]. Furthermore, gene therapy might increase the risk of unintentional germline transfer, even if animal studies to date have not demonstrated significant germline effects [306]. Nonetheless, if prenatal gene therapy is carried out after the 7th week of gestation, when germ cells are already compartmentalized, this risk may be reduced [309] although not discarded. Gene therapy can also cause insertional mutagenesis, observed for instance with an early, non-self-inactivating gamma-retroviral vector used to treat SCID-X1 immune deficiency, in which 5 of 21 children developed malignant tumors [310]. Insertion of the vector into or near active tumor-promoting genes caused transcriptional activation and oncogenesis [310]. If those specific genes are particularly active during fetal development, there may be enhanced risk of insertional mutagenesis from prenatal vector delivery.

Feasibility of IUT in SMA

Despite the current licensed and pipeline therapies, no strategies address the genesis of SMA in utero. It is recognized that the translation of fetal gene therapy to humans is challenging, but the technology is becoming more common and in utero gene therapy remains a very promising avenue for the treatment of genetic diseases arising during gestation. This statement is supported by the conclusions of the International Fetal Transplantation and Immunology Society (IFeTIS)'s recent panel discussion regarding the scientific, clinical and ethical issues related to prenatal gene therapy [311]. Pre-symptomatic delivery of treatment may prevent

development of the SMA phenotype and the irreversible damage that accompanies this, perhaps due to the deficiency being corrected during the period of motor unit maturation [312]. In utero treatment could lessen disease symptoms in the early years of life, slow disease progression, or perhaps even prevent disease onset. Combining prenatal diagnostic screening for SMA with in utero delivery could have the potential to reduce the severity and number of SMA cases presenting to the clinic, thus reducing clinical burden. In light of this, in utero delivery is an attractive opportunity to prevent development of symptoms and potentially allow healthy offspring to be born. The cases of SMA that initially may be considered to treat in utero are those with few *SMN2* copies (one or two) and with previous family history of severe SMA [313].

Mice are a common model used for prenatal vector delivery due to ease of use and handling and the presence of between 6-12 fetuses per pregnancy, each surrounded by their own interior gestation sac allowing each fetus to receive different vector injections. ASO administration by intra-embryonic injection in mice can lead to effective distribution and improved phenotypical functions in pups. Transuterine microinjection of a metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)-targeted ASO, resulted in significantly reduced MALAT1 RNA in multiple tissues of neonatal mice which persisted throughout 30 days after birth [314]. Transuterine injection of a splice-switching ASO into the amniotic cavity immediately surrounding the embryo at E13.5 partially rescued hearing in juvenile Usher syndrome type Ic (Ush1c) mouse mutants, while direct injection into the E12.5 inner ear led to improved therapeutic outcomes, which were sustained well into adulthood, showing that in utero ASO administrations can preemptively correct disease phenotype [315]. Such studies could pave the way for in utero administration of nusinersen ASO. Intravenous administration of nusinersen may be feasible in the fetus, similar to enzyme replacement therapy, as the porous blood-brain barrier in the fetus may allow penetration into the CNS. In addition, the *SMN2* modulator risdiplam could potentially be administered orally to the mother with the aim of crossing the placenta, although this may only achieve low level correction. While administration in the last months of gestation could be possible, it should be stressed that labels for all currently marketed treatments note a lack of data and warn about their use in pregnant women. Experience of successful treatment of cystic fibrosis in utero with modulators has been reported [316]. A fetus with ultrasound findings suggestive of meconium ileus was diagnosed with CF by amniocentesis at 26 weeks and oral maternal therapy with modulators was initiated at 31 weeks. No dilated bowel was observed in the fetus at 39 weeks and no signs of bowel obstruction after birth. Maternal treatment was continued during breastfeeding [316].

These results encouraged application of DMT's to fetuses with SMA. Indeed, preliminary successful results of a single case of prenatal treatment with risdiplam to a fetus with 2 copies

of *SMN2* expected to develop severe SMA has been recently reported [317]. Drug was administered to the mother during the last six weeks of the pregnancy. Pharmacokinetic and pharmacodynamic data supported target engagement and a favorable effect on motor neuron development. A second case of prenatal treatment with risdiplam has occurred in Europe as a compassionate use effort. Results from that experience have not yet been released.
While administration in the last months of gestation could be possible, it should be stressed that labels for all currently marketed treatments note a lack of data and warn about their use in pregnant women. Furthermore, the postnatal observed adverse effects of the type of therapy administered (e.g. hepatotoxicity, off-target effects) should also be considered when treating a fetus with SMA [318]. Idiosyncratic liver injury is common during pregnancy, therefore SMA fetal liver may be more prone to hepatotoxicity or other adverse effects [319].

Integration-deficient lentiviral vector (IDLV) transduction of the spinal cord in rodents *in vivo* [320] and in utero IDLV delivery [321] have been previously described. We have also reported in utero technology with IDLVs, of potential application in SMA. Intraspinal injection of IDLV expressing eGFP at embryonic day 16 (E16) into CD1 wild-type mouse embryos led to complete transduction of the spinal cord at all levels, with eGFP expression sustained for at least seven months post administration. ChAT+ MNs showed 100% transduction efficiency. IDLVs were more efficient at transducing MNs after *in vivo* injection than AAV vectors [322]. More recently, a laboratory has published the first use of IUGT in SMA mice to investigate the efficacy of fetal gene therapy for this disease [302]. This study compared intracerebroventricular (ICV) and intraplacental routes of administration, as well as both single-stranded (ss) and self-complementary (sc) AAV9-*SMN1* vectors, concluding that ICV injection of scAAV9 vectors was optimal. SMNΔ7 mice fetuses injected in this manner at E15 resulted in 43.8% full-term gestation births, with these pups going on to survive for a median of 105 days, compared to 12 days in untreated pups [302]. Atrophied muscle present in untreated SMA mice was rescued in IUGT-treated animals, which also showed increased numbers of MNs in spinal cord sections [302]. This study highlighted three issues of concern. First, supraphysiological levels of SMN protein were found in CNS samples (but not in muscle). This may need to be addressed, as it appears that overproduction of SMN protein from AAV9 may have long-term toxic neuronal effects in an SMA mouse model [133]. Second, low survival to full gestation was observed, with the authors citing a possible inflammatory response to the AAV capsid as a factor. It is tempting to speculate that vector engineering could rectify these issues, but no studies have been completed to address this. Third, the rescued mice still had a shorter median survival, about half of that in wild-type mice. While this study provided proof of principle that IUGT for SMA may be a viable option and should be explored further, a comparison between the phenotypic benefits of in utero and postnatal gene therapy for SMA

should be thoroughly evaluated. Another study investigated in utero viral delivery in the domestic sow, showing AAV9-related fetal rejection, which suggests that in this model preclinical in utero AAV9 gene therapy studies may not be successful [323]. Combinatorial IUT in SMA remains also a topic of future experimental investigation, when more data is available from treating postnatal SMA patients under the afore mentioned circumstances.

Ethical issues

The goal of fetal therapy is not only to improve outcomes for the fetus but also to minimize risks and complications for the mother during pregnancy. A summary of possible ethical risks involved in IUT is provided in Table 11. Complications of fetal therapy can include fetal loss, preterm birth, infection, and maternal immune response or the need for maternal-fetal surgery [282].

There are multiple levels of risk which need to be addressed when IUT is considered. The presence of two patients –mother and fetus – requires special precautions to be included in the protocol. Diagnostic and therapeutic monitoring of the fetus may involve added risk, e.g. percutaneous umbilical cord blood sampling (PUBS) [258]. The mother's health also needs to be carefully monitored for adverse events. In utero therapy, therefore, should be conducted in a center with expertise in the care of the mother and fetus, to include maternal-fetal medicine and neonatologists, where such monitoring can be performed and adverse events addressed promptly. From the three gene-targeted medications approved for post-natal treatment of individuals with SMA, nusinersen is currently administered via lumbar puncture into the CSF and would be a challenge and risk to perform in the fetus. Other means of delivery of an ASO to the fetus are being considered. Risdiplam can be administered to the mother orally in a safe dosage, as recommended for adult patients with SMA. It is known that this drug crosses the placenta in animals and has been demonstrated in one prenatal therapy case [317]. OA carries risk of adverse immune response to capsid proteins or the transgene, which may be tempered in the relatively tolerant fetus but not necessarily in the mother [278]. In addition, animal studies have demonstrated concerns over gene integration into germ cells of the fetus [306] and even fetal loss [324].

In regard to maternal-fetal surgical interventions, which have become relatively common in prenatal care, particularly in neural tube defects or diaphragmatic hernia, there might be uncertainty in predicting the seriousness of disease severity and its consequences; this makes discussions around treatment choices difficult and complex [325]. Gene therapy and genome editing carry the additional risk of the therapeutic vector crossing the placental barrier and causing maternal genotoxic damage or an immune response, or maternal genome editing. Potential risks to the mother from gene therapy thus include insertional mutagenesis, which should be explored in animal models. Studies in mice have shown no evidence of maternal

genome editing after in utero CRISPR nuclease delivery [290], [326]. Germline modification following genetic therapy to the fetus would be an additional risk to future generations, and it would have to be assessed in suitable animal models.

Maternal-fetal surgery, gene therapy or pharmacological intervention can save the life of a fetus that would otherwise die but may result in a child with severe disabilities. One could argue against investing medical and financial resources for sub-optimal treatment. IUT can also raise ethical issues regarding the role of healthcare professionals in relation to maternal decision-making and the fetus' life. In a case of prenatal diagnosis of myelomeningocele, the parents decided to refuse resuscitation measures of a 25-week fetus if emergency delivery was necessary during the open uterine procedure [327]. The pressure to intervene, for instance in the case of SMA, could be increased by the rapid evolution of disease. If IUT were to be carried out in SMA fetuses, it would be imperative for healthcare professionals to carefully navigate this delicate environment by engaging in meaningful discussions with parents, weighing the potential risks and benefits of various treatment options, respecting parental autonomy while safeguarding the best interests of the child and clinical obligations [328]. Prenatal decision-making is therefore complex, emphasizing the need for early engagement and counseling, and it is crucial that informed decisions be made in a supportive environment. In the US, the FDA has special requirements for investigational testing of drugs in a pregnant woman, whether directed to the mother or the fetus as referred in FDA 45 CFR 46: "subpart B – additional protections for pregnant women, human fetuses and neonates involved in research" (https://www.ecfr.gov/current/title-45/subtitle-A/subchapter-A/part-46). The EMA also has a policy for investigational drugs in pregnant women. In all cases, surveillance should follow and efficacy and safety information from studies with predefined outcomes should be collected. Adverse outcome data of fetal exposure comprise both structural malformations that may be detected at birth or in early childhood, and non-structural or long-term functional effects that can be potentially important but also difficult to detect or define (https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/guideline-exposure-medicinal-products-during-pregnancy-need-post-authorisation-data_en.pdf). Most of these guidelines were developed in relation to classic pharmacological compounds. However, the recent booming of advanced therapies has created a thin red line between experimental research success and the possibility of application in a translational way to clinical use [329].

Importantly, genetic therapies are the most expensive drugs currently available. Access to them can be restricted by reimbursement agreements between manufacturers and insurance companies or national health services. In addition to the cost of the drug, it is likely that fetal

interventions will carry a significant price tag from clinical services. Access to this medical care could thus be particularly constrained financially in the case of fetal therapies.

The clinical and therapeutic experience in SMA so far is that the earlier intervention occurs, the better the outcome for the patient. However, there are still shortcomings of present therapies, particularly in severe SMA patients. Neurodevelopmental problems are currently observed in a proportion of treated patients with two *SMN2* copies that need further investigation [330]. Parents are demanding information about long-term effects and prognosis of the disease when treatment is offered. Facing a pregnancy with SMA diagnosis (either by previous family history or prenatal screening) for a fetus with one or two *SMN2* copies, parents may request information about premature delivery to initiate postnatal therapy as early as possible [274] or even request the alternative of initiating IUT [317]. This represents a challenge for physicians in communicating the diagnosis and shared decision of treatment [328].

Despite the risks and ethical concerns, women still express interest in enrolling in clinical trials for fetal gene therapy to achieve a more favorable pregnancy outcome [331], with Schwab et al. [332] reporting favorable attitudes to fetal enzyme replacement therapy for lysosomal storage diseases and SMA gene therapies (ASO, small molecule and AAV). Nonetheless, multidisciplinary approach, prenatal counseling and thorough informed consent will be critical for successful implementation of IUT.

Conclusions

There have been impressive advances in SMA therapy during the last decades, especially after the initiation of the investigational clinical trials in 2011-2012. However, none of the three currently approved therapies offer a complete cure. Early detection by newborn screening followed by pre-symptomatic treatment ameliorates manifestations and delays the initiation of disease in most treated babies. Given that SMA with one or two *SMN2* copies presents with low SMN levels and pathology during prenatal stages, in utero therapy for SMA should be explored as a possible alternative to optimize response to a gene-directed treatment. Therefore, the development of pre-clinical investigations and well-designed clinical trials of IUT in SMA (balancing risk, benefit and ethical issues) should be regarded as an opportunity to approach treatment of the disease as early as possible, maximizing therapeutic benefit.

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Conflict of Interest:

EFT received personal compensation for consultancy from Novartis Gene Therapies, Inc., Biogen, Biologix, Cytokinetics, Novartis, and Roche, and research funding from Biogen/Ionis and Roche.

RSF has received personal compensation for advisory board/data safety monitoring board participation from Novartis Gene Therapies, Inc., Biogen, Catabasis, Capricor, ReveraGen, Roche, and Scholar Rock; editorial fees from Elsevier for co-editing a neurology textbook; license fees from the Children's Hospital of Philadelphia; research funding from Novartis Gene Therapies, Inc., Biogen, Capricor, Catabasis, ReveraGen, Roche, and Scholar Rock; and received personal compensation for serving as a speaker for a workshop with the National Academy of Sciences. EC and RJY-M have filed a patent application for a novel sequence-optimized *SMN1* transgene. GL has no conflicts of interest to disclose.

Figures

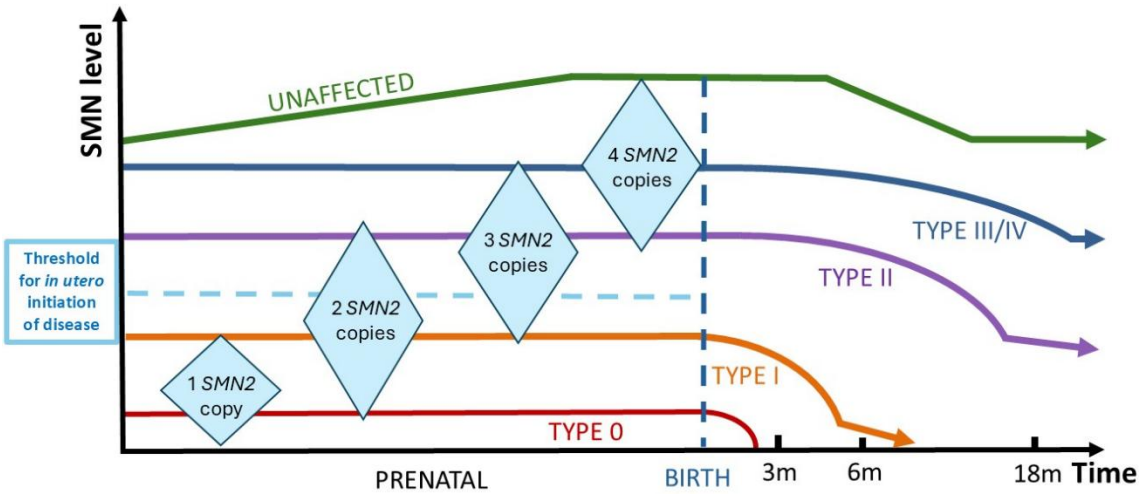

Figure 18: Possible threshold for development of prenatal SMA neuropathology and extraneuronal manifestations. The amount of SMN protein present in SMA correlates with *SMN2* copy number, a key factor in disease severity. Generic SMN levels are presented in two ways in SMA: as straight lines for average levels over time; and as diamonds to account for person to person variation which in some cases, mainly with two or three *SMN2* copies, may overlap considerably. SMN requirements are highest in the 3rd trimester of fetal development and approximately the first 3 months after birth, then reduce (see green curve, based on Pérez-García et al. **[124]** and Ramos et al. **[247]**. Manifestations in individuals with SMA appear depending on the motor neuron pool, generally inversely related to *SMN2* copy number. Cases with one *SMN2* copy usually develop type 0 SMA having cardiac malformation and arthrogryposis, anomalies potentially detectable during the prenatal period. Other problems such as severe hypotonia, weakness, respiratory insufficiency and vascular issues are noted at birth as part of congenital SMA. In fetuses with two *SMN2* copies, predicted to have type I disease, it has been demonstrated that pathology is present in the prenatal period although the consequences usually manifest after birth (<3m). Fetuses with three and four copies may produce SMN above the threshold for prenatal pathology (horizontal dotted blue line) and thus disease may develop and manifest just in the postnatal period, usually after several months of life (>6m in type II, >18m in type III disease). Patients with four copies may remain without symptoms for several months or years. Predicted prenatal levels of SMN are represented according to *SMN2* copy number [19]. Levels after birth represent the evolution according to natural history **[239], [240]**.[m=months]

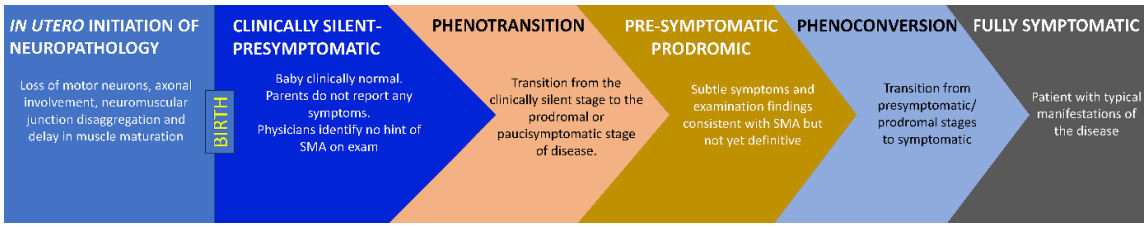

Figure 19: Evolution of clinical stages in SMA babies with two *SMN2* copies from fetal stage to postnatal period. Most fetuses with SMA and two copies of *SMN2* are predicted to have severe type I disease with prenatal initiation of neuropathology. In cases detected by newborn screening the three main stages observable are clinically silent – presymptomatic, prodromic and fully symptomatic. The disease evolution between stages occurs by phenotransition in the first case and phenoconversion in the second. In patients with three *SMN2* copies the clinically

silent phase varies but is typically at >3 months in duration and may be extended over years in four copy individuals. Based on Finkel and Benatar [267] & Tizzano and Zafeiriou [127].

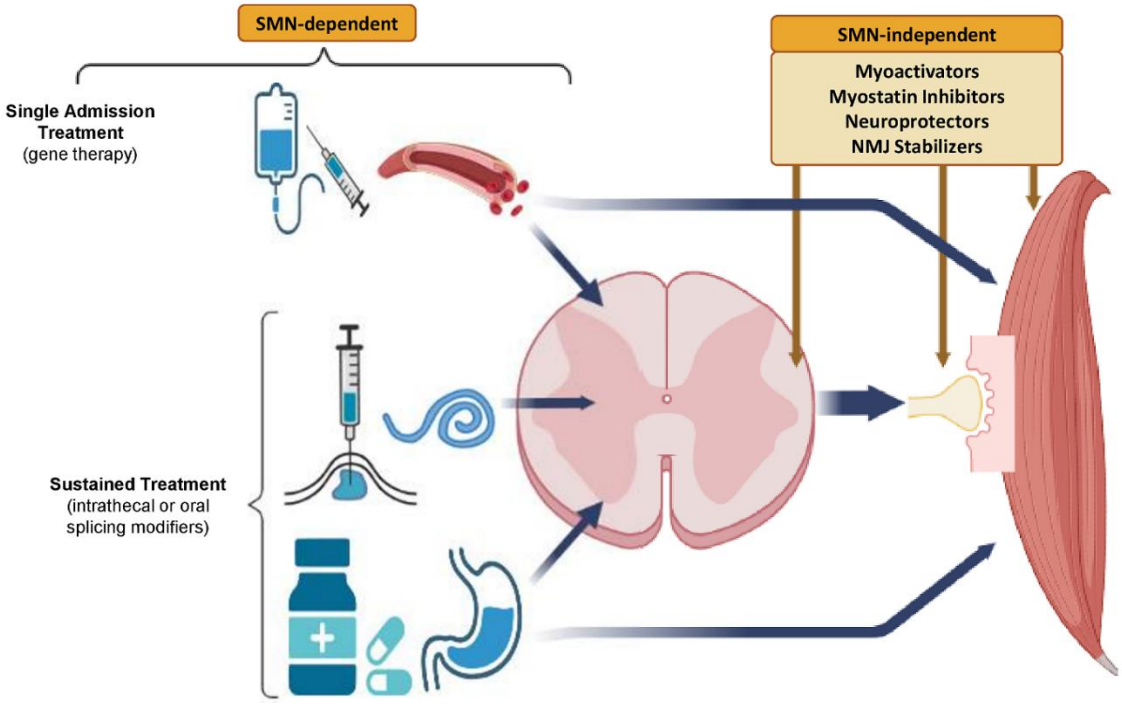

Figure 20: Various approaches to treat SMA (SMN-dependent and SMN-independent) could be complementary. Data from clinical trials and clinical practice will be used to assess whether a multi-pronged approach results in beneficial effects. (see Table 10: Clinical trials in which combination therapies are currently investigated in SMA). At present combination-therapy has yet to be experimentally investigated in the prenatal stage in terms of clinical research. Created in BioRender. Lindner, G. (2025) https://BioRender.com/o5kmjq7

Tables

Clinical Trial (NTC number)	Trial phase	Baseline therapy	Intervention	Mechanism of action	Patient Criteria
RESPOND (NCT04488133)	Phase 4	onasemnogene abeparvovec	nusinersen	*SMN1* gene transfer + ASO to modulate *SMN2*	2 months - 36months
ASCEND (NCT05067790)	Phase 3b	Risdiplam (for at least 6months)	High-dose nusinersen	oral *SMN2* modifier + ASO	15 years - 50 years
TOPAZ (NCT03921528)	Phase 2	Nusinersen treatment initiated in different cohorts with different ages	Apitegromab (IV) every 4 weeks	ASO + anti-promyostatin antibody	>2 years
SAPPHIRE (NCT05156320)	Phase 3	Nusinersen or risdiplam	Apitegromab (IV) every 4 weeks for 1 year	ASO or oral *SMN2* modifier + anti-promyostatin antibody	2 years - 21 years
ONYX (NCT05626855)	Phase 3 (Open-label extension)	Nusinersen or risdiplam	Apitegromab (IV) every 4 weeks for 2 years	ASO or oral *SMN2* modifier + anti-promyostatin antibody	>2 years + Completion of TOPAZ or SAPPHIRE
JEWELFISH (NCT03032172)	Phase 2	RG7800, olesoxime, nusinersen or onasemnogene abeparvovec	Risdiplam for 2 years	any prior therapy + oral *SMN2* modifier	6 months - 60 years
MANATEE (NCT03032172)	Phase 3	Risdiplam	Investigational anti-myostatin antibody	oral *SMN2* modifier + anti-promyostatin antibody	2 years – 25 years

RESILIENT (NCT05337553)	Phase 3	Nusinersen, risdiplam or previously treated with zolgensma	taldefgrobep alfa	any SMN-dependent therapy + anti-promyostatin antibody	4 years – 21 years

Table 10: Clinical trials in which combination therapies are currently investigated in SMA (OA = onasemnogene abeparvovec; ASO = antisense oligonucleotide)

Risk	Ethical issues
Treatment limitations or failure to meet expectations	IUT could lessen disease symptoms, slow disease progression, or perhaps even prevent disease onset. However, treatment might be sub-optimal or have variable levels of success or fail to meet parental expectations of a cure.
Risk-Benefit Considerations/Safety concerns	The disease under consideration for IUGT must have significant pathology that occurs during gestation, whereby waiting to initiate treatment after birth has clear limitations in efficacy, to support equipoise of benefit/risk. Risks can be present for the mother (fetal loss, preterm birth, infection, immune response, maternal-fetal surgery, insertional mutagenesis), the child (disrupted development, insertional mutagenesis) and future generations (unintentional germline transfer).
Restricted access	Cost may vary significantly based on several factors, including the phenotype, severity of the condition, therapeutic drug and selected treatment approach. These variables may determine whether cost can be reimbursed or access be denied. Equitable access may not be possible.

Table 11: Ethical aspects of in utero therapy

The second manuscript is a review in collaboration with Dr. Finkel from the United States and Dr. Ellie Chilcott and Rafael Yáñez from the United Kingdom. This review highlights the important question if IUT could more effectively address SMA pathology by intervening before irreversible MN loss. As previously mentioned, currently existing postnatal therapies such as nusinersen, risdiplam, and OA significantly improve the patients outcome and disease progression, yet particularly the more severe forms of SMA (types 0 and I) already exhibit prenatal onset of neurodegeneration. Prenatal DMT's administered during this developmental window may theoretically offer several advantages over postnatal administration of DMT's. First and foremost, early intervention could potentially prevent MN degeneration to better preserve motor function. Additionally, the tolerogenic fetal immune system could reduce adverse immune responses, as previously seen with OA treatment [278]. Fetal physiology also allows for improved biodistribution, due to a more permeable BBB and a smaller target mass, which may further improve clinical outcome beyond the CNS. In addition to ongoing clinical trials using IUT in humans real-life treatment, while limited, have shown favorable outcomes [283], [285], [286]. Regarding SMA, a case study with risdiplam treatment before birth resulted in favorable outcomes, highlighting the feasibility of IUT [317]. While in theory and practice so far IUT for SMA sounds very promising, considerations regarding fetal and maternal risks should not be disregarded. If IUT is going to become a routinely used method meticulous preclinical studies, robust safety protocols and clear communication with families will be essential before application.

VIII. BIBLIOGRAPHY

[1] S. Ogino and R. B. Wilson, “Genetic testing and risk assessment for spinal muscular atrophy (SMA),” *Hum Genet*, vol. 111, no. 6, pp. 477–500, Dec. 2002, doi: 10.1007/s00439-002-0828-x.

[2] G. Werdnig, “Zwei frühinfantile hereditäre Fälle von progressiver Muskelatrophie unter dem Bilde der Dystrophie, aber anf neurotischer Grundlage,” *Archiv f. Psychiatrie*, vol. 22, no. 2, pp. 437–480, Jun. 1891, doi: 10.1007/BF01776636.

[3] V. Dubowitz, “Ramblings in the history of spinal muscular atrophy,” *Neuromuscul Disord*, vol. 19, no. 1, pp. 69–73, Jan. 2009, doi: 10.1016/j.nmd.2008.10.004.

[4] C. E. BEEVOR, “A CASE OF CONGENITAL SPINAL MUSCULAR ATROPHY (FAMILY TYPE), AND A CASE OF HÆMORRHAGE INTO THE SPINAL CORD AT BIRTH, GIVING SIMILAR SYMPTOMS,” *Brain*, vol. 25, no. 1, pp. 85–108, Jan. 1902, doi: 10.1093/brain/25.1.85.

[5] M. Sylvestre, “Paralysie Flasque de Quatre Membres et Des Muscles Du Tronc (Sauf Le Diaphragme),” 1899.

[6] J. G. GREENFIELD and R. O. STERN, “THE ANATOMICAL IDENTITY OF THE WERDNIG-HOFFMANN AND OPPENHEIM FORMS OF INFANTILE MUSCULAR ATROPHY,” *Brain*, vol. 50, no. 3–4, pp. 652–686, Oct. 1927, doi: 10.1093/brain/50.3-4.652.

[7] V. DUBOWITZ, “INFANTILE MUSCULAR ATROPHY A PROSPECTIVE STUDY WITH PARTICULAR REFERENCE TO A SLOWLY PROGRESSIVE VARIETY,” *Brain*, vol. 87, no. 4, pp. 707–718, Dec. 1964, doi: 10.1093/brain/87.4.707.

[8] E. KUGELBERG and L. WELANDER, “Heredofamilial Juvenile muscular Atrophy Simulating muscular Dystrophy,” *A.M.A. Archives of Neurology & Psychiatry*, vol. 75, no. 5, pp. 500–509, May 1956, doi: 10.1001/archneurpsyc.1956.02330230050005.

[9] L. M. Brzustowicz *et al.*, “Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q1 1.2–13.3,” *Nature*, vol. 344, no. 6266, pp. 540–541, Apr. 1990, doi: 10.1038/344540a0.

[10] T. C. Gilliam *et al.*, “Genetic homogeneity between acute and chronic forms of spinal muscular atrophy,” *Nature*, vol. 345, no. 6278, pp. 823–825, Jun. 1990, doi: 10.1038/345823a0.

[11] J. Melki *et al.*, “Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q,” *Nature*, vol. 344, no. 6268, pp. 767–768, Apr. 1990, doi: 10.1038/344767a0.

[12] J. Melki *et al.*, “Mapping of acute (type I) spinal muscular atrophy to chromosome 5q12-q14,” *The Lancet*, vol. 336, no. 8710, pp. 271–273, Aug. 1990, doi: 10.1016/0140-6736(90)91803-I.

[13] S. Lefebvre *et al.*, “Identification and characterization of a spinal muscular atrophy-determining gene,” *Cell*, vol. 80, no. 1, pp. 155–165, Jan. 1995, doi: 10.1016/0092-8674(95)90460-3.

[14] S. Grotto *et al.*, “Type 0 Spinal Muscular Atrophy: Further Delineation of Prenatal and Postnatal Features in 16 Patients,” *Journal of Neuromuscular Diseases*, vol. 3, no. 4, Art. no. 4, Jan. 2016, doi: 10.3233/JND-160177.

[15] R. Finkel, E. Bertini, F. Muntoni, E. Mercuri, and ENMC SMA Workshop Study Group, “209th ENMC International Workshop: Outcome Measures and Clinical Trial Readiness in Spinal Muscular Atrophy 7-9 November 2014, Heemskerk, The Netherlands,” *Neuromuscul Disord*, vol. 25, no. 7, pp. 593–602, Jul. 2015, doi: 10.1016/j.nmd.2015.04.009.

[16] R. S. Finkel *et al.*, “Observational study of spinal muscular atrophy type I and implications for clinical trials,” *Neurology*, vol. 83, no. 9, Art. no. 9, Aug. 2014, doi: 10.1212/WNL.0000000000000741.

[17] A. von Gontard *et al.*, "Intelligence and cognitive function in children and adolescents with spinal muscular atrophy," *Neuromuscular Disorders*, vol. 12, no. 2, Art. no. 2, Feb. 2002, doi: 10.1016/S0960-8966(01)00274-7.

[18] K. Zerres and K. E. Davies, "59th ENMC International Workshop: Spinal Muscular Atrophies: recent progress and revised diagnostic criteria 17-19 April 1998, Soestduinen, The Netherlands," *Neuromuscul Disord*, vol. 9, no. 4, pp. 272–278, Jun. 1999, doi: 10.1016/s0960-8966(99)00016-4.

[19] M. Calucho *et al.*, "Correlation between SMA type and SMN2 copy number revisited: An analysis of 625 unrelated Spanish patients and a compilation of 2834 reported cases," *Neuromuscul Disord*, vol. 28, no. 3, Art. no. 3, Mar. 2018, doi: 10.1016/j.nmd.2018.01.003.

[20] NINDS, "Spinal Muscular Atrophy | National Institute of Neurological Disorders and Stroke." Accessed: May 23, 2025. [Online]. Available: https://www.ninds.nih.gov/health-information/disorders/spinal-muscular-atrophy

[21] R. S. Finkel *et al.*, "Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy," *N Engl J Med*, vol. 377, no. 18, pp. 1723–1732, Nov. 2017, doi: 10.1056/NEJMoa1702752.

[22] J. R. Mendell *et al.*, "Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy," *N Engl J Med*, vol. 377, no. 18, Art. no. 18, Nov. 2017, doi: 10.1056/NEJMoa1706198.

[23] D. C. Schorling, A. Pechmann, and J. Kirschner, "Advances in Treatment of Spinal Muscular Atrophy – New Phenotypes, New Challenges, New Implications for Care," *J Neuromuscul Dis*, vol. 7, no. 1, pp. 1–13, 2020, doi: 10.3233/JND-190424.

[24] S. Pagliardini *et al.*, "Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord," *Hum Mol Genet*, vol. 9, no. 1, pp. 47–56, Jan. 2000, doi: 10.1093/hmg/9.1.47.

[25] M. Costa-Roger *et al.*, "Complex SMN Hybrids Detected in a Cohort of 31 Patients With Spinal Muscular Atrophy," *Neurol Genet*, vol. 10, no. 4, p. e200175, Jul. 2024, doi: 10.1212/NXG.0000000000200175.

[26] C. F. Rochette, N. Gilbert, and L. R. Simard, "SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens," *Hum Genet*, vol. 108, no. 3, pp. 255–266, Mar. 2001, doi: 10.1007/s004390100473.

[27] N. N. Singh, B. M. Lee, and R. N. Singh, "Splicing regulation in spinal muscular atrophy by an RNA structure formed by long-distance interactions," *Ann N Y Acad Sci*, vol. 1341, pp. 176–187, Apr. 2015, doi: 10.1111/nyas.12727.

[28] T. T. Le *et al.*, "SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN," *Hum Mol Genet*, vol. 14, no. 6, pp. 845–857, Mar. 2005, doi: 10.1093/hmg/ddi078.

[29] M. E. R. Butchbach, "Copy Number Variations in the Survival Motor Neuron Genes: Implications for Spinal Muscular Atrophy and Other Neurodegenerative Diseases," *Front. Mol. Biosci.*, vol. 3, Mar. 2016, doi: 10.3389/fmolb.2016.00007.

[30] J. Seo, N. N. Singh, E. W. Ottesen, B. M. Lee, and R. N. Singh, "A novel human-specific splice isoform alters the critical C-terminus of Survival Motor Neuron protein," *Scientific Reports*, vol. 6, p. 30778, Aug. 2016, doi: 10.1038/srep30778.

[31] M. Gennarelli *et al.*, "Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients," *Biochem Biophys Res Commun*, vol. 213, no. 1, pp. 342–348, Aug. 1995, doi: 10.1006/bbrc.1995.2135.

[32] V. Setola, M. Terao, D. Locatelli, S. Bassanini, E. Garattini, and G. Battaglia, "Axonal-SMN (a-SMN), a protein isoform of the survival motor neuron gene, is specifically involved in axonogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 6, p. 1959, Jan. 2007, doi: 10.1073/pnas.0610660104.

[33] L. Pellizzoni, N. Kataoka, B. Charroux, and G. Dreyfuss, "A Novel Function for SMN, the Spinal Muscular Atrophy Disease Gene Product, in Pre-mRNA Splicing," *Cell*, vol. 95, no. 5, pp. 615–624, Nov. 1998, doi: 10.1016/S0092-8674(00)81632-3.

[34] S. Ahmad, K. Bhatia, A. Kannan, and L. Gangwani, "Molecular Mechanisms of Neurodegeneration in Spinal Muscular Atrophy," *J Exp Neurosci*, vol. 10, pp. 39–49, 2016, doi: 10.4137/JEN.S33122.

[35] J. S. Da Silva, M. Medina, C. Zuliani, A. Di Nardo, W. Witke, and C. G. Dotti, "RhoA/ROCK regulation of neuritogenesis via profilin IIa–mediated control of actin stability," *J Cell Biol*, vol. 162, no. 7, pp. 1267–1279, Sep. 2003, doi: 10.1083/jcb.200304021.

[36] A. Hall, "Rho GTPases and the actin cytoskeleton," *Science*, vol. 279, no. 5350, pp. 509–514, Jan. 1998, doi: 10.1126/science.279.5350.509.

[37] A. V. Schofield and O. Bernard, "Rho-associated coiled-coil kinase (ROCK) signaling and disease," *Crit Rev Biochem Mol Biol*, vol. 48, no. 4, pp. 301–316, 2013, doi: 10.3109/10409238.2013.786671.

[38] M. Bowerman, D. Shafey, and R. Kothary, "Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity," *J Mol Neurosci*, vol. 32, no. 2, pp. 120–131, 2007, doi: 10.1007/s12031-007-0024-5.

[39] E. Coque, C. Raoul, and M. Bowerman, "ROCK inhibition as a therapy for spinal muscular atrophy: understanding the repercussions on multiple cellular targets," *Front Neurosci*, vol. 8, p. 271, 2014, doi: 10.3389/fnins.2014.00271.

[40] N. Hensel, S. Rademacher, and P. Claus, "Chatting with the neighbors: crosstalk between Rho-kinase (ROCK) and other signaling pathways for treatment of neurological disorders," *Front Neurosci*, vol. 9, p. 198, Jun. 2015, doi: 10.3389/fnins.2015.00198.

[41] N. K. Genabai, S. Ahmad, Z. Zhang, X. Jiang, C. A. Gabaldon, and L. Gangwani, "Genetic inhibition of JNK3 ameliorates spinal muscular atrophy," *Human Molecular Genetics*, vol. 24, no. 24, pp. 6986–7004, Dec. 2015, doi: 10.1093/hmg/ddv401.

[42] A. Lin, "Activation of the JNK signaling pathway: Breaking the brake on apoptosis," *BioEssays*, vol. 25, no. 1, pp. 17–24, 2003, doi: 10.1002/bies.10204.

[43] R. Schellino, M. Boido, T. Borsello, and A. Vercelli, "Pharmacological c-Jun NH2-Terminal Kinase (JNK) Pathway Inhibition Reduces Severity of Spinal Muscular Atrophy Disease in Mice," *Front Mol Neurosci*, vol. 11, p. 308, Sep. 2018, doi: 10.3389/fnmol.2018.00308.

[44] O. Biondi *et al.*, "In Vivo NMDA Receptor Activation Accelerates Motor Unit Maturation, Protects Spinal Motor Neurons, and Enhances SMN2 Gene Expression in Severe Spinal Muscular Atrophy Mice," *J. Neurosci.*, vol. 30, no. 34, pp. 11288–11299, Aug. 2010, doi: 10.1523/JNEUROSCI.1764-10.2010.

[45] E. W. Dent and F. B. Gertler, "Cytoskeletal Dynamics and Transport in Growth Cone Motility and Axon Guidance," *Neuron*, vol. 40, no. 2, pp. 209–227, Oct. 2003, doi: 10.1016/S0896-6273(03)00633-0.

[46] G. E. Oprea *et al.*, "Plastin 3 Is a Protective Modifier of Autosomal Recessive Spinal Muscular Atrophy," *Science*, vol. 320, no. 5875, pp. 524–527, Apr. 2008, doi: 10.1126/science.1155085.

[47] S. Jablonka and N. Schäfer, “The SMA Modifier Plastin 3 Targets Cell Membrane-Associated Proteins in Motoneurons,” *Neurosci Insights*, vol. 19, p. 26331055241226623, Jan. 2024, doi: 10.1177/26331055241226623.
[48] A. Kannan, X. Jiang, L. He, S. Ahmad, and L. Gangwani, “ZPR1 prevents R-loop accumulation, upregulates SMN2 expression and rescues spinal muscular atrophy,” *Brain*, vol. 143, no. 1, pp. 69–93, Jan. 2020, doi: 10.1093/brain/awz373.
[49] M. Riessland *et al.*, “Neurocalcin Delta Suppression Protects against Spinal Muscular Atrophy in Humans and across Species by Restoring Impaired Endocytosis,” *Am J Hum Genet*, vol. 100, no. 2, pp. 297–315, Feb. 2017, doi: 10.1016/j.ajhg.2017.01.005.
[50] A. Muiños-Bühl *et al.*, “Long-Term SMN- and Ncald-ASO Combinatorial Therapy in SMA Mice and NCALD-ASO Treatment in hiPSC-Derived Motor Neurons Show Protective Effects,” *Int J Mol Sci*, vol. 24, no. 4, p. 4198, Feb. 2023, doi: 10.3390/ijms24044198.
[51] T. O. Crawford *et al.*, “Continued benefit of nusinersen initiated in the presymptomatic stage of spinal muscular atrophy: 5-year update of the NURTURE study,” *Muscle Nerve*, vol. 68, no. 2, pp. 157–170, Aug. 2023, doi: 10.1002/mus.27853.
[52] Y. O. S. Wijaya *et al.*, “Assessment of Spinal Muscular Atrophy Carrier Status by Determining SMN1 Copy Number Using Dried Blood Spots,” *Int J Neonatal Screen*, vol. 6, no. 2, p. 43, May 2020, doi: 10.3390/ijns6020043.
[53] N. R. Eissa, H. A. Hassan, S. M. Senousy, H. N. Soliman, and M. L. Essawi, “SMA carrier testing using Real-time PCR as a potential preconception screening tool,” *Egyptian Journal of Medical Human Genetics*, vol. 23, no. 1, p. 24, Feb. 2022, doi: 10.1186/s43042-022-00233-9.
[54] E. L. Arkblad *et al.*, “Multiplex ligation-dependent probe amplification improves diagnostics in spinal muscular atrophy,” *Neuromuscular Disorders*, vol. 16, no. 12, pp. 830–838, Dec. 2006, doi: 10.1016/j.nmd.2006.08.011.
[55] L. Alías *et al.*, “Mutation update of spinal muscular atrophy in Spain: molecular characterization of 745 unrelated patients and identification of four novel mutations in the SMN1 gene,” *Hum Genet*, vol. 125, no. 1, pp. 29–39, Feb. 2009, doi: 10.1007/s00439-008-0598-1.
[56] L. Blasco-Pérez *et al.*, “Deep Molecular Characterization of Milder Spinal Muscular Atrophy Patients Carrying the c.859G>C Variant in SMN2,” *Int J Mol Sci*, vol. 23, no. 15, p. 8289, Jul. 2022, doi: 10.3390/ijms23158289.
[57] T. O. Crawford *et al.*, “Evaluation of SMN Protein, Transcript, and Copy Number in the Biomarkers for Spinal Muscular Atrophy (BforSMA) Clinical Study,” *PLoS One*, vol. 7, no. 4, p. e33572, Apr. 2012, doi: 10.1371/journal.pone.0033572.
[58] R. I. Wadman *et al.*, “A Comparative Study of SMN Protein and mRNA in Blood and Fibroblasts in Patients with Spinal Muscular Atrophy and Healthy Controls,” *PLoS One*, vol. 11, no. 11, p. e0167087, Nov. 2016, doi: 10.1371/journal.pone.0167087.
[59] J. Glascock *et al.*, “Identifying Biomarkers of Spinal Muscular Atrophy for Further Development,” *J Neuromuscul Dis*, vol. 10, no. 5, Art. no. 5, Jul. 2024, doi: 10.3233/JND-230054.
[60] C. J. Sumner *et al.*, “SMN mRNA and protein levels in peripheral blood: biomarkers for SMA clinical trials,” *Neurology*, vol. 66, no. 7, pp. 1067–1073, Apr. 2006, doi: 10.1212/01.wnl.0000201929.56928.13.
[61] C. Czech *et al.*, “Biomarker for Spinal Muscular Atrophy: Expression of SMN in Peripheral Blood of SMA Patients and Healthy Controls,” *PLoS One*, vol. 10, no. 10, p. e0139950, Oct. 2015, doi: 10.1371/journal.pone.0139950.

[62] B. T. Darras *et al.*, "Neurofilament as a potential biomarker for spinal muscular atrophy," *Ann Clin Transl Neurol*, vol. 6, no. 5, pp. 932–944, Apr. 2019, doi: 10.1002/acn3.779.

[63] G. Seo, S. Kim, J. C. Byun, S. Kwon, and Y. J. Lee, "Evaluation of the neurofilament light chain as a biomarker in children with spinal muscular atrophy treated with nusinersen," *Brain Dev*, vol. 45, no. 10, pp. 554–563, Nov. 2023, doi: 10.1016/j.braindev.2023.07.005.

[64] C. R. R. Alves *et al.*, "Serum creatinine is a biomarker of progressive denervation in spinal muscular atrophy," *Neurology*, vol. 94, no. 9, pp. e921–e931, Mar. 2020, doi: 10.1212/WNL.0000000000008762.

[65] C. Yanyan, Q. Yujin, B. Jinli, J. Yuwei, W. Hong, and S. Fang, "Correlation of PLS3 expression with disease severity in children with spinal muscular atrophy," *J Hum Genet*, vol. 59, no. 1, pp. 24–27, Jan. 2014, doi: 10.1038/jhg.2013.111.

[66] S. Bernal *et al.*, "Plastin 3 expression in discordant spinal muscular atrophy (SMA) siblings," *Neuromuscul Disord*, vol. 21, no. 6, Art. no. 6, Jun. 2011, doi: 10.1016/j.nmd.2011.03.009.

[67] A. M. D'Silva, D. Kariyawasam, P. Venkat, C. Mayoh, and M. A. Farrar, "Identification of Novel CSF-Derived miRNAs in Treated Paediatric Onset Spinal Muscular Atrophy: An Exploratory Study," *Pharmaceutics*, vol. 15, no. 1, p. 170, Jan. 2023, doi: 10.3390/pharmaceutics15010170.

[68] K.-W. Chen and J.-A. Chen, "Functional Roles of Long Non-coding RNAs in Motor Neuron Development and Disease," *J Biomed Sci*, vol. 27, no. 1, p. 38, Feb. 2020, doi: 10.1186/s12929-020-00628-z.

[69] M. A. Maretina *et al.*, "Identification of specific gene methylation patterns during motor neuron differentiation from spinal muscular atrophy patient-derived iPSC," *Gene*, vol. 811, p. 146109, Feb. 2022, doi: 10.1016/j.gene.2021.146109.

[70] A. Sprenger-Svačina *et al.*, "MRI correlates of motoneuron loss in SMA," *J Neurol*, vol. 270, no. 1, pp. 503–510, Jan. 2023, doi: 10.1007/s00415-022-11326-1.

[71] J. Li, T. R. Geisbush, W. D. Arnold, G. D. Rosen, P. G. Zaworski, and S. B. Rutkove, "A comparison of three electrophysiological methods for the assessment of disease status in a mild spinal muscular atrophy mouse model," *PLoS One*, vol. 9, no. 10, p. e111428, 2014, doi: 10.1371/journal.pone.0111428.

[72] P. B. Kang *et al.*, "The motor neuron response to SMN1 deficiency in spinal muscular atrophy," *Muscle Nerve*, vol. 49, no. 5, pp. 636–644, May 2014, doi: 10.1002/mus.23967.

[73] A. M. Glanzman *et al.*, "The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND): test development and reliability," *Neuromuscul Disord*, vol. 20, no. 3, pp. 155–161, Mar. 2010, doi: 10.1016/j.nmd.2009.11.014.

[74] J. M. O'Hagen *et al.*, "An expanded version of the Hammersmith Functional Motor Scale for SMA II and III patients," *Neuromuscul Disord*, vol. 17, no. 9–10, pp. 693–697, Oct. 2007, doi: 10.1016/j.nmd.2007.05.009.

[75] E. S. Mazzone *et al.*, "Revised upper limb module for spinal muscular atrophy: Development of a new module," *Muscle Nerve*, vol. 55, no. 6, pp. 869–874, Jun. 2017, doi: 10.1002/mus.25430.

[76] C. M. McDonald *et al.*, "The 6-minute walk test as a new outcome measure in Duchenne muscular dystrophy," *Muscle Nerve*, vol. 41, no. 4, pp. 500–510, Apr. 2010, doi: 10.1002/mus.21544.

[77] N. N. Singh, M. D. Howell, E. J. Androphy, and R. N. Singh, "How the discovery of ISS-N1 led to the first medical therapy for spinal muscular atrophy," *Gene Ther*, vol. 24, no. 9, pp. 520–526, Sep. 2017, doi: 10.1038/gt.2017.34.

[78] B. T. Darras *et al.*, "Nusinersen in later-onset spinal muscular atrophy," *Neurology*, vol. 92, no. 21, pp. e2492–e2506, May 2019, doi: 10.1212/WNL.0000000000007527.
[79] R. Günther *et al.*, "Long-term efficacy and safety of nusinersen in adults with 5q spinal muscular atrophy: a prospective European multinational observational study," *Lancet Reg Health Eur*, vol. 39, p. 100862, Apr. 2024, doi: 10.1016/j.lanepe.2024.100862.
[80] D. Chand *et al.*, "Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy," *J Hepatol*, vol. 74, no. 3, pp. 560–566, Mar. 2021, doi: 10.1016/j.jhep.2020.11.001.
[81] H. Ratni *et al.*, "Discovery of Risdiplam, a Selective Survival of Motor Neuron-2 (SMN2) Gene Splicing Modifier for the Treatment of Spinal Muscular Atrophy (SMA)," *J Med Chem*, vol. 61, no. 15, pp. 6501–6517, Aug. 2018, doi: 10.1021/acs.jmedchem.8b00741.
[82] B. T. Darras *et al.*, "Risdiplam-Treated Infants with Type 1 Spinal Muscular Atrophy versus Historical Controls," *N Engl J Med*, vol. 385, no. 5, pp. 427–435, Jul. 2021, doi: 10.1056/NEJMoa2102047.
[83] A. K. Cheung *et al.*, "Discovery of Small Molecule Splicing Modulators of Survival Motor Neuron-2 (SMN2) for the Treatment of Spinal Muscular Atrophy (SMA)," *J Med Chem*, vol. 61, no. 24, pp. 11021–11036, Dec. 2018, doi: 10.1021/acs.jmedchem.8b01291.
[84] K. S. Ojala, E. J. Reedich, C. J. DiDonato, and S. D. Meriney, "In Search of a Cure: The Development of Therapeutics to Alter the Progression of Spinal Muscular Atrophy," *Brain Sciences*, vol. 11, no. 2, Art. no. 2, Feb. 2021, doi: 10.3390/brainsci11020194.
[85] A. Iskenderian *et al.*, "Myostatin and activin blockade by engineered follistatin results in hypertrophy and improves dystrophic pathology in mdx mouse more than myostatin blockade alone," *Skelet Muscle*, vol. 8, p. 34, Oct. 2018, doi: 10.1186/s13395-018-0180-z.
[86] H. Zhou *et al.*, "Myostatin inhibition in combination with antisense oligonucleotide therapy improves outcomes in spinal muscular atrophy," *J Cachexia Sarcopenia Muscle*, vol. 11, no. 3, pp. 768–782, Jun. 2020, doi: 10.1002/jcsm.12542.
[87] R. A. Powis *et al.*, "Systemic restoration of UBA1 ameliorates disease in spinal muscular atrophy," *JCI Insight*, vol. 1, no. 11, Art. no. 11, Jul. 2016, doi: 10.1172/jci.insight.87908.
[88] A. Alrafiah *et al.*, "Plastin 3 Promotes Motor Neuron Axonal Growth and Extends Survival in a Mouse Model of Spinal Muscular Atrophy," *Mol Ther Methods Clin Dev*, vol. 9, pp. 81–89, Jun. 2018, doi: 10.1016/j.omtm.2018.01.007.
[89] K. A. Kaifer *et al.*, "Plastin-3 extends survival and reduces severity in mouse models of spinal muscular atrophy," *JCI Insight*, vol. 2, no. 5, Art. no. 5, Jun. 2023, doi: 10.1172/jci.insight.89970.
[90] M. Riessland *et al.*, "Neurocalcin Delta Suppression Protects against Spinal Muscular Atrophy in Humans and across Species by Restoring Impaired Endocytosis," *Am J Hum Genet*, vol. 100, no. 2, pp. 297–315, Feb. 2017, doi: 10.1016/j.ajhg.2017.01.005.
[91] E. M. Chilcott, E. W. Muiruri, T. C. Hirst, and R. J. Yáñez-Muñoz, "Systematic review and meta-analysis determining the benefits of in vivo genetic therapy in spinal muscular atrophy rodent models," *Gene Ther*, vol. 29, no. 9, Art. no. 9, Sep. 2022, doi: 10.1038/s41434-021-00292-4.

[92] T. O. Crawford *et al.*, “Safety and Efficacy of Apitegromab in Patients With Spinal Muscular Atrophy Types 2 and 3: The Phase 2 TOPAZ Study,” *Neurology*, vol. 102, no. 5, p. e209151, Mar. 2024, doi: 10.1212/WNL.0000000000209151.
[93] C. M. Proud *et al.*, “Combination disease-modifying treatment in spinal muscular atrophy: A proposed classification,” *Ann Clin Transl Neurol*, vol. 10, no. 11, Art. no. 11, Nov. 2023, doi: 10.1002/acn3.51889.
[94] V. Pagliarini, M. Guerra, V. Di Rosa, C. Compagnucci, and C. Sette, “Combined treatment with the histone deacetylase inhibitor LBH589 and a splice-switch antisense oligonucleotide enhances SMN2 splicing and SMN expression in Spinal Muscular Atrophy cells,” *Journal of Neurochemistry*, vol. 153, no. 2, p. e14935, 2020, doi: 10.1111/jnc.14935.
[95] L. E. Marasco *et al.*, “Counteracting chromatin effects of a splicing-correcting antisense oligonucleotide improves its therapeutic efficacy in spinal muscular atrophy,” *Cell*, vol. 185, no. 12, pp. 2057-2070.e15, Jun. 2022, doi: 10.1016/j.cell.2022.04.031.
[96] M. B. Abera *et al.*, “ML372 blocks SMN ubiquitination and improves spinal muscular atrophy pathology in mice,” *JCI Insight*, vol. 1, no. 19, Art. no. 19, Nov. 2016, doi: 10.1172/jci.insight.88427.
[97] S. A. Dumas *et al.*, “A combinatorial approach increases SMN level in SMA model mice,” *Hum Mol Genet*, vol. 31, no. 17, pp. 2989–3000, Aug. 2022, doi: 10.1093/hmg/ddac068.
[98] Y. Harada *et al.*, “Combination molecular therapies for type 1 spinal muscular atrophy,” *Muscle Nerve*, vol. 62, no. 4, pp. 550–554, Oct. 2020, doi: 10.1002/mus.27034.
[99] A. Mirea *et al.*, “Combination Therapy with Nusinersen and Onasemnogene Abeparvovec-xioi in Spinal Muscular Atrophy Type I,” *J Clin Med*, vol. 10, no. 23, p. 5540, Nov. 2021, doi: 10.3390/jcm10235540.
[100] K. F. Oechsel and M. S. Cartwright, “Combination therapy with onasemnogene and risdiplam in spinal muscular atrophy type 1,” *Muscle Nerve*, vol. 64, no. 4, pp. 487–490, Oct. 2021, doi: 10.1002/mus.27375.
[101] E. Tiberi *et al.*, “Nusinersen in type 0 spinal muscular atrophy: should we treat?,” *Ann Clin Transl Neurol*, vol. 7, no. 12, pp. 2481–2483, Nov. 2020, doi: 10.1002/acn3.51126.
[102] S. E. Matesanz *et al.*, “Clinical Course in a Patient With Spinal Muscular Atrophy Type 0 Treated With Nusinersen and Onasemnogene Abeparvovec,” *J Child Neurol*, vol. 35, no. 11, pp. 717–723, Oct. 2020, doi: 10.1177/0883073820928784.
[103] P.-J. H. Zushin, S. Mukherjee, and J. C. Wu, “FDA Modernization Act 2.0: transitioning beyond animal models with human cells, organoids, and AI/ML-based approaches,” *J Clin Invest*, vol. 133, no. 21, p. e175824, 2023, doi: 10.1172/JCI175824.
[104] B. Schrank *et al.*, “Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos,” *Proc Natl Acad Sci U S A*, vol. 94, no. 18, Art. no. 18, Sep. 1997.
[105] U. R. Monani *et al.*, “The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy,” *Hum Mol Genet*, vol. 9, no. 3, pp. 333–339, Feb. 2000, doi: 10.1093/hmg/9.3.333.
[106] H. M. Hsieh-Li *et al.*, “A mouse model for spinal muscular atrophy,” *Nat Genet*, vol. 24, no. 1, pp. 66–70, Jan. 2000, doi: 10.1038/71709.

[107] M. Riessland *et al.*, “SAHA ameliorates the SMA phenotype in two mouse models for spinal muscular atrophy,” *Hum Mol Genet*, vol. 19, no. 8, pp. 1492–1506, Apr. 2010, doi: 10.1093/hmg/ddq023.
[108] M. Bowerman, C. L. Anderson, A. Beauvais, P. P. Boyl, W. Witke, and R. Kothary, “SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis,” *Mol Cell Neurosci*, vol. 42, no. 1, pp. 66–74, Sep. 2009, doi: 10.1016/j.mcn.2009.05.009.
[109] M. Bowerman, A. Beauvais, C. L. Anderson, and R. Kothary, “Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model,” *Human Molecular Genetics*, vol. 19, no. 8, pp. 1468–1478, Apr. 2010, doi: 10.1093/hmg/ddq021.
[110] T. W. Bebee, C. E. Dominguez, and D. S. Chandler, “Mouse models of SMA: tools for disease characterization and therapeutic development,” *Hum Genet*, vol. 131, no. 8, pp. 1277–1293, Aug. 2012, doi: 10.1007/s00439-012-1171-5.
[111] K.-L. Boon *et al.*, “Zebrafish survival motor neuron mutants exhibit presynaptic neuromuscular junction defects,” *Human Molecular Genetics*, vol. 18, no. 19, pp. 3615–3625, Oct. 2009, doi: 10.1093/hmg/ddp310.
[112] M. Briese *et al.*, “Deletion of smn-1, the Caenorhabditis elegans ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan,” *Hum Mol Genet*, vol. 18, no. 1, pp. 97–104, Jan. 2009, doi: 10.1093/hmg/ddn320.
[113] Y. B. Chan *et al.*, “Neuromuscular defects in a Drosophila survival motor neuron gene mutant,” *Hum Mol Genet*, vol. 12, no. 12, pp. 1367–1376, Jun. 2003, doi: 10.1093/hmg/ddg157.
[114] M. L. McWhorter, U. R. Monani, A. H. M. Burghes, and C. E. Beattie, “Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding,” *J Cell Biol*, vol. 162, no. 5, pp. 919–932, Sep. 2003, doi: 10.1083/jcb.200303168.
[115] S. I. Duque *et al.*, “A large animal model of Spinal Muscular Atrophy and correction of phenotype,” *Ann Neurol*, vol. 77, no. 3, pp. 399–414, Mar. 2015, doi: 10.1002/ana.24332.
[116] I. Signoria, W. L. van der Pol, and E. J. N. Groen, “Innovating spinal muscular atrophy models in the therapeutic era,” *Dis Model Mech*, vol. 16, no. 9, p. dmm050352, Oct. 2023, doi: 10.1242/dmm.050352.
[117] R. Adami and D. Bottai, “Spinal Muscular Atrophy Modeling and Treatment Advances by Induced Pluripotent Stem Cells Studies,” *Stem Cell Rev Rep*, vol. 15, no. 6, pp. 795–813, Dec. 2019, doi: 10.1007/s12015-019-09910-6.
[118] W.-F. Chang *et al.*, “Survival Motor Neuron Enhances Pluripotent Gene Expression and Facilitates Cell Reprogramming,” *Stem Cells Dev*, vol. 31, no. 21–22, pp. 696–705, Nov. 2022, doi: 10.1089/scd.2022.0091.
[119] J. Ladewig *et al.*, “Small molecules enable highly efficient neuronal conversion of human fibroblasts,” *Nat Methods*, vol. 9, no. 6, pp. 575–578, Jun. 2012, doi: 10.1038/nmeth.1972.
[120] R. de Jongh, X. M. Spijkers, S. Pasteuning-Vuhman, P. Vulto, and R. J. Pasterkamp, “Neuromuscular junction-on-a-chip: ALS disease modeling and read-out development in microfluidic devices,” *J Neurochem*, vol. 157, no. 3, pp. 393–412, May 2021, doi: 10.1111/jnc.15289.
[121] J.-M. Faustino Martins *et al.*, “Self-Organizing 3D Human Trunk Neuromuscular Organoids,” *Cell Stem Cell*, vol. 26, no. 2, pp. 172-186.e6, Feb. 2020, doi: 10.1016/j.stem.2019.12.007.

[122] J. H. Hor *et al.*, "Cell cycle inhibitors protect motor neurons in an organoid model of Spinal Muscular Atrophy," *Cell Death Dis*, vol. 9, no. 11, p. 1100, Oct. 2018, doi: 10.1038/s41419-018-1081-0.
[123] A. Kurjak *et al.*, "The assessment of fetal neurobehavior by three-dimensional and four-dimensional ultrasound," *J Matern Fetal Neonatal Med*, vol. 21, no. 10, Art. no. 10, Oct. 2008, doi: 10.1080/14767050802212166.
[124] M. J. Pérez-García, L. Kong, C. J. Sumner, and E. F. Tizzano, "Developmental Aspects and Pathological Findings in Spinal Muscular Atrophy," *Spinal Muscular Atrophy: Disease Mechanisms and Therapy*, pp. 21–42, Jan. 2017, doi: 10.1016/B978-0-12-803685-3.00002-1.
[125] J. IJkema-Paassen and A. Gramsbergen, "Development of Postural Muscles and Their Innervation," *Neural Plast*, vol. 12, no. 2–3, pp. 141–151, 2005, doi: 10.1155/NP.2005.141.
[126] M. R. Bennett and A. G. Pettigrew, "The formation of synapses in striated muscle during development," *J Physiol*, vol. 241, no. 2, pp. 515–545, Sep. 1974, doi: 10.1113/jphysiol.1974.sp010670.
[127] E. F. Tizzano and D. Zafeiriou, "Prenatal aspects in spinal muscular atrophy: From early detection to early presymptomatic intervention," *Eur J Paediatr Neurol*, vol. 22, no. 6, Art. no. 6, Nov. 2018, doi: 10.1016/j.ejpn.2018.08.009.
[128] P. Burlet *et al.*, "The distribution of SMN protein complex in human fetal tissues and its alteration in spinal muscular atrophy," *Human Molecular Genetics*, vol. 7, no. 12, Art. no. 12, Nov. 1998, doi: 10.1093/hmg/7.12.1927.
[129] L. Kong *et al.*, "Impaired prenatal motor axon development necessitates early therapeutic intervention in severe SMA," *Science Translational Medicine*, vol. 13, no. 578, Art. no. 578, Jan. 2021, doi: 10.1126/scitranslmed.abb6871.
[130] D. M. Ramos *et al.*, "Age-dependent SMN expression in disease-relevant tissue and implications for SMA treatment," *The Journal of clinical investigation*, vol. 129, no. 11, Art. no. 11, Nov. 2019, doi: 10.1172/JCI124120.
[131] C. Soler-Botija, I. Cuscó, L. Caselles, E. López, M. Baiget, and E. F. Tizzano, "Implication of Fetal SMN2 Expression in Type I SMA Pathogenesis: Protection or Pathological Gain of Function?," *Journal of Neuropathology & Experimental Neurology*, vol. 64, no. 3, Art. no. 3, Mar. 2005, doi: 10.1093/jnen/64.3.215.
[132] L. T. Hao, P. Q. Duy, J. D. Jontes, and C. E. Beattie, "Motoneuron development influences dorsal root ganglia survival and Schwann cell development in a vertebrate model of spinal muscular atrophy," *Hum Mol Genet*, vol. 24, no. 2, pp. 346–360, Jan. 2015, doi: 10.1093/hmg/ddu447.
[133] G. Z. Mentis *et al.*, "Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy," *Neuron*, vol. 69, no. 3, pp. 453–467, Feb. 2011, doi: 10.1016/j.neuron.2010.12.032.
[134] C. Soler-Botija, I. Ferrer, I. Gich, M. Baiget, and E. F. Tizzano, "Neuronal death is enhanced and begins during foetal development in type I spinal muscular atrophy spinal cord," *Brain*, vol. 125, no. Pt 7, Art. no. Pt 7, Jul. 2002, doi: 10.1093/brain/awf155.
[135] R. Martínez-Hernández *et al.*, "Synaptic defects in type I spinal muscular atrophy in human development," *The Journal of Pathology*, vol. 229, no. 1, Art. no. 1, 2013, doi: 10.1002/path.4080.
[136] R. Martínez-Hernández *et al.*, "The developmental pattern of myotubes in spinal muscular atrophy indicates prenatal delay of muscle maturation," *J Neuropathol Exp Neurol*, vol. 68, no. 5, Art. no. 5, May 2009, doi: 10.1097/NEN.0b013e3181a10ea1.

[137] R. Martínez-Hernández, S. Bernal, L. Alias, and E. F. Tizzano, “Abnormalities in Early Markers of Muscle Involvement Support a Delay in Myogenesis in Spinal Muscular Atrophy,” *Journal of Neuropathology & Experimental Neurology*, vol. 73, no. 6, Art. no. 6, Jun. 2014, doi: 10.1097/NEN.0000000000000078.
[138] T. W. Prior *et al.*, “A Positive Modifier of Spinal Muscular Atrophy in the SMN2 Gene,” *Am J Hum Genet*, vol. 85, no. 3, pp. 408–413, Sep. 2009, doi: 10.1016/j.ajhg.2009.08.002.
[139] W. Müller-Felber *et al.*, “Infants Diagnosed with Spinal Muscular Atrophy and 4 SMN2 Copies through Newborn Screening – Opportunity or Burden?1,” *J Neuromuscul Dis*, vol. 7, no. 2, Art. no. 2, Mar. 2024, doi: 10.3233/JND-200475.
[140] B. N. Harding *et al.*, “Spectrum of neuropathophysiology in spinal muscular atrophy type I,” *J Neuropathol Exp Neurol*, vol. 74, no. 1, pp. 15–24, Jan. 2015, doi: 10.1097/NEN.0000000000000144.
[141] H. Rindt *et al.*, “Astrocytes influence the severity of spinal muscular atrophy,” *Hum Mol Genet*, vol. 24, no. 14, pp. 4094–4102, Jul. 2015, doi: 10.1093/hmg/ddv148.
[142] M. Shababi, C. L. Lorson, and S. S. Rudnik-Schöneborn, “Spinal muscular atrophy: a motor neuron disorder or a multi-organ disease?,” *J Anat*, vol. 224, no. 1, pp. 15–28, Jan. 2014, doi: 10.1111/joa.12083.
[143] C. J. J. Yeo and B. T. Darras, “Overturning the Paradigm of Spinal Muscular Atrophy as Just a Motor Neuron Disease,” *Pediatric Neurology*, vol. 109, pp. 12–19, Aug. 2020, doi: 10.1016/j.pediatrneurol.2020.01.003.
[144] P. Mathew and B. Bordoni, “Embryology, Heart,” in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2025. Accessed: Jul. 02, 2025. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK537313/
[145] N. A. Blom, A. C. Gittenberger-de Groot, M. C. DeRuiter, R. E. Poelmann, M. M. Mentink, and J. Ottenkamp, “Development of the cardiac conduction tissue in human embryos using HNK-1 antigen expression: possible relevance for understanding of abnormal atrial automaticity,” *Circulation*, vol. 99, no. 6, pp. 800–806, Feb. 1999, doi: 10.1161/01.cir.99.6.800.
[146] G. K. Maxwell, E. Szunyogova, H. K. Shorrock, T. H. Gillingwater, and S. H. Parson, “Developmental and degenerative cardiac defects in the Taiwanese mouse model of severe spinal muscular atrophy,” *J Anat*, vol. 232, no. 6, pp. 965–978, Jun. 2018, doi: 10.1111/joa.12793.
[147] C. A. Wijngaarde, A. C. Blank, M. Stam, R. I. Wadman, L. H. van den Berg, and W. L. van der Pol, “Cardiac pathology in spinal muscular atrophy: a systematic review,” *Orphanet Journal of Rare Diseases*, vol. 12, no. 1, p. 67, Apr. 2017, doi: 10.1186/s13023-017-0613-5.
[148] A. Palladino *et al.*, “Cardiac involvement in patients with Spinal Muscular Atrophies,” *Acta Myol*, vol. 30, no. 3, pp. 175–178, Dec. 2011.
[149] A. Bhatia, R. A. Shatanof, and B. Bordoni, “Embryology, Gastrointestinal,” in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2024. Accessed: Dec. 19, 2024. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK537172/
[150] I. S. Moriyama, “[Development of fetal organs and adaptation to extrauterine life],” *Nihon Sanka Fujinka Gakkai Zasshi*, vol. 38, no. 8, pp. 1227–1237, Aug. 1986.
[151] C. H. Wang *et al.*, “Consensus statement for standard of care in spinal muscular atrophy,” *J Child Neurol*, vol. 22, no. 8, pp. 1027–1049, Aug. 2007, doi: 10.1177/0883073807305788.
[152] P. Sintusek *et al.*, “Histopathological Defects in Intestine in Severe Spinal Muscular Atrophy Mice Are Improved by Systemic Antisense Oligonucleotide

Treatment," *PLoS One*, vol. 11, no. 5, p. e0155032, May 2016, doi: 10.1371/journal.pone.0155032.
[153] J. Schreml *et al.*, "Severe SMA mice show organ impairment that cannot be rescued by therapy with the HDACi JNJ-26481585," *Eur J Hum Genet*, vol. 21, no. 6, pp. 643–652, Jun. 2013, doi: 10.1038/ejhg.2012.222.
[154] S. E. Gombash *et al.*, "SMN deficiency disrupts gastrointestinal and enteric nervous system function in mice," *Hum Mol Genet*, vol. 24, no. 13, pp. 3847–3860, Jul. 2015, doi: 10.1093/hmg/ddv127.
[155] A. K. Datta, *Essentials of Human Embryology: General Embryology, Special Embryology and Human Genetics*. Current Books International., 1995.
[156] R. Zhao and S. A. Duncan, "Embryonic development of the liver," *Hepatology*, vol. 41, no. 5, Art. no. 5, May 2005, doi: 10.1002/hep.20691.
[157] B. M. Carlson, "Development of the Vascular System," in *Reference Module in Biomedical Sciences*, Elsevier, 2014. doi: 10.1016/B978-0-12-801238-3.05459-3.
[158] P. Van Eyken, R. Sciot, and V. Desmet, "Intrahepatic bile duct development in the rat: a cytokeratin-immunohistochemical study," *Lab Invest*, vol. 59, no. 1, Art. no. 1, Jul. 1988.
[159] P. Russo, "Liver," in *Color Atlas of Fetal and Neonatal Histology*, L. M. Ernst, E. D. Ruchelli, and D. S. Huff, Eds., New York, NY: Springer, 2011, pp. 67–77. doi: 10.1007/978-1-4614-0019-6_4.
[160] A. M. Zorn, "Liver development," in *StemBook*, Cambridge (MA): Harvard Stem Cell Institute, 2008. Accessed: Jul. 03, 2025. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK27068/
[161] D. D. Coovert *et al.*, "The survival motor neuron protein in spinal muscular atrophy," *Hum Mol Genet*, vol. 6, no. 8, Art. no. 8, Aug. 1997, doi: 10.1093/hmg/6.8.1205.
[162] E. Szunyogova *et al.*, "Survival Motor Neuron (SMN) protein is required for normal mouse liver development," *Sci Rep*, vol. 6, p. 34635, Oct. 2016, doi: 10.1038/srep34635.
[163] M. Deguise *et al.*, "Abnormal fatty acid metabolism is a core component of spinal muscular atrophy," *Ann Clin Transl Neurol*, vol. 6, no. 8, pp. 1519–1532, Jul. 2019, doi: 10.1002/acn3.50855.
[164] K. L. Moore and T. V. N. Persaud, *The Developing Human: Clinically Oriented Embryology*. Saunders, 2003.
[165] S. J. Pandol, "Pancreatic Embryology and Development," in *The Exocrine Pancreas*, Morgan & Claypool Life Sciences, 2010. Accessed: Jul. 03, 2025. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK54135/
[166] G. Da Silva Xavier, "The Cells of the Islets of Langerhans," *J Clin Med*, vol. 7, no. 3, p. 54, Mar. 2018, doi: 10.3390/jcm7030054.
[167] M. Bowerman *et al.*, "Glucose Metabolism and Pancreatic Defects in Spinal Muscular Atrophy," *Ann Neurol*, vol. 72, no. 2, pp. 256–268, Aug. 2012, doi: 10.1002/ana.23582.
[168] S. Rehman and D. Ahmed, "Embryology, Kidney, Bladder, and Ureter," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2025. Accessed: Jul. 02, 2025. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK547747/
[169] M. Nagata, "Glomerulogenesis and the role of endothelium," *Curr Opin Nephrol Hypertens*, vol. 27, no. 3, pp. 159–164, May 2018, doi: 10.1097/MNH.0000000000000402.
[170] K. K. Upadhyay and D. M. Silverstein, "Renal development: a complex process dependent on inductive interaction," *Curr Pediatr Rev*, vol. 10, no. 2, pp. 107–114, 2014, doi: 10.2174/1573396310021405131019 50.

[171] R. Viana *et al.*, "The development of the bladder trigone, the center of the anti-reflux mechanism," *Development*, vol. 134, no. 20, pp. 3763–3769, Oct. 2007, doi: 10.1242/dev.011270.
[172] H. Allardyce *et al.*, "Renal pathology in a mouse model of severe Spinal Muscular Atrophy is associated with downregulation of Glial Cell-Line Derived Neurotrophic Factor (GDNF)," *Human Molecular Genetics*, vol. 29, no. 14, pp. 2365–2378, Aug. 2020, doi: 10.1093/hmg/ddaa126.
[173] F. C. Nery *et al.*, "Impaired kidney structure and function in spinal muscular atrophy," *Neurol Genet*, vol. 5, no. 5, p. e353, Aug. 2019, doi: 10.1212/NXG.0000000000000353.
[174] G. M. Crane, Y.-C. Liu, and A. Chadburn, "Spleen: Development, anatomy and reactive lymphoid proliferations," *Seminars in Diagnostic Pathology*, vol. 38, no. 2, pp. 112–124, Mar. 2021, doi: 10.1053/j.semdp.2020.06.003.
[175] *Larsen's Human Embryology*. 2008. Accessed: Jul. 03, 2025. [Online]. Available: https://shop.elsevier.com/books/larsens-human-embryology/schoenwolf/978-0-443-06811-9
[176] V. Kapila, C. J. Wehrle, and F. Tuma, "Physiology, Spleen," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2023. Accessed: Jan. 03, 2024. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK537307/
[177] S. M. Lewis, A. Williams, and S. C. Eisenbarth, "Structure-function of the immune system in the spleen," *Sci Immunol*, vol. 4, no. 33, p. eaau6085, Mar. 2019, doi: 10.1126/sciimmunol.aau6085.
[178] V. Bronte and M. J. Pittet, "The spleen in local and systemic regulation of immunity," *Immunity*, vol. 39, no. 5, pp. 806–818, Nov. 2013, doi: 10.1016/j.immuni.2013.10.010.
[179] M.-O. Deguise, Y. De Repentigny, E. McFall, N. Auclair, S. Sad, and R. Kothary, "Immune dysregulation may contribute to disease pathogenesis in spinal muscular atrophy mice," *Hum Mol Genet*, vol. 26, no. 4, pp. 801–819, Feb. 2017, doi: 10.1093/hmg/ddw434.
[180] M.-T. Khairallah, J. Astroski, S. K. Custer, E. J. Androphy, C. L. Franklin, and C. L. Lorson, "SMN deficiency negatively impacts red pulp macrophages and spleen development in mouse models of spinal muscular atrophy," *Hum Mol Genet*, vol. 26, no. 5, pp. 932–941, Mar. 2017, doi: 10.1093/hmg/ddx008.
[181] T. Boehm, I. Hess, and J. B. Swann, "Evolution of lymphoid tissues," *Trends in Immunology*, vol. 33, no. 6, pp. 315–321, Jun. 2012, doi: 10.1016/j.it.2012.02.005.
[182] P. Thapa and D. L. Farber, "The Role of the Thymus in the Immune Response," *Thorac Surg Clin*, vol. 29, no. 2, pp. 123–131, May 2019, doi: 10.1016/j.thorsurg.2018.12.001.
[183] J. Perera and H. Huang, "The development and function of thymic B cells," *Cell Mol Life Sci*, vol. 72, no. 14, pp. 2657–2663, Jul. 2015, doi: 10.1007/s00018-015-1895-1.
[184] A. H. Jobe and M. Ikegami, "Lung development and function in preterm infants in the surfactant treatment era," *Annu Rev Physiol*, vol. 62, pp. 825–846, 2000, doi: 10.1146/annurev.physiol.62.1.825.
[185] W. M. Thurlbeck, "Postnatal human lung growth.," *Thorax*, vol. 37, no. 8, pp. 564–571, Aug. 1982, doi: 10.1136/thx.37.8.564.
[186] F. Trucco *et al.*, "Respiratory Trajectories in Type 2 and 3 Spinal Muscular Atrophy in the iSMAC Cohort Study," *Neurology*, vol. 96, no. 4, pp. e587–e599, Jan. 2021, doi: 10.1212/WNL.0000000000011051.

[187] G. Hamilton and T. H. Gillingwater, “Spinal muscular atrophy: going beyond the motor neuron,” *Trends Mol Med*, vol. 19, no. 1, pp. 40–50, Jan. 2013, doi: 10.1016/j.molmed.2012.11.002.

[188] M. Bowerman, J.-P. Michalski, A. Beauvais, L. M. Murray, Y. DeRepentigny, and R. Kothary, “Defects in pancreatic development and glucose metabolism in SMN-depleted mice independent of canonical spinal muscular atrophy neuromuscular pathology,” *Hum Mol Genet*, vol. 23, no. 13, pp. 3432–3444, Jul. 2014, doi: 10.1093/hmg/ddu052.

[189] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, “NIH Image to ImageJ: 25 years of image analysis,” *Nat Methods*, vol. 9, no. 7, Art. no. 7, Jul. 2012, doi: 10.1038/nmeth.2089.

[190] N. Watanabe *et al.*, “Hassall’s corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus,” *Nature*, vol. 436, no. 7054, pp. 1181–1185, Aug. 2005, doi: 10.1038/nature03886.

[191] W. D. Arnold, D. Kassar, and J. T. Kissel, “Spinal muscular atrophy: diagnosis and management in a new therapeutic era,” *Muscle Nerve*, vol. 51, no. 2, pp. 157–167, Feb. 2015, doi: 10.1002/mus.24497.

[192] C. A. Stuart *et al.*, “Myosin content of individual human muscle fibers isolated by laser capture microdissection,” *Am J Physiol Cell Physiol*, vol. 310, no. 5, pp. C381-389, Mar. 2016, doi: 10.1152/ajpcell.00317.2015.

[193] C. R. Vanderburg and M. S. F. Clarke, “Laser capture microdissection of metachromatically stained skeletal muscle allows quantification of fiber type specific gene expression,” *Mol Cell Biochem*, vol. 375, no. 1–2, pp. 159–170, Mar. 2013, doi: 10.1007/s11010-012-1538-x.

[194] R. Martínez-Hernández *et al.*, “Synaptic defects in type I spinal muscular atrophy in human development,” *The Journal of Pathology*, vol. 229, no. 1, Art. no. 1, 2013, doi: 10.1002/path.4080.

[195] E. Abati, A. Manini, G. P. Comi, and S. Corti, “Inhibition of myostatin and related signaling pathways for the treatment of muscle atrophy in motor neuron diseases,” *Cell Mol Life Sci*, vol. 79, no. 7, p. 374, Jun. 2022, doi: 10.1007/s00018-022-04408-w.

[196] M. Marotta, Y. Sarria, C. Ruiz-Roig, F. Munell, and M. Roig-Quilis, “Laser microdissection-based expression analysis of key genes involved in muscle regeneration in mdx mice,” *Neuromuscul Disord*, vol. 17, no. 9–10, pp. 707–718, Oct. 2007, doi: 10.1016/j.nmd.2007.05.007.

[197] R. A. Irizarry *et al.*, “Exploration, normalization, and summaries of high density oligonucleotide array probe level data,” *Biostatistics*, vol. 4, no. 2, pp. 249–264, Apr. 2003, doi: 10.1093/biostatistics/4.2.249.

[198] Y. Benjamini and Y. Hochberg, “Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing,” *Journal of the Royal Statistical Society. Series B (Methodological)*, vol. 57, no. 1, pp. 289–300, 1995.

[199] R. Gentleman, V. Carey, W. Huber, R. Irizarry, and S. Dudoit, *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. 2005. doi: 10.1007/0-387-29362-0.

[200] R. Sartori, V. Romanello, and M. Sandri, “Mechanisms of muscle atrophy and hypertrophy: implications in health and disease,” *Nat Commun*, vol. 12, no. 1, p. 330, Jan. 2021, doi: 10.1038/s41467-020-20123-1.

[201] R. A. Maselli *et al.*, “Mutations in MUSK causing congenital myasthenic syndrome impair MuSK–Dok-7 interaction,” *Hum Mol Genet*, vol. 19, no. 12, pp. 2370–2379, Jun. 2010, doi: 10.1093/hmg/ddq110.

[202] M. Buckingham and F. Relaix, “PAX3 and PAX7 as upstream regulators of myogenesis,” *Semin Cell Dev Biol*, vol. 44, pp. 115–125, Aug. 2015, doi: 10.1016/j.semcdb.2015.09.017.
[203] F. Chemello *et al.*, “Degenerative and regenerative pathways underlying Duchenne muscular dystrophy revealed by single-nucleus RNA sequencing,” *Proc Natl Acad Sci U S A*, vol. 117, no. 47, pp. 29691–29701, Nov. 2020, doi: 10.1073/pnas.2018391117.
[204] M. Sandri *et al.*, “Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy,” *Cell*, vol. 117, no. 3, pp. 399–412, Apr. 2004, doi: 10.1016/s0092-8674(04)00400-3.
[205] M. R. Emmert-Buck *et al.*, “Laser capture microdissection,” *Science*, vol. 274, no. 5289, pp. 998–1001, Nov. 1996, doi: 10.1126/science.274.5289.998.
[206] L. Cheng *et al.*, “Molecular Characterization of Testicular Germ Cell Tumors Using Tissue Microdissection,” *Methods Mol Biol*, vol. 2195, pp. 31–47, 2021, doi: 10.1007/978-1-0716-0860-9_3.
[207] A. P. Fuller, D. Palmer-Toy, M. G. Erlander, and D. C. Sgroi, “Laser capture microdissection and advanced molecular analysis of human breast cancer,” *J Mammary Gland Biol Neoplasia*, vol. 8, no. 3, pp. 335–345, Jul. 2003, doi: 10.1023/b:jomg.0000010033.49464.0c.
[208] A. Thennavan, M. Sharma, C. Chandrashekar, K. Hunter, and R. Radhakrishnan, “Exploring the potential of laser capture microdissection technology in integrated oral biosciences,” *Oral Dis*, vol. 23, no. 6, pp. 737–748, Sep. 2017, doi: 10.1111/odi.12578.
[209] S. H. Chung and W. Shen, “Laser capture microdissection: from its principle to applications in research on neurodegeneration,” *Neural Regen Res*, vol. 10, no. 6, pp. 897–898, Jun. 2015, doi: 10.4103/1673-5374.158346.
[210] D. G. Standaert, “Applications of laser capture microdissection in the study of neurodegenerative disease,” *Arch Neurol*, vol. 62, no. 2, pp. 203–205, Feb. 2005, doi: 10.1001/archneur.62.2.203.
[211] Z. Cao, J. Wanagat, S. H. McKiernan, and J. M. Aiken, “Mitochondrial DNA deletion mutations are concomitant with ragged red regions of individual, aged muscle fibers: analysis by laser-capture microdissection,” *Nucleic Acids Res*, vol. 29, no. 21, pp. 4502–4508, Nov. 2001, doi: 10.1093/nar/29.21.4502.
[212] J. Wanagat, Z. Cao, P. Pathare, and J. M. Aiken, “Mitochondrial DNA deletion mutations colocalize with segmental electron transport system abnormalities, muscle fiber atrophy, fiber splitting, and oxidative damage in sarcopenia,” *FASEB J*, vol. 15, no. 2, pp. 322–332, Feb. 2001, doi: 10.1096/fj.00-0320com.
[213] D. Pistilli *et al.*, “Detection of deleted mitochondrial DNA in Kearns-Sayre syndrome using laser capture microdissection,” *Human Pathology*, vol. 34, no. 10, pp. 1058–1061, Oct. 2003, doi: 10.1053/S0046-8177(03)00344-7.
[214] N. G. Gokey *et al.*, “Molecular analyses of mtDNA deletion mutations in microdissected skeletal muscle fibers from aged rhesus monkeys,” *Aging Cell*, vol. 3, no. 5, pp. 319–326, 2004, doi: 10.1111/j.1474-9728.2004.00122.x.
[215] M. Milkiewicz and T. L. Haas, “EFFECT OF MECHANICAL STRETCH ON HIF-1α AND MMP-2 EXPRESSION IN CAPILLARIES ISOLATED FROM OVERLOADED SKELETAL MUSCLES: LASER-CAPTURE MICRODISSECTION STUDY,” *https://doi.org/10.1152/ajpheart.00284.2005*, May 2005, doi: 10.1152/ajpheart.00284.2005.
[216] M. Hofbauer *et al.*, “Clonal tracking of autoaggressive T cells in polymyositis by combining laser microdissection, single-cell PCR, and CDR3-spectratype

analysis," *Proc Natl Acad Sci U S A*, vol. 100, no. 7, pp. 4090–4095, Apr. 2003, doi: 10.1073/pnas.0236183100.

[217] T. Chiba *et al.*, "Identification of fasting-induced genes in the rat hypothalamus: relationship with neuroprotection," *Ann N Y Acad Sci*, vol. 1119, pp. 216–226, Nov. 2007, doi: 10.1196/annals.1404.013.

[218] A. D. Posey, A. Demonbreun, and E. M. McNally, "Ferlin proteins in myoblast fusion and muscle growth," *Curr Top Dev Biol*, vol. 96, pp. 203–230, 2011, doi: 10.1016/B978-0-12-385940-2.00008-5.

[219] M. Buckingham *et al.*, "The formation of skeletal muscle: from somite to limb," *J Anat*, vol. 202, no. 1, pp. 59–68, Jan. 2003, doi: 10.1046/j.1469-7580.2003.00139.x.

[220] C. E. Davis, J. B. Harris, and L. V. Nicholson, "Myosin isoform transitions and physiological properties of regenerated and re-innervated soleus muscles of the rat," *Neuromuscul Disord*, vol. 1, no. 6, pp. 411–421, 1991, doi: 10.1016/0960-8966(91)90004-c.

[221] M. Ecob-Prince, M. Hill, and W. Brown, "Immunocytochemical demonstration of myosin heavy chain expression in human muscle," *J Neurol Sci*, vol. 91, no. 1–2, pp. 71–78, Jun. 1989, doi: 10.1016/0022-510x(89)90076-2.

[222] R. G. Whalen, "Myosin isoenzymes as molecular markers for muscle physiology," *J Exp Biol*, vol. 115, pp. 43–53, Mar. 1985, doi: 10.1242/jeb.115.1.43.

[223] A. Lek, F. J. Evesson, R. B. Sutton, K. N. North, and S. T. Cooper, "Ferlins: regulators of vesicle fusion for auditory neurotransmission, receptor trafficking and membrane repair," *Traffic*, vol. 13, no. 2, pp. 185–194, Feb. 2012, doi: 10.1111/j.1600-0854.2011.01267.x.

[224] R. Han and K. P. Campbell, "Dysferlin and Muscle Membrane Repair," *Curr Opin Cell Biol*, vol. 19, no. 4, pp. 409–416, Aug. 2007, doi: 10.1016/j.ceb.2007.07.001.

[225] S.-J. Lee, "Targeting the myostatin signaling pathway to treat muscle loss and metabolic dysfunction," *J Clin Invest*, vol. 131, no. 9, pp. e148372, 148372, May 2021, doi: 10.1172/JCI148372.

[226] F. F. Rose, V. B. Mattis, H. Rindt, and C. L. Lorson, "Delivery of recombinant follistatin lessens disease severity in a mouse model of spinal muscular atrophy," *Hum Mol Genet*, vol. 18, no. 6, pp. 997–1005, Mar. 2009, doi: 10.1093/hmg/ddn426.

[227] A. L. A. de Albuquerque *et al.*, "Serum myostatin as a candidate disease severity and progression biomarker of spinal muscular atrophy," *Brain Commun*, vol. 6, no. 2, p. fcae062, 2024, doi: 10.1093/braincomms/fcae062.

[228] T. M. DeChiara *et al.*, "The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo," *Cell*, vol. 85, no. 4, pp. 501–512, May 1996, doi: 10.1016/s0092-8674(00)81251-9.

[229] M. Boido *et al.*, "Increasing Agrin Function Antagonizes Muscle Atrophy and Motor Impairment in Spinal Muscular Atrophy," *Front Cell Neurosci*, vol. 12, p. 17, Jan. 2018, doi: 10.3389/fncel.2018.00017.

[230] M. B. Tan-Sindhunata *et al.*, "Identification of a Dutch founder mutation in MUSK causing fetal akinesia deformation sequence," *Eur J Hum Genet*, vol. 23, no. 9, pp. 1151–1157, Sep. 2015, doi: 10.1038/ejhg.2014.273.

[231] Z. Feng *et al.*, "Activation of Muscle-Specific Kinase (MuSK) Reduces Neuromuscular Defects in the Delta7 Mouse Model of Spinal Muscular Atrophy (SMA)," *Int J Mol Sci*, vol. 22, no. 15, p. 8015, Jul. 2021, doi: 10.3390/ijms22158015.

[232] E. A. Sugarman *et al.*, "Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens," *Eur J Hum Genet*, vol. 20, no. 1, Art. no. 1, Jan. 2012, doi: 10.1038/ejhg.2011.134.
[233] C. L. Lorson, E. Hahnen, E. J. Androphy, and B. Wirth, "A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy," *Proc Natl Acad Sci U S A*, vol. 96, no. 11, pp. 6307–6311, May 1999, doi: 10.1073/pnas.96.11.6307.
[234] R. N. Singh, M. D. Howell, E. W. Ottesen, and N. N. Singh, "Diverse role of Survival Motor Neuron Protein," *Biochim Biophys Acta*, vol. 1860, no. 3, pp. 299–315, Mar. 2017, doi: 10.1016/j.bbagrm.2016.12.008.
[235] S. Jablonka, S. Wiese, and M. Sendtner, "Axonal defects in mouse models of motoneuron disease," *J Neurobiol*, vol. 58, no. 2, pp. 272–286, Feb. 2004, doi: 10.1002/neu.10313.
[236] S. Pagliardini *et al.*, "Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord," *Hum Mol Genet*, vol. 9, no. 1, pp. 47–56, Jan. 2000, doi: 10.1093/hmg/9.1.47.
[237] E. Mercuri, C. J. Sumner, F. Muntoni, B. T. Darras, and R. S. Finkel, "Spinal muscular atrophy," *Nat Rev Dis Primers*, vol. 8, no. 1, p. 52, Aug. 2022, doi: 10.1038/s41572-022-00380-8.
[238] J. M. Cobben, H. H. Lemmink, I. Snoeck, P. A. Barth, J. H. van der Lee, and M. de Visser, "Survival in SMA type I: a prospective analysis of 34 consecutive cases," *Neuromuscul Disord*, vol. 18, no. 7, pp. 541–544, Jul. 2008, doi: 10.1016/j.nmd.2008.05.008.
[239] R. Finkel, E. Bertini, F. Muntoni, and E. Mercuri, "209th ENMC International Workshop: Outcome Measures and Clinical Trial Readiness in Spinal Muscular Atrophy 7–9 November 2014, Heemskerk, The Netherlands," *Neuromuscular Disorders*, vol. 25, no. 7, Art. no. 7, Jul. 2015, doi: 10.1016/j.nmd.2015.04.009.
[240] S. J. Kolb and J. T. Kissel, "Spinal Muscular Atrophy: A Timely Review," *Archives of Neurology*, vol. 68, no. 8, Art. no. 8, Aug. 2011, doi: 10.1001/archneurol.2011.74.
[241] K. Zerres and S. Rudnik-Schöneborn, "Natural history in proximal spinal muscular atrophy. Clinical analysis of 445 patients and suggestions for a modification of existing classifications," *Arch Neurol*, vol. 52, no. 5, pp. 518–523, May 1995, doi: 10.1001/archneur.1995.00540290108025.
[242] P. Kaufmann *et al.*, "Prospective cohort study of spinal muscular atrophy types 2 and 3," *Neurology*, vol. 79, no. 18, pp. 1889–1897, Oct. 2012, doi: 10.1212/WNL.0b013e318271f7e4.
[243] C. A. Wijngaarde *et al.*, "Natural course of scoliosis and lifetime risk of scoliosis surgery in spinal muscular atrophy," *Neurology*, vol. 93, no. 2, pp. e149–e158, Jul. 2019, doi: 10.1212/WNL.0000000000007742.
[244] M. E. R. Butchbach, "Genomic Variability in the Survival Motor Neuron Genes (SMN1 and SMN2): Implications for Spinal Muscular Atrophy Phenotype and Therapeutics Development," *Int J Mol Sci*, vol. 22, no. 15, Art. no. 15, Jul. 2021, doi: 10.3390/ijms22157896.
[245] M. Costa-Roger, L. Blasco-Pérez, I. Cuscó, and E. F. Tizzano, "The Importance of Digging into the Genetics of SMN Genes in the Therapeutic Scenario of Spinal Muscular Atrophy," *Int J Mol Sci*, vol. 22, no. 16, Art. no. 16, Aug. 2021, doi: 10.3390/ijms22169029.
[246] L. Kong *et al.*, "Impaired prenatal motor axon development necessitates early therapeutic intervention in severe SMA," *Science Translational Medicine*, vol. 13, no. 578, Art. no. 578, Jan. 2021, doi: 10.1126/scitranslmed.abb6871.

[247] D. M. Ramos *et al.*, “Age-dependent SMN expression in disease-relevant tissue and implications for SMA treatment,” *The Journal of clinical investigation*, vol. 129, no. 11, Art. no. 11, Nov. 2019, doi: 10.1172/JCI124120.

[248] C. Soler-Botija, I. Cuscó, L. Caselles, E. López, M. Baiget, and E. F. Tizzano, “Implication of Fetal SMN2 Expression in Type I SMA Pathogenesis: Protection or Pathological Gain of Function?,” *Journal of Neuropathology & Experimental Neurology*, vol. 64, no. 3, Art. no. 3, Mar. 2005, doi: 10.1093/jnen/64.3.215.

[249] A. Totzeck *et al.*, “Neurofilament Heavy Chain and Tau Protein Are Not Elevated in Cerebrospinal Fluid of Adult Patients with Spinal Muscular Atrophy during Loading with Nusinersen,” *Int J Mol Sci*, vol. 20, no. 21, p. 5397, Oct. 2019, doi: 10.3390/ijms20215397.

[250] R. Martínez-Hernández *et al.*, “The developmental pattern of myotubes in spinal muscular atrophy indicates prenatal delay of muscle maturation,” *J Neuropathol Exp Neurol*, vol. 68, no. 5, Art. no. 5, May 2009, doi: 10.1097/NEN.0b013e3181a10ea1.

[251] K. Ma *et al.*, “Real-world evidence: Risdiplam in a patient with spinal muscular atrophy type I with a novel splicing mutation and one SMN2 copy,” *Human Molecular Genetics*, vol. 33, no. 13, pp. 1120–1130, Jul. 2024, doi: 10.1093/hmg/ddae052.

[252] C. Votsi *et al.*, “Spinal muscular atrophy type I associated with a novel SMN1 splicing variant that disrupts the expression of the functional transcript,” *Front Neurol*, vol. 14, p. 1241195, 2023, doi: 10.3389/fneur.2023.1241195.

[253] C. Chen *et al.*, “Noninvasive prenatal testing of α-thalassemia and β-thalassemia through population-based parental haplotyping,” *Genome Med*, vol. 13, no. 1, p. 18, Feb. 2021, doi: 10.1186/s13073-021-00836-8.

[254] M. Parks *et al.*, “Non-invasive prenatal diagnosis of spinal muscular atrophy by relative haplotype dosage,” *Eur J Hum Genet*, vol. 25, no. 4, Art. no. 4, Apr. 2017, doi: 10.1038/ejhg.2016.195.

[255] A. Girardet, C. Fernandez, and M. Claustres, “Efficient strategies for preimplantation genetic diagnosis of spinal muscular atrophy,” *Fertil Steril*, vol. 90, no. 2, p. 443.e7–12, Aug. 2008, doi: 10.1016/j.fertnstert.2007.07.1305.

[256] C. Serra-Juhe and E. F. Tizzano, “Perspectives in genetic counseling for spinal muscular atrophy in the new therapeutic era: early pre-symptomatic intervention and test in minors,” *Eur J Hum Genet*, vol. 27, no. 12, pp. 1774–1782, Dec. 2019, doi: 10.1038/s41431-019-0415-4.

[257] M. G. Pino, K. A. Rich, and S. J. Kolb, “Update on Biomarkers in Spinal Muscular Atrophy,” *Biomark Insights*, vol. 16, p. 11772719211035643, Aug. 2021, doi: 10.1177/11772719211035643.

[258] N. C. Peddi, C. Avanthika, S. Vuppalapati, R. Balasubramanian, J. Kaur, and C. D. N, “A Review of Cordocentesis: Percutaneous Umbilical Cord Blood Sampling,” *Cureus*, vol. 13, no. 7, p. e16423, Jul. 2021, doi: 10.7759/cureus.16423.

[259] H. Schmalbruch, “The effect of peripheral nerve injury on immature motor and sensory neurons and on muscle fibres. Possible relation to the histogenesis of Werdnig-Hoffmann disease,” *Rev Neurol (Paris)*, vol. 144, no. 11, pp. 721–729, 1988.

[260] E. Udina, C. T. Putman, L. R. Harris, N. Tyreman, V. E. Cook, and T. Gordon, “Compensatory axon sprouting for very slow axonal die-back in a transgenic model of spinal muscular atrophy type III,” *J Physiol*, vol. 595, no. 5, pp. 1815–1829, Mar. 2017, doi: 10.1113/JP273404.

[261] V. Vacchiano *et al.*, "Motor unit number estimation via MScanFit MUNE in spinal muscular atrophy," *Muscle Nerve*, vol. 70, no. 1, pp. 71–81, Jul. 2024, doi: 10.1002/mus.28091.
[262] R. S. Finkel *et al.*, "RESTORE: A Prospective Multinational Registry of Patients with Genetically Confirmed Spinal Muscular Atrophy - Rationale and Study Design," *J Neuromuscul Dis*, vol. 7, no. 2, pp. 145–152, 2020, doi: 10.3233/JND-190451.
[263] A. Pechmann *et al.*, "SMArtCARE - A platform to collect real-life outcome data of patients with spinal muscular atrophy," *Orphanet J Rare Dis*, vol. 14, no. 1, p. 18, Jan. 2019, doi: 10.1186/s13023-019-0998-4.
[264] E. F. Tizzano, "Treating neonatal spinal muscular atrophy: A 21st century success story?," *Early Hum Dev*, vol. 138, p. 104851, Nov. 2019, doi: 10.1016/j.earlhumdev.2019.104851.
[265] M. Pane *et al.*, "Neurological assessment of newborns with spinal muscular atrophy identified through neonatal screening," *Eur J Pediatr*, vol. 181, no. 7, pp. 2821–2829, Jul. 2022, doi: 10.1007/s00431-022-04470-3.
[266] M. Khalil *et al.*, "Neurofilaments as biomarkers in neurological disorders," *Nat Rev Neurol*, vol. 14, no. 10, Art. no. 10, Oct. 2018, doi: 10.1038/s41582-018-0058-z.
[267] R. S. Finkel and M. Benatar, "Pre-symptomatic spinal muscular atrophy: a proposed nosology," *Brain*, vol. 145, no. 7, pp. 2247–2249, Jul. 2022, doi: 10.1093/brain/awac125.
[268] M. Van Alstyne *et al.*, "Gain of toxic function by long-term AAV9-mediated SMN overexpression in the sensorimotor circuit," *Nat Neurosci*, vol. 24, no. 7, Art. no. 7, Jul. 2021, doi: 10.1038/s41593-021-00827-3.
[269] S. Jablonka, L. Hennlein, and M. Sendtner, "Therapy development for spinal muscular atrophy: perspectives for muscular dystrophies and neurodegenerative disorders," *Neurol Res Pract*, vol. 4, no. 1, p. 2, Jan. 2022, doi: 10.1186/s42466-021-00162-9.
[270] R. I. Wadman *et al.*, "Drug treatment for spinal muscular atrophy types II and III," *Cochrane Database of Systematic Reviews*, no. 1, Art. no. 1, 2020, doi: 10.1002/14651858.CD006282.pub5.
[271] I. Papaioannou, J. S. Owen, and R. J. Yáñez-Muñoz, "Clinical applications of gene therapy for rare diseases: A review," *International Journal of Experimental Pathology*, vol. n/a, no. n/a, Art. no. n/a, Jun. 2023, doi: 10.1111/iep.12478.
[272] L. Ferrante, A. Melendez-Zaidi, W. Lindsey, and T. Lotze, "Novel use of nusinersen as a therapeutic bridge to onasemnogene abeparvovec-xioi in a premature neonate with type 1 spinal muscular atrophy," *Muscle Nerve*, vol. 66, no. 2, pp. E8–E10, Aug. 2022, doi: 10.1002/mus.27648.
[273] R. Trollmann *et al.*, "Postnatal management of preterm infants with spinal muscular atrophy: experience from German newborn screening," *Orphanet J Rare Dis*, vol. 19, no. 1, p. 353, Sep. 2024, doi: 10.1186/s13023-024-03362-z.
[274] S. M. Brown *et al.*, "Onasemnogene-abeparvovec administration to premature infants with spinal muscular atrophy," *Ann Clin Transl Neurol*, vol. 11, no. 11, pp. 3042–3046, Nov. 2024, doi: 10.1002/acn3.52213.
[275] C. M. Proud *et al.*, "Combination disease-modifying treatment in spinal muscular atrophy: A proposed classification," *Ann Clin Transl Neurol*, vol. 10, no. 11, pp. 2155–2160, Nov. 2023, doi: 10.1002/acn3.51889.
[276] L. Servais *et al.*, "Real-World Outcomes in Patients with Spinal Muscular Atrophy Treated with Onasemnogene Abeparvovec Monotherapy: Findings from the RESTORE Registry," *J Neuromuscul Dis*, vol. 11, no. 2, pp. 425–442, 2024, doi: 10.3233/JND-230122.

[277] G. Prat-Ortega *et al.*, "First-in-human study of epidural spinal cord stimulation in individuals with spinal muscular atrophy," *Nat Med*, Feb. 2025, doi: 10.1038/s41591-024-03484-8.
[278] A. Herzeg *et al.*, "Prenatal Somatic Cell Gene Therapies: Charting a Path Toward Clinical Applications (Proceedings of the CERSI-FDA Meeting)," *J Clin Pharmacol*, vol. 62 Suppl 1, no. Suppl 1, Art. no. Suppl 1, Sep. 2022, doi: 10.1002/jcph.2127.
[279] H. D. Baumgarten and A. W. Flake, "Fetal Surgery," *Pediatr Clin North Am*, vol. 66, no. 2, pp. 295–308, Apr. 2019, doi: 10.1016/j.pcl.2018.12.001.
[280] F. Shakuntulla and S. E. Chiarella, "Safety of Biologics for Atopic Diseases During Pregnancy," *J Allergy Clin Immunol Pract*, vol. 10, no. 12, Art. no. 12, Dec. 2022, doi: 10.1016/j.jaip.2022.08.013.
[281] T. C. MacKenzie *et al.*, "In Utero Stem Cell Transplantation in Patients with Alpha Thalassemia Major: Interim Results of a Phase 1 Clinical Trial," *Blood*, vol. 136, no. Supplement 1, p. 1, Nov. 2020, doi: 10.1182/blood-2020-142698.
[282] R. Sagar *et al.*, "Fetal and Maternal Safety Considerations for In Utero Therapy Clinical Trials: iFeTiS Consensus Statement," *Molecular Therapy*, vol. 28, no. 11, pp. 2316–2319, Nov. 2020, doi: 10.1016/j.ymthe.2020.10.012.
[283] J. L. Cohen *et al.*, "In Utero Enzyme-Replacement Therapy for Infantile-Onset Pompe's Disease," *N Engl J Med*, vol. 387, no. 23, pp. 2150–2158, Dec. 2022, doi: 10.1056/NEJMoa2200587.
[284] H. Schneider *et al.*, "Protocol for the Phase 2 EDELIFE Trial Investigating the Efficacy and Safety of Intra-Amniotic ER004 Administration to Male Subjects with X-Linked Hypohidrotic Ectodermal Dysplasia," *Genes (Basel)*, vol. 14, no. 1, p. 153, Jan. 2023, doi: 10.3390/genes14010153.
[285] H. Schneider, C. Schweikl, F. Faschingbauer, S. Hadj-Rabia, and P. Schneider, "A Causal Treatment for X-Linked Hypohidrotic Ectodermal Dysplasia: Long-Term Results of Short-Term Perinatal Ectodysplasin A1 Replacement," *Int J Mol Sci*, vol. 24, no. 8, p. 7155, Apr. 2023, doi: 10.3390/ijms24087155.
[286] H. Schneider *et al.*, "Prenatal Correction of X-Linked Hypohidrotic Ectodermal Dysplasia," *N Engl J Med*, vol. 378, no. 17, pp. 1604–1610, Apr. 2018, doi: 10.1056/NEJMoa1714322.
[287] D. P. Chauhan, A. S. Srivastava, M. E. Moustafa, S. Shenouda, and E. Carrier, "In utero gene therapy: prospect and future," *Curr Pharm Des*, vol. 10, no. 29, Art. no. 29, 2004, doi: 10.2174/1381612043382828.
[288] S. N. Waddington, N. L. Kennea, S. M. K. Buckley, L. G. Gregory, M. Themis, and C. Coutelle, "Fetal and neonatal gene therapy: benefits and pitfalls," *Gene Ther*, vol. 11 Suppl 1, pp. S92-97, Oct. 2004, doi: 10.1038/sj.gt.3302375.
[289] S. N. Waddington *et al.*, "In utero gene therapy: current challenges and perspectives," *Mol Ther*, vol. 11, no. 5, Art. no. 5, May 2005, doi: 10.1016/j.ymthe.2005.01.015.
[290] A. C. Rossidis *et al.*, "In utero CRISPR-mediated therapeutic editing of metabolic genes," *Nat Med*, vol. 24, no. 10, Art. no. 10, Oct. 2018, doi: 10.1038/s41591-018-0184-6.
[291] C. T. Nicolas *et al.*, "In vivo lentiviral vector gene therapy to cure hereditary tyrosinemia type 1 and prevent development of precancerous and cancerous lesions," *Nature Communications*, vol. 13, 2022, doi: 10.1038/s41467-022-32576-7.
[292] A. S. Ricciardi *et al.*, "In utero nanoparticle delivery for site-specific genome editing," *Nat Commun*, vol. 9, no. 1, Art. no. 1, Jun. 2018, doi: 10.1038/s41467-018-04894-2.

[293] P. Shangaris *et al.*, "In Utero Gene Therapy (IUGT) Using GLOBE Lentiviral Vector Phenotypically Corrects the Heterozygous Humanised Mouse Model and Its Progress Can Be Monitored Using MRI Techniques," *Sci Rep*, vol. 9, no. 1, Art. no. 1, Aug. 2019, doi: 10.1038/s41598-019-48078-4.

[294] J. K. Y. Chan *et al.*, "Therapeutic expression of human clotting factors IX and X following adeno-associated viral vector-mediated intrauterine gene transfer in early-gestation fetal macaques," *FASEB J*, vol. 33, no. 3, Art. no. 3, Mar. 2019, doi: 10.1096/fj.201801391R.

[295] C. N. Z. Mattar *et al.*, "Stable human FIX expression after 0.9G intrauterine gene transfer of self-complementary adeno-associated viral vector 5 and 8 in macaques," *Mol Ther*, vol. 19, no. 11, Art. no. 11, Nov. 2011, doi: 10.1038/mt.2011.107.

[296] C. N. Z. Mattar *et al.*, "In Utero Transfer of Adeno-Associated Viral Vectors Produces Long-Term Factor IX Levels in a Cynomolgus Macaque Model," *Mol Ther*, vol. 25, no. 8, Art. no. 8, Aug. 2017, doi: 10.1016/j.ymthe.2017.04.003.

[297] G. Massaro *et al.*, "Fetal gene therapy for neurodegenerative disease of infants," *Nat Med*, vol. 24, no. 9, Art. no. 9, Sep. 2018, doi: 10.1038/s41591-018-0106-7.

[298] M. T. Clarke *et al.*, "Prenatal delivery of a therapeutic antisense oligonucleotide achieves broad biodistribution in the brain and ameliorates Angelman syndrome phenotype in mice," *Mol Ther*, vol. 32, no. 4, pp. 935–951, Apr. 2024, doi: 10.1016/j.ymthe.2024.02.004.

[299] D. J. Carr *et al.*, "Uteroplacental adenovirus vascular endothelial growth factor gene therapy increases fetal growth velocity in growth-restricted sheep pregnancies," *Hum Gene Ther*, vol. 25, no. 4, Art. no. 4, Apr. 2014, doi: 10.1089/hum.2013.214.

[300] A. L. David *et al.*, "Local delivery of VEGF adenovirus to the uterine artery increases vasorelaxation and uterine blood flow in the pregnant sheep," *Gene Ther*, vol. 15, no. 19, pp. 1344–1350, Oct. 2008, doi: 10.1038/gt.2008.102.

[301] A. M. Swanson *et al.*, "Maternal Therapy with Ad.VEGF-A165 Increases Fetal Weight at Term in a Guinea-Pig Model of Fetal Growth Restriction," *Hum Gene Ther*, vol. 27, no. 12, Art. no. 12, Dec. 2016, doi: 10.1089/hum.2016.046.

[302] A. Rashnonejad *et al.*, "Fetal Gene Therapy Using a Single Injection of Recombinant AAV9 Rescued SMA Phenotype in Mice," *Mol Ther*, vol. 27, no. 12, Art. no. 12, Dec. 2019, doi: 10.1016/j.ymthe.2019.08.017.

[303] S. Niewiesk, "Maternal antibodies: clinical significance, mechanism of interference with immune responses, and possible vaccination strategies," *Front Immunol*, vol. 5, p. 446, 2014, doi: 10.3389/fimmu.2014.00446.

[304] R. Calcedo *et al.*, "Adeno-Associated Virus Antibody Profiles in Newborns, Children, and Adolescents ▿," *Clin Vaccine Immunol*, vol. 18, no. 9, pp. 1586–1588, Sep. 2011, doi: 10.1128/CVI.05107-11.

[305] D. E. Sabatino *et al.*, "Persistent expression of hF.IX After tolerance induction by in utero or neonatal administration of AAV-1-F.IX in hemophilia B mice," *Mol Ther*, vol. 15, no. 9, pp. 1677–1685, Sep. 2007, doi: 10.1038/sj.mt.6300219.

[306] M. G. Davey *et al.*, "Induction of Immune Tolerance to Foreign Protein via Adeno-Associated Viral Vector Gene Transfer in Mid-Gestation Fetal Sheep," *PLoS One*, vol. 12, no. 1, Art. no. 1, 2017, doi: 10.1371/journal.pone.0171132.

[307] B. Borges *et al.*, "Prenatal AAV9-GFP administration in fetal lambs results in transduction of female germ cells and maternal exposure to virus," *Molecular Therapy Methods & Clinical Development*, vol. 32, no. 2, Jun. 2024, doi: 10.1016/j.omtm.2024.101263.

[308] T. C. MacKenzie *et al.*, "Efficient transduction of liver and muscle after in utero injection of lentiviral vectors with different pseudotypes," *Mol Ther*, vol. 6, no. 3, Art. no. 3, Sep. 2002, doi: 10.1006/mthe.2002.0681.
[309] C. Coutelle and R. Ashcroft, "Risks, benefits and ethical, legal, and societal considerations for translation of prenatal gene therapy to human application," *Methods Mol Biol*, vol. 891, pp. 371–387, 2012, doi: 10.1007/978-1-61779-873-3_17.
[310] M. Cavazzana, E. Six, C. Lagresle-Peyrou, I. André-Schmutz, and S. Hacein-Bey-Abina, "Gene Therapy for X-Linked Severe Combined Immunodeficiency: Where Do We Stand?," *Human Gene Therapy*, vol. 27, no. 2, Art. no. 2, Feb. 2016, doi: 10.1089/hum.2015.137.
[311] G. Almeida-Porada, S. N. Waddington, J. K. Y. Chan, W. H. Peranteau, T. MacKenzie, and C. D. Porada, "In Utero Gene Therapy Consensus Statement from the IFeTIS," *Mol Ther*, vol. 27, no. 4, Art. no. 4, Apr. 2019, doi: 10.1016/j.ymthe.2019.02.015.
[312] M. A. Farrar *et al.*, "Emerging therapies and challenges in spinal muscular atrophy," *Ann Neurol*, vol. 81, no. 3, Art. no. 3, Mar. 2017, doi: 10.1002/ana.24864.
[313] C. Baptiste and D. C. De Vivo, "Spinal Muscular Atrophy: A Potential Target for In Utero Therapy," *Clin Obstet Gynecol*, vol. 64, no. 4, pp. 917–925, Dec. 2021, doi: 10.1097/GRF.0000000000000654.
[314] F. F. Depreux *et al.*, "Antisense oligonucleotides delivered to the amniotic cavity in utero modulate gene expression in the postnatal mouse," *Nucleic Acids Res*, vol. 44, no. 20, pp. 9519–9529, Nov. 2016, doi: 10.1093/nar/gkw867.
[315] L. Wang *et al.*, "Fetal antisense oligonucleotide therapy for congenital deafness and vestibular dysfunction," *Nucleic Acids Res*, vol. 48, no. 9, pp. 5065–5080, May 2020, doi: 10.1093/nar/gkaa194.
[316] E. Gómez-Montes *et al.*, "Prenatal Cystic Fibrosis Transmembrane Conductance Regulator Modulator Therapy: A Promising Way to Change the Impact of Cystic Fibrosis," *Fetal Diagn Ther*, vol. 50, no. 2, Art. no. 2, 2023, doi: 10.1159/000530261.
[317] R. S. Finkel *et al.*, "Risdiplam for Prenatal Therapy of Spinal Muscular Atrophy," *N Engl J Med*, vol. 392, no. 11, pp. 1138–1140, Mar. 2025, doi: 10.1056/NEJMc2300802.
[318] C. J. J. Yeo, E. F. Tizzano, and B. T. Darras, "Challenges and opportunities in spinal muscular atrophy therapeutics," *Lancet Neurol*, vol. 23, no. 2, pp. 205–218, Feb. 2024, doi: 10.1016/S1474-4422(23)00419-2.
[319] P. Kamath, A. Kamath, and S. D. Ullal, "Liver injury associated with drug intake during pregnancy," *World J Hepatol*, vol. 13, no. 7, pp. 747–762, Jul. 2021, doi: 10.4254/wjh.v13.i7.747.
[320] H. Peluffo *et al.*, "Efficient gene expression from integration-deficient lentiviral vectors in the spinal cord," *Gene Ther*, vol. 20, no. 6, Art. no. 6, Jun. 2013, doi: 10.1038/gt.2012.78.
[321] A. A. Rahim *et al.*, "Efficient gene delivery to the adult and fetal CNS using pseudotyped non-integrating lentiviral vectors," *Gene Ther*, vol. 16, no. 4, Art. no. 4, Apr. 2009, doi: 10.1038/gt.2008.186.
[322] S. G. Ahmed, S. N. Waddington, M. G. Boza-Morán, and R. J. Yáñez-Muñoz, "High-efficiency transduction of spinal cord motor neurons by intrauterine delivery of integration-deficient lentiviral vectors," *Journal of Controlled Release*, vol. 273, pp. 99–107, Mar. 2018, doi: 10.1016/j.jconrel.2017.12.029.

[323] K. A. Rich *et al.*, "Premature delivery in the domestic sow in response to in utero delivery of AAV9 to fetal piglets," *Gene Ther*, vol. 29, no. 9, Art. no. 9, Sep. 2022, doi: 10.1038/s41434-021-00305-2.
[324] C. N. Z. Mattar, W. L. Chew, and P. S. Lai, "Embryo and fetal gene editing: Technical challenges and progress toward clinical applications," *Mol Ther Methods Clin Dev*, vol. 32, no. 2, p. 101229, Jun. 2024, doi: 10.1016/j.omtm.2024.101229.
[325] F. R. De Bie, T. Tate, and R. M. Antiel, "Maternal-fetal surgery as part of pediatric palliative care," *Semin Fetal Neonatal Med*, vol. 28, no. 3, p. 101440, Jun. 2023, doi: 10.1016/j.siny.2023.101440.
[326] S. K. Bose, P. Menon, and W. H. Peranteau, "In utero gene therapy: Progress and Challenges," *Trends Mol Med*, vol. 27, no. 8, Art. no. 8, Aug. 2021, doi: 10.1016/j.molmed.2021.05.007.
[327] I. D. Wolfe, J. B. Lillegard, and B. S. Carter, "Parental request for non-resuscitation in fetal myelomeningocele repair: an analysis of the novel ethical tensions in fetal intervention," *J Perinatol*, vol. 42, no. 7, Art. no. 7, Jul. 2022, doi: 10.1038/s41372-022-01317-5.
[328] E. Rovira-Moreno *et al.*, "The diagnosis communication process in spinal muscular atrophy: A cross-cutting view of the new challenges facing the therapeutic era," *Genetics in Medicine Open*, vol. 1, no. 1, p. 100825, Jan. 2023, doi: 10.1016/j.gimo.2023.100825.
[329] C. W. Lederer *et al.*, "Catching Them Early: Framework Parameters and Progress for Prenatal and Childhood Application of Advanced Therapies," *Pharmaceutics*, vol. 14, no. 4, p. 793, Apr. 2022, doi: 10.3390/pharmaceutics14040793.
[330] G. Baranello and Neurodevelopment in SMA Working Group, "The emerging spectrum of neurodevelopmental comorbidities in early-onset Spinal Muscular Atrophy," *Eur J Paediatr Neurol*, vol. 48, pp. 67–68, Jan. 2024, doi: 10.1016/j.ejpn.2023.11.006.
[331] M. Sheppard, R. N. Spencer, R. Ashcroft, A. L. David, and EVERREST Consortium, "Ethics and social acceptability of a proposed clinical trial using maternal gene therapy to treat severe early-onset fetal growth restriction," *Ultrasound Obstet Gynecol*, vol. 47, no. 4, pp. 484–491, Apr. 2016, doi: 10.1002/uog.15880.
[332] M. E. Schwab *et al.*, "Investigating attitudes toward prenatal diagnosis and fetal therapy for spinal muscular atrophy," *Prenat Diagn*, vol. 42, no. 11, pp. 1409–1419, Oct. 2022, doi: 10.1002/pd.6228.
[333] E. F. Tizzano, G. Lindner, E. Chilcott, R. S. Finkel, and R. J. Yáñez-Muñoz, "In utero therapy for spinal muscular atrophy: closer to clinical translation," *Brain*, p. awaf123, Apr. 2025, doi: 10.1093/brain/awaf123.
[334] M. Gaballa and C. A. Ramos, "Overview of Normal Hematopoiesis," in *Handbook of Benign Hematology*, M. P. Mims, C. Miller-Chism, and I. R. Sosa, Eds., New York: Springer Publishing Company, 2019, pp. 1–24. doi: 10.1891/9780826149879.0001.
[335] J. A. Chasis and N. Mohandas, "Red Blood Cell Glycophorins," *Blood*, vol. 80, no. 8, Art. no. 8, Oct. 1992, doi: 10.1182/blood.V80.8.1869.1869.
[336] B. Wan, P. Feng, Z. Guan, L. Sheng, Z. Liu, and Y. Hua, "A severe mouse model of spinal muscular atrophy develops early systemic inflammation," *Hum Mol Genet*, vol. 27, no. 23, pp. 4061–4076, Dec. 2018, doi: 10.1093/hmg/ddy300.
[337] E. D. Ruchelli and T. R. Bhatti, "Pancreas," in *Color Atlas of Human Fetal and Neonatal Histology*, L. M. Ernst, E. D. Ruchelli, C. K. Carreon, and D. S. Huff, Eds., Cham: Springer International Publishing, 2019, pp. 91–97. doi: 10.1007/978-3-030-11425-1_6.

[338] D. Bosco *et al.*, “Unique arrangement of alpha- and beta-cells in human islets of Langerhans,” *Diabetes*, vol. 59, no. 5, pp. 1202–1210, May 2010, doi: 10.2337/db09-1177.
[339] S. A. Djordjevic *et al.*, “Glucose and lipid metabolism disorders in children and adolescents with spinal muscular atrophy types 2 and 3,” *Neuromuscular Disorders*, vol. 31, no. 4, pp. 291–299, Apr. 2021, doi: 10.1016/j.nmd.2021.02.002.
[340] M.-O. Deguise *et al.*, “Abnormal fatty acid metabolism is a core component of spinal muscular atrophy,” *Ann Clin Transl Neurol*, vol. 6, no. 8, pp. 1519–1532, Aug. 2019, doi: 10.1002/acn3.50855.
[341] M. Miletić *et al.*, “Glucose and Lipid Metabolism Disorders in Adults with Spinal Muscular Atrophy Type 3,” *Diagnostics*, vol. 14, no. 18, Art. no. 18, Jan. 2024, doi: 10.3390/diagnostics14182078.
[342] K. G. Margolis and J. A. Picoraro, “90 - Development of Gastrointestinal Motility,” in *Fetal and Neonatal Physiology (Fifth Edition)*, R. A. Polin, S. H. Abman, D. H. Rowitch, W. E. Benitz, and W. W. Fox, Eds., Elsevier, 2017, pp. 881-888.e2. doi: 10.1016/B978-0-323-35214-7.00090-1.
[343] A. Corsello *et al.*, “Nutritional, Gastrointestinal and Endo-Metabolic Challenges in the Management of Children with Spinal Muscular Atrophy Type 1,” *Nutrients*, vol. 13, no. 7, p. 2400, Jul. 2021, doi: 10.3390/nu13072400.
[344] E. Boland, F. Quondamatteo, and T. Van Agtmael, “The role of basement membranes in cardiac biology and disease,” *Biosci Rep*, vol. 41, no. 8, p. BSR20204185, Aug. 2021, doi: 10.1042/BSR20204185.
[345] L. Lagae, M. Proesmans, M. Van den Hauwe, F. Vermeulen, L. De Waele, and M. Boon, “Respiratory morbidity in patients with spinal muscular atrophy-a changing world in the light of disease-modifying therapies,” *Front Pediatr*, vol. 12, p. 1366943, 2024, doi: 10.3389/fped.2024.1366943.
[346] B. Fauroux *et al.*, “Respiratory management of children with spinal muscular atrophy (SMA),” *Arch Pediatr*, vol. 27, no. 7S, pp. 7S29-27S34, Dec. 2020, doi: 10.1016/S0929-693X(20)30274-8.
[347] A. Gök, S. K. Saygılı, S. Kuruğoğlu, S. Saltık, and N. Canpolat, “Children With Type 1 Spinal Muscular Atrophy Are at Increased Risk for Nephrolithiasis,” *Pediatr Neurol*, vol. 150, pp. 32–36, Jan. 2024, doi: 10.1016/j.pediatrneurol.2023.10.006.
[348] M. F. Cesta, “Normal structure, function, and histology of the spleen,” *Toxicol Pathol*, vol. 34, no. 5, pp. 455–465, 2006, doi: 10.1080/01926230600867743.
[349] M. Wieczorek *et al.*, “Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation,” *Front Immunol*, vol. 8, p. 292, Mar. 2017, doi: 10.3389/fimmu.2017.00292.
[350] S. Kong *et al.*, “Mechanisms of low MHC I expression and strategies for targeting MHC I with small molecules in cancer immunotherapy,” *Cancer Letters*, vol. 611, p. 217432, Feb. 2025, doi: 10.1016/j.canlet.2024.217432.
[351] K. L. Rock, E. Reits, and J. Neefjes, “Present Yourself! By MHC Class I and MHC Class II Molecules,” *Trends Immunol*, vol. 37, no. 11, pp. 724–737, Nov. 2016, doi: 10.1016/j.it.2016.08.010.
[352] M. T. Ramieri, E. Gallo, and M. Marino, “Immunohistochemistry of Normal Thymus,” in *Atlas of Thymic Pathology*, D. Jain, J. A. Bishop, and M. R. Wick, Eds., Singapore: Springer, 2020, pp. 11–21. doi: 10.1007/978-981-15-3164-4_2.
[353] G. Pearse, “Normal structure, function and histology of the thymus,” *Toxicol Pathol*, vol. 34, no. 5, pp. 504–514, 2006, doi: 10.1080/01926230600865549.
[354] S. Nuñez *et al.*, “The human thymus perivascular space is a functional niche for viral-specific plasma cells,” *Sci Immunol*, vol. 1, no. 6, p. eaah4447, Dec. 2016, doi: 10.1126/sciimmunol.aah4447.

[355] A. Lev *et al.*, “Thymic function in MHC class II-deficient patients,” *J Allergy Clin Immunol*, vol. 131, no. 3, pp. 831–839, Mar. 2013, doi: 10.1016/j.jaci.2012.10.040.
[356] J. A. Tarbox *et al.*, “Elevated Double Negative T Cells in Pediatric Autoimmunity,” *J Clin Immunol*, vol. 34, no. 5, pp. 594–599, Jul. 2014, doi: 10.1007/s10875-014-0038-z.
[357] M. Deguise and R. Kothary, “New insights into SMA pathogenesis: immune dysfunction and neuroinflammation,” *Ann Clin Transl Neurol*, vol. 4, no. 7, pp. 522–530, May 2017, doi: 10.1002/acn3.423.