

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

MIOCINAS COMO BIOMARCADORES DA ATROFIA MUSCULAR ESPINHAL

ANA LETÍCIA AMORIM DE ALBUQUERQUE

Porto Alegre

2024

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

MIOCINAS COMO BIOMARCADORES DA ATROFIA MUSCULAR ESPINHAL

ANA LETÍCIA AMORIM DE ALBUQUERQUE

Orientador: Prof. Dr. Jonas Alex Morales Saute.
Tese de Doutorado apresentada como requisito parcial para obtenção de título de Doutor em Medicina: Ciências Médicas, da Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Medicina: Ciências Médicas.

Porto Alegre

2024

CIP - Catalogação na Publicação

Amorim de Albuquerque, Ana Letícia
MIOCINAS COMO BIOMARCADORES DA ATROFIA MUSCULAR
ESPINHAL / Ana Letícia Amorim de Albuquerque. -- 2024.
81 f.
Orientador: Jonas Alex Morales Saute.

Tese (Doutorado) -- Universidade Federal do Rio
Grande do Sul, Faculdade de Medicina, Programa de
Pós-Graduação em Medicina: Ciências Médicas, Porto
Alegre, BR-RS, 2024.

1. Atrofia Muscular Espinhal. 2. AME-5q. 3.
Biomarcadores. 4. Miocinas. 5. Miostatina. I. Morales
Saute, Jonas Alex, orient. II. Título.

Agradecimentos

Gostaria de agradecer primeiramente a Deus e à minha família por todo apoio durante os meus anos de formação, especialmente meus pais, Audenes e Sandra, aos meus irmãos, Álvaro e Ana Clara, e aos meus avós (*in memoriam*), José e Maria de Lourdes, por terem sempre estado ao meu lado, me incentivando a concretizar meus sonhos. Eu jamais teria chegado aqui sem vocês.

Agradeço também aos meus professores, meus mestres que me ajudaram durante o colégio, universidade e na residência médica. Deixo meu agradecimento especial ao meu professor e orientador Dr Jonas Saute, pela confiança, pela ajuda e por construir esse trabalho junto comigo. Agradeço também aos meus colegas de NeMUG, especialmente à minha dupla, Júlia Kersting, por ter estado comigo, lado a lado, durante esses três anos de projeto.

A todos, muito obrigada!

RESUMO

Introdução: A Atrofia Muscular Espinhal (AME) é uma doença neurodegenerativa autossômica recessiva, caracterizada pela degeneração progressiva e irreversível dos neurônios motores do corno anterior da medula espinhal, o que resulta em atrofia e fraqueza muscular proximal progressiva. Nos últimos anos, avanços significativos foram realizados no entendimento da fisiopatologia da AME que culminaram com a emergência de drogas modificadoras de doença altamente eficazes. Desta forma, a descrição da eficácia dessas drogas em contexto de mundo real, bem como a identificação de biomarcadores que possam prever a progressão, gravidade e resposta às novas terapias modificadoras da doença passaram a ser questões cruciais a serem respondidas. **Objetivo:** Descrever a Casuística da AME em um serviço de referência regional para doenças raras no Sul do Brasil, descrevendo os casos e relatando experiência de mundo real com tratamentos modificadores de doença. Além de investigar o papel dos níveis séricos de miostatina e follistatina como biomarcadores para a atrofia muscular espinhal, considerando a atrofia muscular secundária à desnervação como a principal manifestação clínica da doença. **Metodologia:** O primeiro estudo trata-se de uma coorte histórica unicêntrica que incluiu todos os pacientes com diagnóstico de atrofia muscular espinhal vinculados ao Hospital de Clínicas de Porto Alegre. O segundo estudo inicialmente avaliou a expressão gênica diferencial de miostatina e follistatina em um modelo lesional de desnervação do gastrocnêmio em camundongos, assim como em uma meta-análise de três conjuntos de dados em modelos transgênicos de camundongos de atrofia muscular espinhal e em dois estudos envolvendo humanos com atrofia muscular espinhal. Posteriormente, foi conduzido um estudo caso-controle envolvendo 27 pacientes com atrofia muscular espinhal e 27 controles, seguido por um estudo de coorte de 12 meses com 25 desses pacientes. **Resultados:** Identificamos que 74 pacientes estão atualmente em acompanhamento no centro de referência do Rio Grande do Sul (5,4% pré-sintomáticos, 24,3% AME tipo 1, 28,4% tipo 2, 36,5% tipo 3 e 5,4% tipo 4), dos quais, 41,9% receberam terapia específica para AME, e, destes, 96,8% receberam Nusinersen, com 19,35% deles mudando para terapia gênica com Onasemnogene Apeparvovec, e 6,4% iniciaram Risdiplam. Dos tratados, 80% apresentaram melhora no desempenho motor ao longo do período de acompanhamento, com menor resposta terapêutica associada ao início tardio do tratamento e baixos escores de função motora no início do estudo. No segundo estudo, a expressão gênica da miostatina no músculo esquelético diminuiu e a da follistatina aumentou após a denervação muscular lesional em camundongos, consistente com os achados na meta-análise de camundongos transgênicos SMA e no músculo

iliopsoas de 5 pacientes com SMA tipo 1. Os níveis medianos de miostatina sérica foram significativamente mais baixos em pacientes com atrofia muscular espinhal (98 pg/mL; 5-157) em comparação com controles (412 pg/mL; 299-730) ($P < 0.001$). Níveis mais baixos de miostatina estavam associados a maior gravidade da doença com base em desfechos avaliados pelo clínico ($Rho = 0.493-0.812$; $P < 0.05$). Após 12 meses, houve uma redução adicional nos níveis de miostatina entre os casos de atrofia muscular espinhal ($P = 0.021$). Os níveis de follistatina não diferiram entre casos e controles, e não foram observadas mudanças significativas ao longo do tempo. A razão follistatina:miostatina aumentou significativamente em sujeitos com atrofia muscular espinhal e correlacionou-se inversamente com a gravidade motora. **Conclusão:** O nosso estudo do mundo real reforça a eficácia das terapias modificadoras da doença para AME no Brasil e no contexto de países com perfis socioeconômicos semelhantes, que é maior quanto mais precoce e melhor for o estado funcional do paciente. Além disso, os níveis séricos de miostatina mostram-se promissores como um novo biomarcador para avaliar a gravidade e progressão da AME.

PALAVRAS CHAVE: Atrofia Muscular Espinhal; AME-5q; Biomarcadores; Miocinas; Miostatina; Follistatina; Irisina.

ABSTRACT

Introduction: Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease characterized by progressive and irreversible degeneration of motor neurons in the anterior horn of the spinal cord, resulting in progressive proximal muscle atrophy and weakness. In recent years, significant advances have been made in understanding the pathophysiology of SMA, culminating in the emergence of highly effective disease-modifying drugs. Consequently, describing the effectiveness of these drugs in real-world contexts, as well as identifying biomarkers that can predict disease progression, severity, and response to new disease-modifying therapies, has become crucial. **Objective:** To describe the casuistry of SMA in a regional reference center for rare diseases in southern Brazil, describing cases and reporting real-world experience with disease-modifying treatments. Additionally, to investigate the role of serum levels of myostatin and follistatin as biomarkers for spinal muscular atrophy, considering muscle atrophy secondary to denervation as the main clinical manifestation of the disease. **Methodology:** The first study is a unicentric historical cohort that included all patients diagnosed with spinal muscular atrophy linked to the Hospital de Clínicas de Porto Alegre. The second study initially evaluated the differential gene expression of myostatin and follistatin in a denervation model of the gastrocnemius in mice, as well as in a meta-analysis of three datasets in transgenic mouse models of spinal muscular atrophy and in two studies involving humans with spinal muscular atrophy. Subsequently, a case-control study involving 27 patients with spinal muscular atrophy and 27 controls was conducted, followed by a 12-month cohort study with 25 of these patients. **Results:** We identified that 74 patients are currently under follow-up at the reference center in Rio Grande do Sul (5.4% pre-symptomatic, 24.3% SMA type 1, 28.4% type 2, 36.5% type 3, and 5.4% type 4), of which 41.9% received specific therapy for SMA, and of these, 96.8% received Nusinersen, with 19.35% switching to gene therapy with Onasemnogene Apeparvovec, and 6.4% initiated Risdiplam. Of those treated, 80% showed improvement in motor performance over the follow-up period, with poorer therapeutic response associated with late initiation of treatment and low motor function scores at study initiation. In the second study, myostatin gene expression in skeletal muscle decreased and follistatin increased after muscle denervation injury in mice, consistent with findings in the meta-analysis of SMA transgenic mice and in the iliopsoas muscle of 5 patients with SMA type 1. Median serum myostatin levels were significantly lower in patients with spinal muscular atrophy (98 pg/mL; 5-157) compared to controls (412 pg/mL; 299-730) ($P < 0.001$). Lower levels of myostatin were associated with greater disease severity based on clinician-assessed

outcomes ($\text{Rho} = 0.493\text{-}0.812$; $P < 0.05$). After 12 months, there was a further reduction in myostatin levels among spinal muscular atrophy cases ($P = 0.021$). Follistatin levels did not differ between cases and controls, and no significant changes over time were observed. The follistatin:myostatin ratio significantly increased in subjects with spinal muscular atrophy and correlated inversely with motor severity. **Conclusion:** Our real-world study reinforces the effectiveness of disease-modifying therapies for SMA in Brazil and in the context of countries with similar socioeconomic profiles, which is greater the earlier and better the patient's functional status. Additionally, serum myostatin levels show promise as a new biomarker for assessing the severity and progression of SMA.

KEY WORDS: Spinal Muscular Atrophy; AME-5q; Biomarkers; Myokines; Myostatin; Follistatin; Irisin.

LISTA DE FIGURAS

Figura 1: Fluxograma - Seleção dos estudos para elaboração do projeto.

Figura 2: Esquema dos genes humanos *SMN2* e *SMN1* e seus transcritos resultantes.

Figura 3: Modelo de camundongo *Smn*^{-/-}.

Figura 4: Modelo de camundongo *SMN* Δ 7.

Figura 5: Marco Conceitual.

Figura 6: Prêmio no CBGM em 2023.

Figura 7: Prêmio Destaque no I Encontro Científico do PPGCM (2023).

Figura 8: Jornal da Universidade (2024).

LISTA DE ABREVIATURAS E SIGLAS

AME	Atrofia Muscular Espinhal
ANVISA	Agência Nacional de Vigilância Sanitária
CEP	Comitê de Ética em Pesquisa
CHOP INTEND	<i>Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders</i>
EMBASE	<i>Excerpta Medica dataBASE</i>
ENMG	Eletroneuromiografia
FDA	<i>Food and Drug Administration</i>
FNDC5	Domínio da Fibronectina tipo III contendo a Proteína 5
FSTN	Folistatina
GDF	<i>Growth Differentiation Factor</i>
GEE	<i>Generalized Estimating Equations</i>
HCPA	Hospital de Clínicas de Porto Alegre
HFMSE	<i>Hammersmith Functional Motor Scale–Expanded</i>
HINE	<i>Hammer-smith Infant Neurological Examination</i>
MeSH	<i>Medical Subjects Headings</i>
MLPA	<i>Multiplex Ligation-dependent Probe Amplification</i>
mRNA	Ácido Ribonucleico mensageiro
NGS	Sequenciamento de Próxima Geração
PubMed	<i>Public/Publisher MEDLINE</i>
qPCR	Reação em Cadeia da Polimerase Quantitativa
RULM	<i>Revised Upper Limb Module</i>
SMN	Sobrevivência do Neurônio Motor
SRM	<i>Standardized Response Mean</i>
TCLE	Termo de Consentimento Livre e Esclarecido
TLL2	<i>Tolloid-like protein 2</i>

SUMÁRIO

1. INTRODUÇÃO	12
2. REVISÃO DA LITERATURA	14
3. ATUALIZAÇÃO DA REVISÃO DA LITERATURA	15
3.1 Aspectos Históricos e Características Clínicas da AME.....	16
3.2 Epidemiologia.....	17
3.3 Genética Molecular e Fisiopatologia.....	17
3.3.1 Modelos Animais.....	19
3.4 Diagnóstico.....	20
3.5 Tratamento.....	21
3.6 Biomarcadores na AME.....	22
3.7 Miocinas.....	24
4. JUSTIFICATIVA	26
5. MARCO CONCEITUAL	27
6. OBJETIVO	28
6.1 Objetivo Geral:.....	28
6.2 Objetivos Específicos:.....	28
7. REFERÊNCIAS BIBLIOGRÁFICAS	29
8. ARTIGOS	36
8.1 ARTIGO I.....	36
8.2 ARTIGO II.....	36
9. CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS	58
10. ANEXOS	59
10.1 Termo de Consentimento Livre e Esclarecido.....	59
10.2 STROBE Checklist.....	63
10.3 Destaques.....	67
10.3.1 Destaque no Congresso Brasileiro de Neurogenética (2023).....	67
10.3.2 Destaque no I Encontro Científico do PPGCM (2023).....	68
10.3.3 Publicação no Jornal da UFRGS em janeiro de 2024.....	69

1. INTRODUÇÃO

A Atrofia Muscular Espinhal (AME) é uma doença neurodegenerativa autossômica recessiva, caracterizada pela degeneração progressiva e irreversível dos neurônios motores do corno anterior da medula espinhal, o que resulta em atrofia e fraqueza muscular proximal progressiva (1). Sua incidência estimada é de 1 em 6.000 a 1 em 10.000 nascidos vivos, e a prevalência estimada de portadores da mutação é 1 em 40 a 1 em 60 indivíduos na população geral (2–5). É considerada a principal causa hereditária de óbito infantil, e é a segunda doença autossômica recessiva fatal mais comum, sendo a primeira a fibrose cística (6).

A AME é causada por variantes patogênicas no gene de sobrevivência do neurônio motor 1 (*SMN1*), que foi descrito em 1990 por Melki e colaboradores no cromossomo 5q13 (7,8), sendo também chamada de AME-5q, para diferenciar este tipo de atrofia muscular espinhal de outras formas descritas mais recentemente causadas alterações em outros genes. Entretanto, utilizaremos o termo AME como sinônimo da AME-5q ao longo desta tese. Em seres humanos, duas formas do gene sobrevivência do neurônio motor (*SMN*) existem em cada alelo: a forma telomérica (*SMN1*) e a forma centromérica (*SMN2*). A principal diferença entre *SMN2* e *SMN1* é um único nucleotídeo, a substituição do nucleotídeo C pelo T na posição 840, resultando na exclusão do éxon 7 durante a transcrição, o que resulta na formação de uma proteína *SMN* instável, pouco funcional e rapidamente degradada (9).

A proteína *SMN* é uma proteína multifuncional que é expressa na maioria das células somáticas, apresentando um papel essencial nas vias de homeostase celular. Estudos mostram que essa proteína participa da montagem dos spliceossomos, da biogênese das ribonucleoproteínas, do transporte e tradução do RNA mensageiro (mRNA), da dinâmica do citoesqueleto, endocitose, autofagia, além de exercer influência na mitocôndria e nas vias bioenergéticas, havendo maior suscetibilidade a degeneração dos neurônios motores do corno anterior da medula na perda de sua função (10).

Os pacientes com AME têm um não funcionamento do gene *SMN1* e se tornarão dependentes do gene *SMN2*, que é ineficiente em produzir proteína *SMN* suficiente para o adequado funcionamento dos motoneurônios (11). A perda do *SMN1* é essencial para a patogênese da AME, mas é o número de cópias de *SMN2* que determina a severidade da doença. Há uma relação inversa entre o número de cópias de *SMN2* e a gravidade do quadro (12).

A doença é dividida em 5 fenótipos principais (AME 0, 1, 2, 3, 4). Cerca de 80% dos pacientes com AME tipo 1 tem uma ou duas cópias do *SMN2*, já 82% dos pacientes com AME

tipo 2 carregam três cópias do *SMN2*, enquanto os pacientes com AME tipo 3 ou 4, geralmente carregam três ou quatro cópias desse gene (6,12).

No último anos, avanços foram realizados no entendimento da fisiopatologia da AME, possibilitando o surgimento de propostas terapêuticas para a condição. Atualmente, há três terapias já aprovadas, inclusive em território nacional pela ANVISA, para o tratamento da AME. Em 2016, o órgão regulatório americano, FDA (Food and Drug Administration), aprovou a Nusinersena (Spiranza), um oligonucleotídeo antisense (ASO) (13,14). Dois anos após, em 2018, surgiu o Onasemnogene abeparvovec (15) e, mais recentemente, em agosto de 2020, surgiu a proposta oral de tratamento para a AME, o Risdiplam (Evrysdi) (16,17).

Os tratamentos modificadores de doença em geral são mais eficazes quanto mais precocemente administrados e quanto melhor o status funcional no início do tratamento (18). No entanto, há variabilidade fenotípica da AME e sua evolução e progressão muitas vezes diferem da história natural usual da doença, e alguns pacientes irão responder melhor do que outros independentemente da idade de início da terapia (19,20). Deste modo, a busca por biomarcadores que possam indicar a evolução da doença e o prognóstico de resposta a essas novas terapias farmacológicas, serão de grande importância para o entendimento e para o tratamento da AME.

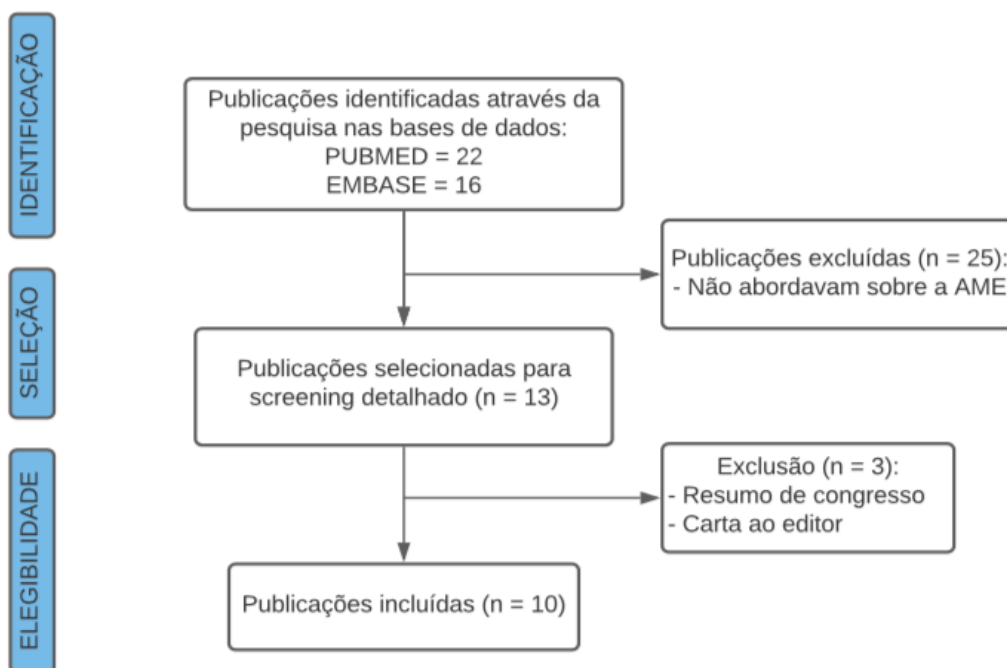
Portanto, é nesse contexto, e também considerando a AME como uma doença de repercussão multissistêmica (21), com manifestação sobretudo de fraqueza e atrofia muscular, que nos propomos a estudar as citocinas musculares (miocinas) como possíveis biomarcadores dessa doença. Poderiam essas moléculas funcionar como bons biomarcadores de dano muscular na AME? Poderiam ser marcadores da progressão da doença e/ou resposta ao tratamento? Como veremos na revisão sistematizada da literatura, ainda não há trabalhos que possam responder essas perguntas de forma consistente.

2. REVISÃO DA LITERATURA

Realizamos uma revisão sistematizada de publicações sobre Miocinas na Atrofia Muscular Espinhal. Para a busca dos estudos foram consultados dois bancos de dados: PubMed e EMBASE, no mês de novembro de 2020. Utilizou-se os seguintes descritores fornecidos pelo MeSH (*Medical Subjects Headings*):

- (**Spinal Muscular Atrophy** OR Atrophy, Spinal Muscular OR Spinal Amyotrophy OR Amyotrophies, Spinal OR Amyotrophy, Spinal OR Spinal Amyotrophies OR Spinal Muscular Atrophy OR Hereditary Motor Neuronopathy OR Hereditary Motor Neuronopathies OR Motor Neuronopathies, Hereditary OR Motor Neuronopathy, Hereditary OR Neuronopathies, Hereditary Motor OR Neuronopathy, Hereditary Motor OR Progressive Myelopathic Muscular Atrophy OR Myelopathic Muscular Atrophy, Progressive OR Progressive Proximal Myelopathic Muscular Atrophy OR Proximal Myelopathic Muscular Atrophy, Progressive OR Myelopathic Muscular Atrophy OR Atrophy, Myelopathic Muscular OR Muscular Atrophy, Myelopathic OR Adult-Onset Spinal Muscular Atrophy OR Adult Onset Spinal Muscular Atrophy OR Muscular Atrophy, Adult Spinal OR Adult Spinal Muscular Atrophy) AND ((**Myostatin** OR Growth Differentiation Factor 8 OR GDF-8) OR (**Follistatin** OR Activin-Binding Protein) OR (**Irisin** OR FRCP2 protein, human OR irisin, human OR fibronectin type III domain-containing protein 5 precursor, human)).

Fluxograma 1 – Seleção dos estudos para elaboração do projeto.



No PubMed obteve-se 22 resultados, dos quais 11 foram excluídos por não serem sobre AME. Restaram 11 trabalhos relevantes ao nosso tema, mas 2 eram cartas ao editor, tendo sido também excluídos desta revisão. Por fim, 9 artigos foram incluídos por serem relevantes. A maioria deles sobre as miocinas em modelos de animais com AME (22,23), alguns deles mostrando o papel promissor da inibição da miostatina e da ação da follistatina na promoção do aumento da massa muscular esquelética e na melhora da atrofia muscular (24–29). O único artigo sobre miocinas em humanos trata-se de um relato de caso, que sugere que a presença da mutação do gene *TLL2* (*Tolloid-like protein 2*), um ativador da miostatina, pode funcionar com um possível amenizador da severidade da AME (30).

A busca no EMBASE resultou em 16 achados, dos quais apenas dois eram relevantes ao tema estudado. Desses dois trabalhos, um se tratava de um estudo sobre um anticorpo monoclonal inibidor da miostatina, que está sendo investigado com uma opção terapêutica na AME, mas é apenas um resumo, não havendo ainda um artigo completo publicado sobre o tema (31). Por fim, o artigo mais relevante encontrado e incluído na nossa revisão foi sobre o papel da regulação da miostatina em doenças neuromusculares, incluindo a AME (32), este trabalho também traz descrição anedótica sobre os níveis de miostatina em 4 pacientes com AME, comparando-nos com níveis em outras doenças musculares.

3. ATUALIZAÇÃO DA REVISÃO DA LITERATURA

Realizamos uma atualização da revisão sistematizada de publicações, a partir de 2021, sobre Miocinas na Atrofia Muscular Espinhal. Para a busca dos estudos foram consultados dois bancos de dados: PubMed e EMBASE, em fevereiro de 2024. Utilizou-se os mesmos descritores fornecidos pelo *MeSH* (*Medical Subjects Headings*) descritos previamente.

No PubMed encontramos 9 novos resultados, dos quais 5 foram excluídos por não se tratarem de AME. Dos 4 incluídos, dois são ensaios clínicos sobre a miostatina como alvo terapêutico na AME (33,34), e os outros dois são revisões sobre o papel da miostatina em doenças neuromusculares (35,36).

No EMBASE encontramos 4 novos resultados, dos quais 2 foram excluídos por não se tratarem de AME (37,38), e os outros 2 por não serem sobre miocinas (39,40).

Em buscas manuais recentes na literatura também encontramos um resumo de congresso com uma análise exploratória do nível sérico dessa molécula em pacientes com AME (41). Foram encontradas também duas outras publicações sobre o papel da miostatina em outras doenças musculares, incluindo as miopatias (42,43), que serão detalhados na seção 3.7,

específica sobre as miocinas. Antes disso, realizaremos uma descrição mais detalhada da condição.

3.1 Aspectos Históricos e Características Clínicas da AME

A primeira descrição de AME-5q infantil ocorreu em 1891, pelo médico austríaco Guido Werdnig, após a realização de necropsia em duas crianças afetadas. Johan Hoffman foi o segundo pesquisador a descrever a doença, publicando dois relatos independentes, em 1891 e 1892. Sendo assim, a AME foi inicialmente conhecida como doença Wernig-Hoffman, em homenagem aos seus descobridores. No entanto, foi apenas na década de 50 que os cientistas, Kugelberg e Welander, através das descrições de seus casos necropsiados, puderam contribuir para identificar a fisiopatologia da doença, como sendo degeneração do corno anterior da medula e atrofia muscular severa (9). Em 1990, pesquisadores descreveram pela primeira vez que a doença envolvia o cromossomo 5q13 (7,8). Melki e seus colaboradores, descobriram o gene envolvido nessa condição e o nomearam de “sobrevivência do neurônio motor” (*SMN*).

Em 1961, através da descrição de uma série de 52 casos, evidenciou-se pela primeira vez o quão variado pode ser o espectro fenotípico desta doença (44), mas foi apenas 30 anos após, em 1991, no Consórcio internacional de Atrofia Muscular Espinhal, que se formalizou um esquema de classificação da doença em 3 tipos, o qual foi atualizado em 2007, quando se acrescentou os tipos 0 e 4 (11,45). Dessa forma, atualmente, tem-se 5 fenótipos para a AME: Tipo 0, Tipo 1 (doença de Wernig-Hoffmann), Tipo 2 (forma intermediária), Tipo 3 (doença de Kugelberg-Welander) e tipo 4 (11,45,46).

As principais características clínicas da AME são a fraqueza e atrofia muscular, sendo essa fraqueza geralmente simétrica, acometendo mais a musculatura proximal. A AME tipo 0, é a forma mais severa, presente no período neonatal, caracterizada por fraqueza e hipotonia, com relato de movimentos fetais reduzidos, e levando a óbito até 6 meses de idade. No tipo 1, são crianças hipotônicas, com paralisia flácida, sem controle do movimento da cabeça e que nunca adquirem a capacidade de se sentar sozinhas, os sintomas iniciam antes dos 6 meses de idade e o óbito geralmente ocorre antes de 2 anos de vida previamente a disponibilidade das terapias modificadoras de doença. No tipo 2, os sintomas surgem entre 7 e 18 meses de idade, são crianças que conseguem sentar sem ajuda, mas nunca conseguem andar sozinhas. O tipo 3, é uma forma mais heterogênea, são crianças ou jovens que conseguem andar sozinhos em algum momento da sua vida, mas com a progressão da fraqueza nos membros, evoluem para

dependência de cadeira de rodas. Nesse grupo a capacidade cognitiva e a expectativa de vida estão preservados. O tipo 4, é a forma mais leve e mais rara, cuja doença inicia após os 18 anos de idade, geralmente após os 30 anos (1,47,48).

3.2 Epidemiologia

Poucos estudos tentaram avaliar a prevalência da Atrofia Muscular Espinhal, dessa forma os dados podem ser subestimados. Estima-se que a prevalência seja aproximadamente 1 a 2 para cada 100.000 pessoas, e que a incidência seja de 1 em 6000 a 1 em 11.000 nascidos vivos (2,4,48–51). Alguns estudos mostram uma prevalência que chega a 6.56 para cada 100.000 indivíduos (52), indicando que existem diferenças regionais na incidência da doença. Acredita-se, também, que a frequência de portadores de variantes patogênicas no *SMN1* seja de 1 em 40 a 1 em 60 indivíduos na população geral (5).

A AME-5q é uma doença autossômica recessiva, causada pela mutação do gene *SMN1* localizado no cromossomo 5q13. A maioria dos pacientes (98%) tem uma alteração em homozigose do *SMN1*, seja deleção, rearranjo ou mutação, mas em raros casos, também pode decorrer de uma mutação de ponto em dos alelos desse gene (53). O tipo mais frequente de AME é o 1 (doença de Wednig-Hoffman), cerca de 60% dos doentes apresentam essa forma. A segunda forma mais frequente é o tipo 2, com 27%, seguida da AME tipo 3, com cerca de 13% dos casos (5).

3.3 Genética Molecular e Fisiopatologia

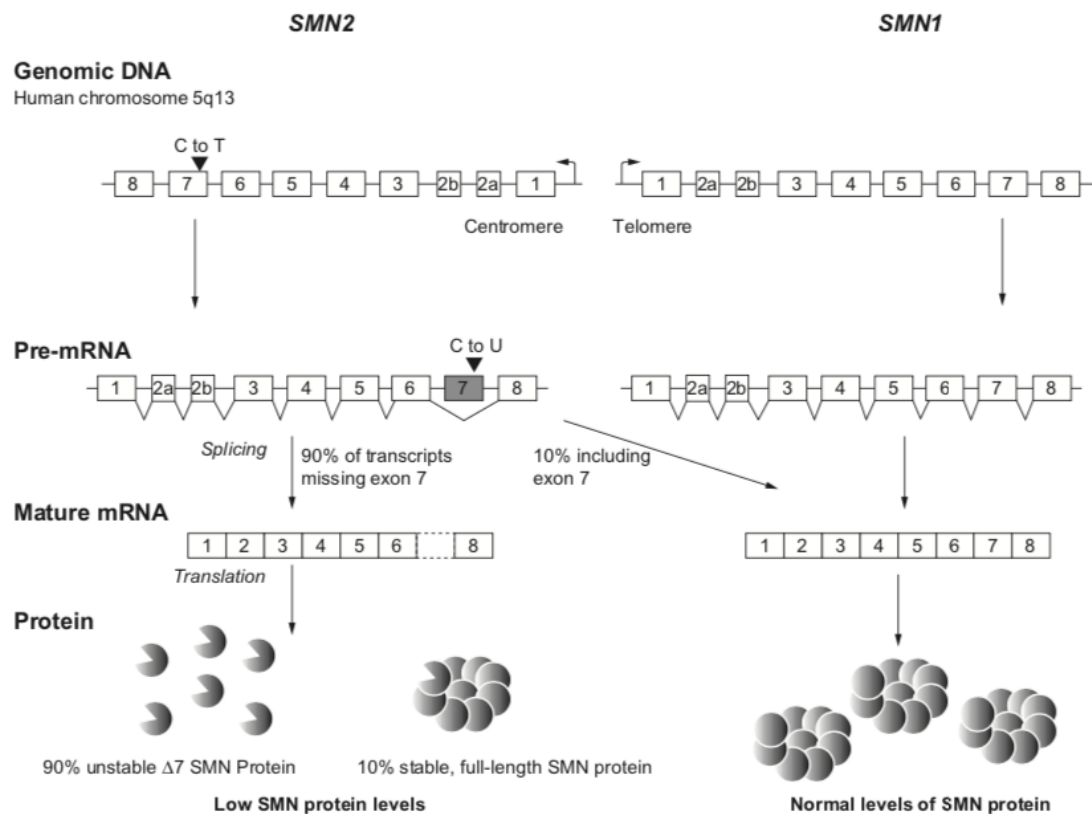
A Atrofia Muscular Espinhal é uma doença autossômica recessiva, causada por anormalidades no gene *SMN1* localizado no cromossomo 5q13, uma região que tem um alto grau de instabilidade genômica. *SMN1* é uma cópia telomérica do gene, que difere do *SMN2*, cópia centromérica, por apenas 5 pares de bases, e uma sequência codificadora que difere por um único nucleotídeo, troca do nucleotídeo C pelo T na posição 840 (54,55).

A proteína SMN é um polipeptídeo de 294 aminoácidos, essencial para a manutenção dos motoneurônios, sendo que níveis insuficientes dessa proteína resultam em degeneração dos neurônios motores do tronco encefálico e da medula espinhal, levando à progressiva atrofia e fraqueza muscular (9,10).

Na cópia centromérica do gene *SMN* há a exclusão do éxon 7 durante a transcrição do gene, levando à divisão aberrante do pré-mRNA (ácido ribonucleico pré-mensageiro) do *SMN2*, resultando na formação de uma proteína SMN instável, pouco funcional e rapidamente

degradada (Figura 2). Apenas 10% do *SMN2* consegue produzir uma proteína SMN funcional. São esses transcritos que parcialmente compensam pela perda dos *SMN1*, e por isso que o número de cópias de *SMN2* será o principal determinante genética da gravidade da doença, sendo quanto maior o número de cópias de *SMN2*, menos grave a apresentação clínica da doença (1,56).

Figura 2: Esquema dos genes humanos *SMN2* e *SMN1* e seus transcritos resultantes.



Adaptado do Handbook of Clinical Neurology, Vol. 148, 2018(9).

Cerca de 98% dos pais de uma criança doente são heterozigotos, sendo assim, apenas 2% dos pais não são portadores de uma variante patogênica do *SMN1*, e os filhos desses casais apresentam uma variante patogênica *de novo*. Cada filho de um indivíduo afetado tem uma chance aproximada de 25% de ser doente, 25% de chance de ser saudável, e 50% de ser portador assintomático (47).

3.3.1 Modelos Animais

Modelos animais de doenças são essenciais, pois permitem melhor entendimento da fisiopatologia de cada condição, através da análise mais detalhada da doença com a possibilidade de proporcionar avanços terapêuticos que podem chegar na fase clínica.

Dentre os modelos animais da AME, temos os roedores (camundongos), estes têm apenas o gene *SMN1*, cuja deleção leva à letalidade embrionária precoce. Desta forma, para a geração de modelos animais de roedores que reproduzissem a doença e não fossem letais, foi necessário introduzir cópias humanas transgênicas do *SMN2* mediante técnicas de engenharia genética. Os modelos dos camundongos com *Smn1*^{-/-} (Figura 3) e *SMN2*^{Tg/Tg} foram criados usando a inserção de uma a duas cópias do *SMN2* transgênico humano (57). Outro modelo de camundongos é o *SMNΔ7* (Figura 4), no qual os ratos recebem o transgene *SMN2* com éxon 7 ausente (*SMNΔ7*) (58).

Figura 3: Modelo de camundongo *Smn*^{-/-}.



Camundongo *Smn*^{-/-} à esquerda. Adaptado de *Human Molecular Genetics*, Volume 9, Issue 3, 12 February 2000(57).

Figura 4: Modelo de camundongo *SMNΔ7*.

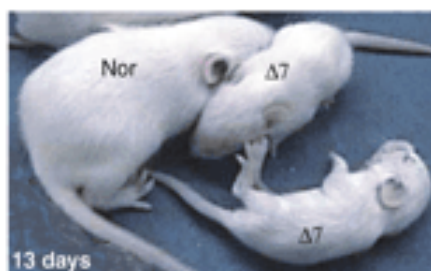


Imagem mostrando um camundongo não SMA e dois camundongos *SMNΔ7* SMA. Adaptado *Human Molecular Genetics*, Volume 14, Issue 6, 15 March 2005(58).

Também existem modelos animais com *Drosophila larvae*, que mostraram que a deficiência de *SMN* reduz a expressão de um subconjunto de genes contendo íntron U12, contribuindo, assim, para disfunção no circuito de atividade motora desses animais (9,59).

Pesquisadores também criaram modelo transgênico de *zebrafish* que carrega o gene *SMN2* humano, que pode ajudar no entendimento da disfunção dos motoneurônios na AME, podendo ser utilizado para abordagens moleculares ou testes farmacológicos (60).

3.4 Diagnóstico

O diagnóstico da atrofia muscular espinhal foi definido no Consenso de 2007, em que foi definido que o teste diagnóstico para pacientes com suspeição da doença deveria ser o teste genético da deleção do gene *SMN* (61). A não ser que haja história familiar, geralmente a suspeição da doença é induzida pelas manifestações clínicas do recém nascido.

Assim, o diagnóstico é baseado na testagem molecular para *SMN1/SMN2*, sendo esta a primeira linha de investigação (62). O padrão ouro de testagem molecular é a análise quantitativa do *SMN1* e *SMN2*, usando a amplificação ligadura-dependente multiplex da ponta de prova (*multiplex ligation-dependent probe amplification* - MLPA), reação em cadeia da polimerase quantitativa (qPCR) ou sequenciamento massivo paralelo (SMP) (12,63,64).

A ausência das duas cópias completas do *SMN1* permite o diagnóstico. Se houver uma cópia completa do *SMN1* presente, mas o paciente tem clínica compatível com AME, deve-se procurar variantes de ponto no gene *SMN1* remanescente por técnicas de sequenciamento. Quando as duas cópias completas do *SMN1* estão presentes, o diagnóstico de AME é improvável, no entanto, se o paciente tem o fenótipo compatível e for filho de casal consanguíneo, o sequenciamento do *SMN1* também deve ser realizado(62).

Embora seja a presença do gene *SMN1* o definidor do diagnóstico, a quantificação das cópias do *SMN2* é importante, por influenciar na severidade da doença, tendo, portanto, implicação prognóstica (12,65,66).

Exames complementares podem incluir: dosagem da enzima muscular creatina quinase, que pode estar normal ou levemente aumentada; teste eletrofisiológico, como a eletroneuromiografia (ENMG), que pode ser usado nas formas mais crônicas no qual o fenótipo da doença é menos evidente (67); e estudo da condução nervosa com estimulação repetitiva (62,68). Estudo recente mostrou que o ultrassom do músculo parece ser um método de ótima acurácia e boa estratégia de screening para AME de início tardio(67).

3.5 Tratamento

Até 2016 o tratamento da AME consistia apenas em medidas de suporte, mas avanços recentes foram realizados no entendimento da fisiopatologia da AME, possibilitando o surgimento de novas propostas terapêuticas.

Em dezembro de 2016, o órgão regulatório americano, FDA (Food and Drug Administration), aprovou a Nusinersena (Spiranza), um oligonucleotídeo antisense (ASO) para o tratamento da AME. A Nusinersena é uma sequência de fita única de ácido nucleico designada a regiões específicas do pré-RNA_m (ácido ribonucleico pré-mensageiro), modulando a expressão genética. Ele permite que o éxon 7 seja incluído durante o processamento do RNA_m transcrito a partir do gene *SMN2*, garantindo a leitura e a tradução correta deste gene, potencializando a síntese de proteínas SMN funcionais (13,14). Em 2019, o Onasemnogene abeparvovec (Zolgesma) foi aprovado nos Estados Unidos para o tratamento de crianças com AME menores de 2 anos de idade e com mutação bi-alélica no gene *SMN1*. Onasemnogene abeparvovec é um vetor adenoviral designado a entregar uma cópia humana funcional do gene *SMN* aos neurônios motores de pacientes com AME (15). Mais recentemente, em agosto de 2020, o Risdiplam (Evrysdi) que é um modificador do processo transcricional do RNA direcionado ao gene *SMN2*, também foi aprovado pelo FDA como tratamento de pacientes com AME maiores de 2 meses de idade. Este fármaco, cuja administração é via oral, atua aumentando a inclusão do éxon 7 nos transcritos do RNA_m (ácido ribonucleico mensageiro) do gene *SMN2*, impulsionando a capacidade deste gene produzir proteínas SMN funcionais (16,17).

No entanto, nenhum dos tratamentos existentes possibilita a cura da doença. Pesquisas em andamento têm buscado potenciais agentes para o desenvolvimento de novos compostos farmacológicos para o tratamento da AME (56). A variação da severidade clínica da doença é um desafio adicional para decisão sobre o melhor momento de início do tratamento para se obter o melhor benefício. Nos indivíduos em tratamento, também não há um consenso sobre a melhor medida ou marcador para avaliar a resposta ao tratamento (20).

Atualmente, alguns estudos têm focado no músculo como alvo terapêutico na AME, já que é a perda de força a característica mais marcante da doença. Poderiam biomarcadores musculares prever o curso clínico da doença e da resposta ao tratamento? É nesse contexto que entram as miocinas, citocinas que têm seu papel na massa e funcionamento muscular (69). Não há muitos trabalhos nesse tópico, mas há um estudo que está em andamento para avaliar

um anticorpo monoclonal – *Apitegromab* que funcionaria como inibidor da miostatina, com o objetivo de avaliar seu potencial terapêutico em indivíduos com AME (31,33,34,56).

3.6 Biomarcadores na AME

Biomarcadores são usados para monitorar e avaliar o desenvolvimento de uma doença, do diagnóstico ao óbito, podendo ser moleculares ou tecnologias, bem como exames que ajudam a avaliar as mudanças na fisiologia da condição. Podem ser classificados em biomarcadores de: diagnóstico, prognóstico, progressão de doença, e de resposta ao tratamento (70).

De forma mais ampla, as escalas clínicas, ou desfechos de avaliação clínica (COA da sigla do inglês), também podem ser considerados como marcadores biológicos. Diferentes COAs foram validados na AME para acompanhamento dos pacientes, sendo utilizados como desfechos de eficácia nos diversos ensaios clínicos que avaliaram terapias modificadoras da doença. Para AME de início precoce (tipo 1) temos: Children’s Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) e Hammer-smith Infant Neurological Examination (HFMSE), e para AME de início tardio, temos as seguintes escalas: Hammersmith Functional Motor Scale–Expanded (HFMSE) e Revised Upper Limb Module (RULM) (71,72). A seguir faremos uma breve revisão de cada um desses COAs.

A CHOP INTEND é uma escala que avalia as habilidades motoras das crianças com AME tipo 1 (ou que não possuem capacidade de sentar). O instrumento contém 16 itens (movimentos espontâneos em membros superiores; movimentos espontâneos em membros inferiores; preensão voluntária; movimentação da cabeça; adução do quadril; rolar sem auxílio das pernas; rolar sem auxílio dos braços; flexão dos ombros e cotovelos e abdução horizontal; flexão dos ombros e cotovelos; extensão dos joelhos; flexão do quadril e dorsiflexão dos pés; sustentação da cabeça; flexão do ombro; flexão do pescoço; extensão do pescoço e cabeça; curvatura da coluna), cada um com uma pontuação que varia de 0 a 4. A pontuação máxima da escala varia de 0 a 64, quanto maior, melhor o desenvolvimento motor da criança (73–75). A HINE é uma avaliação quantificável do funcionamento neurológico da criança, validada para crianças entre dois e 24 meses de idade. Contém 3 seções e 37 itens. A primeira seção inclui 26 itens que avaliam a função dos nervos cranianos, postura, movimentos, tônus e reflexos. A

segunda secção contém 8 itens que avaliam o desenvolvimento da função motora, através da observação da aquisição dos marcos motores (preensão voluntária, chutes, controle da cabeça, rolar, sentar, engatinhar, ficar em pé e andar), a pontuação varia de 0 a 26, com os maiores escores representando um melhor funcionamento motor, sendo esta seção a que é utilizada principalmente nos estudos em AME. A última seção avalia o comportamento, através de 3 itens (73,76,77). A HFMSE é uma escala que foi desenvolvida para crianças com AME tipo 2 e 3, particularmente aquelas com mobilidade reduzida, para fornecer informações sobre as habilidades motoras e progressão clínica da doença. Foi criada em 2003 (78) e atualizada em 2007 (79). A HFMS inicialmente avaliava 20 atividades, cada uma delas pontuando de 0 a 2 (0: incapacidade de realizar a atividade; 1: realiza com assistência, 2: realiza sem ajuda). A pontuação total da escala variava de 0, se o indivíduo falhasse em todas as atividades, à 40, se realizasse todas as atividades. Em 2007, essa escala foi refinada com o acréscimo de 13 novos itens de avaliação, sendo assim, a pontuação da HFMSE atualmente varia de 0 a 66, sendo valores mais altos, representativos de melhor função motora (72,80). A RULM foi criada em 2011, sendo destinada à avaliação da função dos membros superiores em pacientes com AME incapazes de deambular, incluindo crianças pequenas ou aqueles pacientes com contraturas severas (81). Em 2016 houve a revisão dessa escala, que agora conta com 20 itens, mas o item de entrada que serve como identificação da classe funcional, não contribui para a pontuação total. Cada item é classificado de 0 a 2 (0: incapaz; 1: capaz, com modificação; 2: capaz, sem dificuldade), exceto o primeiro item que pontua no máximo 1, sendo, assim, 37 é a pontuação máxima (72,82).

Medidas eletrofisiológicas, como potencial de ação muscular composto (CMAP), estimativa do número de unidades motoras (MUNE) e miografia de impedância elétrica (EIM) parecem ser sensíveis o suficiente para detectar alterações neuromusculares antes da manifestação clínica evidente, tornando-as biomarcadores de progressão e resposta ao tratamento na AME. O índice de número de unidade motora (MUNIX) e o índice de tamanho de unidade motora (MUSIX) também podem funcionar como biomarcadores de progressão. Já a ressonância magnética e a ultrassonografia do músculo são úteis para o diagnóstico, e este último método também é um potencial marcador de progressão e severidade da AME (70,83,84).

Outros estudos examinaram marcadores sanguíneos, como mRNA e níveis proteicos de SMN (85), perfil de metilação do DNA de leucócitos (85,86) e proteínas plasmáticas identificadas através de estudos proteômicos não enviesados, no entanto, esses marcadores não possuem diferenciação clara entre os subtipos de AME e mostraram associação limitada com a gravidade da doença, reduzindo sua utilidade clínica (87). Os níveis plasmáticos da cadeia pesada do neurofilamento (pNF-H) (88,89) e da cadeia leve (NfL)(90–93) demonstraram potencial como biomarcadores para dano axonal, refletindo a gravidade da doença e a resposta ao tratamento na AME de início infantil. No entanto, esses achados não foram reproduzíveis em pacientes com AME tipos 2 e 3, nem em estágios crônicos de AME (91–93). Recentemente outros autores focaram no envolvimento marcado do tecido muscular na doença para buscar biomarcadores, tendo encontrado que a creatina quinase (CK) e creatinina (Cr) correlacionaram-se com a função motora e severidade de doença, não sendo claro o seu papel como biomarcadores de resposta ao tratamento (94).

3.7 Miocinas

As miocinas são citocinas ou peptídeos sintetizados e liberados pelos miócitos em resposta à contração muscular. O termo miocinas surgiu em 2007, mas sua primeira descrição foi feita em 1997, e desde então tem-se investigado o papel dessas substâncias na massa e na função muscular (69,95,96).

A miostatina, um membro da família do fator transformador de crescimento β (TGF- β), é expressa e secretada principalmente pelo músculo esquelético e serve como regulador negativo do crescimento muscular (69). Estudos demonstraram que há níveis reduzidos de miostatina em diversas doenças musculares, incluindo distrofias musculares, distúrbios miotônicos e miopatias de corpos de inclusão (42,43). Há também um estudo anterior que se propõe a avaliar os níveis séricos de miostatina em pacientes com doenças musculares, e também foram avaliados quatro pacientes com AME de início infantil, demonstrando uma redução significativa em comparação aos controles e àqueles pacientes com miopatias primárias (32). Um resumo apresentado recentemente em 2021 no Congresso da World Muscle Society (WMS), posteriormente a escrita do projeto da tese, relatou estudo multicêntrico no qual os níveis séricos de miostatina foram avaliados em pacientes com AME não tratados, encontrando níveis reduzidos de miostatina em pacientes com AME tipo 2 quando comparados ao tipo 3 e correlações significativas dos níveis reduzidos de miostatina com

comprometimentos nos escores de função motora (41). Estudos recentes com modelos animais também têm mostrado que a inibição da miostatina, poderia aumentar a massa, a função e a performance muscular em modelos de ratos com AME (27–29,32,56).

A folistatina, que é um antagonista endógeno da miostatina, também já foi descrita em modelo de ratos com AME como uma substância que promoveria aumento da massa muscular esquelética e melhoraria a atrofia muscular (24–26). Ao contrário da miostatina, a folistatina apresenta um perfil de expressão mais amplo, sendo a expressão hepática mais de 8 vezes maior que a expressão no músculo esquelético (97), conseqüentemente, o aumento da expressão da folistatina no músculo pode não resultar necessariamente em níveis séricos elevados desta miocina.

Outra miocina, a irisina, é um hormônio induzido pelo exercício, resultado da clivagem de seu precursor, domínio da fibronectina tipo III contendo a proteína 5 (FNDC5), sendo secretado pelas células da musculatura esquelética após o exercício. Estudos, inclusive em modelo animal, evidenciaram que essa molécula é capaz de atenuar a perda da massa muscular, induzindo hipertrofia muscular, recuperando as atrofias induzidas por desnervação, e contribuindo para regeneração da musculatura (98–101).

4. JUSTIFICATIVA

A atrofia muscular espinhal é uma doença genética que embora seja rara (incidência de cerca de 1 para cada 10.000 nascidos vivos), é segunda doença autossômica recessiva fatal mais comum (6), sendo assim, um entendimento completo dessa doença e fatores associados é essencial para desenvolvimento de estratégias terapêuticas.

Essa doença neuromuscular é primariamente central, afeta os neurônios motores, levando a uma profunda atrofia muscular esquelética, mas será que as miocinas poderiam funcionar como bons biomarcadores de dano muscular na AME? Poderiam ser marcadores da progressão da doença e/ou resposta ao tratamento? São essas perguntas que tentaremos responder com esse trabalho.

Nossos serviços de Neurologia e Genética Médica, do Hospital de Clínicas de Porto Alegre (HCPA), são o centro de referência estadual do Rio Grande do Sul para o tratamento de pacientes com AME. Em janeiro de 2021 o serviço acompanhava 52 indivíduos com AME, entretanto os dados clínicos e epidemiológicos de nosso centro, bem como a experiência do mundo real com as novas terapias no contexto do sistema único de saúde brasileiro no Sul do Brasil ainda não haviam sido relatadas.

Desta forma, a presente tese iniciará pela descrição detalhada de nossa casuística e da resposta às novas terapias modificadoras de doença. Tais dados também servirão como base para justificar a necessidade da identificação de biomarcadores de gravidade e progressão de doença e farmacodinâmicos. Após esta caracterização e aproveitando do seguimento longitudinal realizados pelos nossos serviços, realizaremos um estudo caso-controle, seguido de um estudo de coorte para a descoberta de novos biomarcadores. Considerando que as miocinas possam ser marcadores de grande potencial ainda não estudados em pacientes com AME, e baseando-se em modelos animais (26–29,32,56) que já demonstram que as miocinas tem um potencial papel terapêutico na atrofia muscular espinhal, o estudo original principal deste tese versará sobre o estudo dessas citocinas, objetivando ampliar o conhecimento sobre o papel como biomarcadores de gravidade, progressão e farmacodinâmicos na AME.

5. MARCO CONCEITUAL



AME, Atrofia Muscular Espinhal; CHOP INTEND, Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; HINE, Hammer-smith Infant Neurological Examination; HFMSE, Hammersmith Functional Motor Scale-Expanded; RULM, Revised Upper Limb Module.

6. OBJETIVO

6.1 Objetivo Geral:

Caracterizar o potencial das miocinas como marcadores biológicos de gravidade e de progressão da Atrofia Muscular Espinhal.

6.2 Objetivos Específicos:

6.2.1 Descrever a Casuística da AME em um serviço de referência regional para doenças raras. Descrevendo os casos e relatando experiência de mundo real com tratamentos modificadores de doença para esta condição.

6.2.2 Analisar dados de expressão gênica diferencial através de estudo de bioinformática para investigar potenciais biomarcadores da AME.

6.2.3 Caracterizar o perfil dos marcadores séricos: Miostatina e Folistatina na AME-5q.

6.2.4 Caracterizar o perfil dos marcadores séricos: Miostatina e Folistatina nos diferentes tipos de AME-5q.

6.2.5 Avaliar a progressão após 12 meses dos níveis séricos desses biomarcadores.

6.2.6 Comparar a sensibilidade à mudança dos marcadores biológicos em comparação com as escalas clínicas.

7. REFERÊNCIAS BIBLIOGRÁFICAS

1. Kolb SJ, Kissel JT. Spinal Muscular Atrophy [Internet]. Vol. 33, Neurologic Clinics. 2015. p. 831–46. Available from: <http://dx.doi.org/10.1016/j.ncl.2015.07.004>
2. Pearn J. Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. *J Med Genet.* 1978 Dec;15(6):409–13.
3. Ogino S, Leonard DGB, Rennert H, Ewens WJ, Wilson RB. Genetic risk assessment in carrier testing for spinal muscular atrophy. *Am J Med Genet.* 2002 Jul 15;110(4):301–7.
4. Prior TW, Snyder PJ, Rink BD, Pearl DK, Pyatt RE, Mihal DC, et al. Newborn and carrier screening for spinal muscular atrophy. *Am J Med Genet A.* 2010 Jul;152A(7):1608–16.
5. Sugarman EA, Nagan N, Zhu H, Akmaev VR, Zhou Z, Rohlfes EM, et al. Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. *Eur J Hum Genet.* 2012 Jan;20(1):27–32.
6. D’Amico A, Mercuri E, Tiziano FD, Bertini E. Spinal muscular atrophy. *Orphanet J Rare Dis.* 2011 Nov 2;6:71.
7. Brzustowicz LM, Lehner T, Castilla LH, Penchaszadeh GK, Wilhelmsen KC, Daniels R, et al. Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. *Nature.* 1990 Apr 5;344(6266):540–1.
8. Melki J, Abdelhak S, Sheth P, Bachelot MF, Burlet P, Marcadet A, et al. Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. *Nature.* 1990 Apr 19;344(6268):767–8.
9. Arnold ES, Fischbeck KH. Spinal muscular atrophy. *Handb Clin Neurol.* 2018;148:591–601.
10. Chaytow H, Huang YT, Gillingwater TH, Faller KME. The role of survival motor neuron protein (SMN) in protein homeostasis. *Cell Mol Life Sci.* 2018 Nov;75(21):3877–94.
11. Russman BS. Spinal muscular atrophy: clinical classification and disease heterogeneity. *J Child Neurol.* 2007 Aug;22(8):946–51.
12. Feldkötter M, Schwarzer V, Wirth R, Wienker TF, Wirth B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet.* 2002 Feb;70(2):358–68.
13. Goodkey K, Aslesh T, Maruyama R, Yokota T. Nusinersen in the Treatment of Spinal Muscular Atrophy. *Methods Mol Biol.* 2018;1828:69–76.
14. Rao VK, Kapp D, Schroth M. Gene Therapy for Spinal Muscular Atrophy: An Emerging Treatment Option for a Devastating Disease. *J Manag Care Spec Pharm.* 2018 Dec;24(12-a Suppl):S3–16.
15. Hoy SM. Onasemnogene Apeparovvec: First Global Approval. *Drugs.* 2019 Jul;79(11):1255–62.
16. Messina S, Sframeli M. New Treatments in Spinal Muscular Atrophy: Positive Results and New Challenges. *J Clin Med Res [Internet].* 2020 Jul 13;9(7). Available from: <http://dx.doi.org/10.3390/jcm9072222>
17. Dhillon S. Risdiplam: First Approval. *Drugs.* 2020 Nov;80(17):1853–8.

18. Farrar MA, Kiernan MC. The Genetics of Spinal Muscular Atrophy: Progress and Challenges. *Neurotherapeutics*. 2015 Apr;12(2):290–302.
19. Schorling DC, Pechmann A, Kirschner J. Advances in Treatment of Spinal Muscular Atrophy - New Phenotypes, New Challenges, New Implications for Care. *J Neuromuscul Dis*. 2020;7(1):1–13.
20. Waldrop MA, Elsheikh BH. Spinal Muscular Atrophy in the Treatment Era. *Neurol Clin*. 2020 Aug;38(3):505–18.
21. Yeo CJJ, Darras BT. Overturning the Paradigm of Spinal Muscular Atrophy as Just a Motor Neuron Disease. *Pediatr Neurol*. 2020 Aug;109:12–9.
22. Sumner CJ, Wee CD, Warsing LC, Choe DW, Ng AS, Lutz C, et al. Inhibition of myostatin does not ameliorate disease features of severe spinal muscular atrophy mice. *Hum Mol Genet*. 2009 Sep 1;18(17):3145–52.
23. Rindt H, Buckley DM, Vale SM, Krogman M, Rose FF Jr, Garcia ML, et al. Transgenic inactivation of murine myostatin does not decrease the severity of disease in a model of Spinal Muscular Atrophy. *Neuromuscul Disord*. 2012 Mar;22(3):277–85.
24. Rose FF Jr, Mattis VB, Rindt H, Lorson CL. Delivery of recombinant follistatin lessens disease severity in a mouse model of spinal muscular atrophy. *Hum Mol Genet*. 2009 Mar 15;18(6):997–1005.
25. Harris AW, Butchbach MER. The effect of the DcpS inhibitor D156844 on the protective action of follistatin in mice with spinal muscular atrophy. *Neuromuscul Disord*. 2015 Sep;25(9):699–705.
26. Feng Z, Ling KKY, Zhao X, Zhou C, Karp G, Welch EM, et al. Pharmacologically induced mouse model of adult spinal muscular atrophy to evaluate effectiveness of therapeutics after disease onset. *Hum Mol Genet*. 2016 Mar 1;25(5):964–75.
27. Liu M, Hammers DW, Barton ER, Sweeney HL. Activin Receptor Type IIB Inhibition Improves Muscle Phenotype and Function in a Mouse Model of Spinal Muscular Atrophy. *PLoS One*. 2016 Nov 21;11(11):e0166803.
28. Long KK, O’Shea KM, Khairallah RJ, Howell K, Paushkin S, Chen KS, et al. Specific inhibition of myostatin activation is beneficial in mouse models of SMA therapy. *Hum Mol Genet*. 2019 Apr 1;28(7):1076–89.
29. Zhou H, Meng J, Malerba A, Catapano F, Sintusek P, Jarmin S, et al. Myostatin inhibition in combination with antisense oligonucleotide therapy improves outcomes in spinal muscular atrophy. *J Cachexia Sarcopenia Muscle*. 2020 Jun;11(3):768–82.
30. Jiang J, Huang J, Gu J, Cai X, Zhao H, Lu H. Genomic analysis of a spinal muscular atrophy (SMA) discordant family identifies a novel mutation in TLL2, an activator of growth differentiation factor 8 (myostatin): a case report. *BMC Med Genet*. 2019 Dec 30;20(1):204.
31. Long K, Cote S, Wawersik. P.270 Myostatin dynamics in health and disease: Pharmacologic effects of SRK-015, a highly selective monoclonal antibody inhibitor of myostatin activation [Internet]. *Abstracts/Neuromuscular Disorders* 30; 2020. Available from: [https://www.nmd-journal.com/article/S0960-8966\(20\)30466-1/fulltext](https://www.nmd-journal.com/article/S0960-8966(20)30466-1/fulltext)
32. Mariot V, Joubert R, Hourdé C, Féasson L, Hanna M, Muntoni F, et al. Downregulation of myostatin pathway in neuromuscular diseases may explain challenges of anti-myostatin

therapeutic approaches. *Nat Commun.* 2017 Nov 30;8(1):1859.

33. Welsh BT, Cote SM, Meshulam D, Jackson J, Pal A, Lansita J, et al. Preclinical Safety Assessment and Toxicokinetics of Apitegromab, an Antibody Targeting Proforms of Myostatin for the Treatment of Muscle-Atrophiying Disease. *Int J Toxicol.* 2021 Jul-Aug;40(4):322–36.
34. Barrett D, Bilic S, Chyung Y, Cote SM, Iarrobino R, Kacena K, et al. A Randomized Phase 1 Safety, Pharmacokinetic and Pharmacodynamic Study of the Novel Myostatin Inhibitor Apitegromab (SRK-015): A Potential Treatment for Spinal Muscular Atrophy. *Adv Ther.* 2021 Jun;38(6):3203–22.
35. Abati E, Manini A, Comi GP, Corti S. Inhibition of myostatin and related signaling pathways for the treatment of muscle atrophy in motor neuron diseases. *Cell Mol Life Sci.* 2022 Jun 21;79(7):374.
36. Lee SJ. Targeting the myostatin signaling pathway to treat muscle loss and metabolic dysfunction. *J Clin Invest [Internet].* 2021 May 3;131(9). Available from: <http://dx.doi.org/10.1172/JCI148372>
37. Lee YA, Kwon A, Kim JH, Nam HK, Yoo JH, Lim JS, et al. Clinical practice guidelines for optimizing bone health in Korean children and adolescents. *Ann Pediatr Endocrinol Metab.* 2022 Mar;27(1):5–14.
38. Świtońska-Kurkowska K, Krist B, Delimata J, Figiel M. Juvenile Huntington’s Disease and Other PolyQ Diseases, Update on Neurodevelopmental Character and Comparative Bioinformatic Review of Transcriptomic and Proteomic Data. *Front Cell Dev Biol.* 2021 Jul 1;9:642773.
39. Järvillehto J, Harjuhaahto S, Palu E, Auranen M, Kvist J, Zetterberg H, et al. Serum Creatine, Not Neurofilament Light, Is Elevated in CHCHD10-Linked Spinal Muscular Atrophy. *Front Neurol.* 2022 Feb 17;13:793937.
40. Chang WF, Peng M, Hsu J, Xu J, Cho HC, Hsieh-Li HM, et al. Effects of Survival Motor Neuron Protein on Germ Cell Development in Mouse and Human. *Int J Mol Sci [Internet].* 2021 Jan 11;22(2). Available from: <http://dx.doi.org/10.3390/ijms22020661>
41. Hellbach N, Karrer TM, Moal ML, et al. Characterizing novel exploratory biomarkers in a longitudinal natural history study in patients with Types 2 and 3 SMA. In 2021.
42. Mariot V, Le Guiner C, Barthélémy I, Montus M, Blot S, Torelli S, et al. Myostatin Is a Quantifiable Biomarker for Monitoring Pharmaco-gene Therapy in Duchenne Muscular Dystrophy. *Mol Ther Methods Clin Dev.* 2020 Sep 11;18:415–21.
43. Burch PM, Pogoryelova O, Palandra J, Goldstein R, Bennett D, Fitz L, et al. Reduced serum myostatin concentrations associated with genetic muscle disease progression. *J Neurol.* 2017 Mar;264(3):541–53.
44. Byers RK, Banker BQ. Infantile muscular atrophy. *Arch Neurol.* 1961 Aug;5:140–64.
45. Munsat TL. Workshop Report: International collaboration. In *Neuromuscul Disord*; 1991. p. 1:81–3.
46. Zerres K, Davies KE. 59th ENMC International Workshop: Spinal Muscular Atrophies: recent progress and revised diagnostic criteria 17–19 April 1998, Soestduinen, The Netherlands. *Neuromuscul Disord.* 1999 Jun;9(4):272–8.
47. Prior TW, Leach ME, Finanger E. Spinal Muscular Atrophy. In: Adam MP, Ardinger HH, Pagon

RA, Wallace SE, Bean LJH, Stephens K, et al., editors. GeneReviews. Seattle (WA): University of Washington, Seattle; 2000.

48. Lunn MR, Wang CH. Spinal muscular atrophy. *Lancet*. 2008 Jun;371(9630):2120–33.
49. Pearn J. Classification of Spinal Muscular Atrophies. *Lancet*. 1980 Apr;315(8174):919–22.
50. Ogino S, Wilson RB. Genetic testing and risk assessment for spinal muscular atrophy (SMA). *Hum Genet*. 2002 Dec;111(6):477–500.
51. Oliveira Netto AB, Brusius-Facchin AC, Lemos JF, Pasetto FB, Brasil CS, Trapp FB, et al. Neonatal screening for spinal muscular atrophy: A pilot study in Brazil. *Genet Mol Biol*. 2023 Dec 11;46(3 Suppl 1):e20230126.
52. Merlini L, Bonfiglioli Stagni S, Marri E, Granata C. Epidemiology of neuromuscular disorders in the under-20 population in Bologna province, Italy. *Neuromuscul Disord*. 1992 Jan;2(3):197–200.
53. Verhaart IEC, Robertson A, Wilson IJ, Aartsma-Rus A, Cameron S, Jones CC, et al. Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - a literature review. *Orphanet J Rare Dis*. 2017 Jul 4;12(1):124.
54. Arnold WD, Kassar D, Kissel JT. Spinal muscular atrophy: diagnosis and management in a new therapeutic era. *Muscle Nerve*. 2015 Feb;51(2):157–67.
55. Hoy SM. Nusinersen: A Review in 5q Spinal Muscular Atrophy. *CNS Drugs*. 2018 Jul;32(7):689–96.
56. Chen TH. New and Developing Therapies in Spinal Muscular Atrophy: From Genotype to Phenotype to Treatment and Where Do We Stand? *Int J Mol Sci [Internet]*. 2020 May 7;21(9). Available from: <http://dx.doi.org/10.3390/ijms21093297>
57. Monani UR, Sendtner M, Coover DD, Parsons DW, Andreassi C, Le TT, 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*. 2000 Feb 12;9(3):333–9.
58. Le TT, Pham LT, Butchbach MER, Zhang HL, Monani UR, Coover DD, et al. SMN Δ 7, 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*. 2005 Mar 15;14(6):845–57.
59. Lotti F, Imlach WL, Saieva L, Beck ES, Hao LT, Li DK, et al. An SMN-dependent U12 splicing event essential for motor circuit function. *Cell*. 2012 Oct 12;151(2):440–54.
60. Hao LT, Burghes AH, Beattie CE. Generation and Characterization of a genetic zebrafish model of SMA carrying the human SMN2 gene. *Mol Neurodegener*. 2011 Mar 28;6(1):24.
61. Wang CH, Finkel RS, Bertini ES, Schroth M, Simonds A, Wong B, et al. Consensus statement for standard of care in spinal muscular atrophy. *J Child Neurol*. 2007 Aug;22(8):1027–49.
62. Mercuri E, Finkel RS, Muntoni F, Wirth B, Montes J, Main M, et al. Diagnosis and management of spinal muscular atrophy: Part 1: Recommendations for diagnosis, rehabilitation, orthopedic and nutritional care. *Neuromuscul Disord*. 2018 Feb;28(2):103–15.
63. Wirth B, Herz M, Wetter A, Moskau S, Hahnen E, Rudnik-Schöneborn S, et al. Quantitative analysis of survival motor neuron copies: identification of subtle SMN1 mutations in patients with spinal muscular atrophy, genotype-phenotype correlation, and implications for genetic

- counseling. *Am J Hum Genet.* 1999 May;64(5):1340–56.
64. Finkel R, Bertini E, Muntoni F, Mercuri E, 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.* 2015 Jul;25(7):593–602.
 65. McAndrew PE, Parsons DW, Simard LR, Rochette C, Ray PN, Mendell JR, et al. Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *Am J Hum Genet.* 1997 Jun;60(6):1411–22.
 66. Wirth B, Brichta L, Schrank B, Lochmüller H, Blick S, Baasner A, et al. Mildly affected patients with spinal muscular atrophy are partially protected by an increased SMN2 copy number. *Hum Genet.* 2006 May;119(4):422–8.
 67. Dos Santos MAR, Brighente SF, Massignan A, Tenório RB, Makariewicz LL, Moreira AL, et al. Accuracy of muscle fasciculations for the diagnosis of later-onset spinal muscle atrophy. *Neuromuscul Disord.* 2022 Sep;32(9):763–8.
 68. Baioni MTC, Ambiel CR. Spinal muscular atrophy: diagnosis, treatment and future prospects. *J Pediatr.* 2010 Jul;86(4):261–70.
 69. Lee JH, Jun HS. Role of Myokines in Regulating Skeletal Muscle Mass and Function. *Front Physiol.* 2019 Jan 30;10:42.
 70. Pino MG, Rich KA, Kolb SJ. Update on Biomarkers in Spinal Muscular Atrophy. *Biomark Insights.* 2021 Aug 14;16:11772719211035643.
 71. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, et al. Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N Engl J Med.* 2017 Nov 2;377(18):1723–32.
 72. Mercuri E, Darras BT, Chiriboga CA, Day JW, Campbell C, Connolly AM, et al. Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy. *N Engl J Med.* 2018 Feb 15;378(7):625–35.
 73. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, et al. Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N Engl J Med.* 2017 Nov 2;377(18):1723–32.
 74. Glanzman AM, Mazzone E, Main M, Pelliccioni M, Wood J, Swoboda KJ, et al. The Children’s Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND): test development and reliability. *Neuromuscul Disord.* 2010 Mar;20(3):155–61.
 75. Glanzman AM, McDermott MP, Montes J, Martens WB, Flickinger J, Riley S, et al. Validation of the Children’s Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND). *Pediatr Phys Ther.* 2011 Winter;23(4):322–6.
 76. Haataja L, Mercuri E, Regev R, Cowan F, Rutherford M, Dubowitz V, et al. Optimality score for the neurologic examination of the infant at 12 and 18 months of age. *J Pediatr.* 1999 Aug;135(2):153–61.
 77. Bishop KM, Montes J, Finkel RS. Motor milestone assessment of infants with spinal muscular atrophy using the hammersmith infant neurological Exam-Part 2: Experience from a nusinersen clinical study. *Muscle Nerve.* 2018 Jan;57(1):142–6.
 78. Main M, Kairon H, Mercuri E, Muntoni F. The Hammersmith Functional Motor Scale for

Children with Spinal Muscular Atrophy: a Scale to Test Ability and Monitor Progress in Children with Limited Ambulation. *Eur J Paediatr Neurol*. 2003 Jul;7(4):155–9.

79. O'Hagen JM, Glanzman AM, McDermott MP, Ryan PA, Flickinger J, Quigley J, et al. An expanded version of the Hammersmith Functional Motor Scale for SMA II and III patients. *Neuromuscul Disord*. 2007 Oct;17(9-10):693–7.
80. Glanzman AM, O'Hagen JM, McDermott MP, Martens WB, Flickinger J, Riley S, et al. Validation of the Expanded Hammersmith Functional Motor Scale in spinal muscular atrophy type II and III. *J Child Neurol*. 2011 Dec;26(12):1499–507.
81. Mazzone E, Bianco F, Martinelli D, Glanzman AM, Messina S, De Sanctis R, et al. Assessing upper limb function in nonambulant SMA patients: development of a new module. *Neuromuscul Disord*. 2011 Jun;21(6):406–12.
82. Mazzone ES, Mayhew A, Montes J, Ramsey D, Fanelli L, Young SD, et al. Revised upper limb module for spinal muscular atrophy: Development of a new module. *Muscle Nerve*. 2017 Jun;55(6):869–74.
83. Pelosi L, Rodrigues M, Zhong C, Patel S, Roxburgh R. Quantitative muscle ultrasound in adult spinal muscular atrophy. A pilot study. *Muscle Nerve*. 2024 Mar;69(3):349–53.
84. Moreira AL, Mendonça RH, Polido GJ, Oliveira MCB, Silva AMS, Zanoteli E. Muscle Ultrasound Changes Correlate With Functional Impairment in Spinal Muscular Atrophy. *Ultrasound Med Biol*. 2023 Jul;49(7):1569–74.
85. Sumner CJ, Kolb SJ, Harmison GG, Jeffries NO, Schadt K, Finkel RS, et al. SMN mRNA and protein levels in peripheral blood: biomarkers for SMA clinical trials. *Neurology*. 2006 Apr 11;66(7):1067–73.
86. Zheleznyakova GY, Voisin S, Kiselev AV, Sällman Almén M, Xavier MJ, Maretina MA, et al. Genome-wide analysis shows association of epigenetic changes in regulators of Rab and Rho GTPases with spinal muscular atrophy severity. *Eur J Hum Genet*. 2013 Sep;21(9):988–93.
87. Finkel RS, Crawford TO, Swoboda KJ, Kaufmann P, Juhasz P, Li X, et al. Candidate proteins, metabolites and transcripts in the Biomarkers for Spinal Muscular Atrophy (BforSMA) clinical study. *PLoS One*. 2012 Apr 27;7(4):e35462.
88. Navarrete-Opazo A, Garrison S, Waite M. Molecular Biomarkers for Spinal Muscular Atrophy: A Systematic Review. *Neurol Clin Pract*. 2021 Aug;11(4):e524–36.
89. Darras BT, Crawford TO, Finkel RS, Mercuri E, De Vivo DC, Oskoui M, et al. Neurofilament as a potential biomarker for spinal muscular atrophy. *Ann Clin Transl Neurol*. 2019 May;6(5):932–44.
90. Olsson B, Alberg L, Cullen NC, Michael E, Wahlgren L, Kroksmark AK, et al. NFL is a marker of treatment response in children with SMA treated with nusinersen. *J Neurol*. 2019 Sep;266(9):2129–36.
91. Wurster CD, Steinacker P, Günther R, Koch JC, Lingor P, Uzelac Z, et al. Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen. *J Neurol*. 2020 Jan;267(1):36–44.
92. Rich KA, Fox A, Yalvac M, Heintzman S, Tellez M, Bartlett A, et al. Neurofilament Levels in CSF and Serum in an Adult SMA Cohort Treated with Nusinersen. *J Neuromuscul Dis*. 2022;9(1):111–9.

93. Seo G, Kim S, Byun JC, Kwon S, Lee YJ. Evaluation of the neurofilament light chain as a biomarker in children with spinal muscular atrophy treated with nusinersen. *Brain Dev.* 2023 Nov;45(10):554–63.
94. Deutsch L, Osredkar D, Plavec J, Stres B. Spinal Muscular Atrophy after Nusinersen Therapy: Improved Physiology in Pediatric Patients with No Significant Change in Urine, Serum, and Liquor 1H-NMR Metabolomes in Comparison to an Age-Matched, Healthy Cohort. *Metabolites* [Internet]. 2021 Mar 30;11(4). Available from: <http://dx.doi.org/10.3390/metabo11040206>
95. Pedersen BK, Steensberg A, Fischer C, Keller C, Keller P, Plomgaard P, et al. Searching for the exercise factor: is IL-6 a candidate? *J Muscle Res Cell Motil.* 2003;24(2-3):113–9.
96. Pedersen BK, Akerström TCA, Nielsen AR, Fischer CP. Role of myokines in exercise and metabolism. *J Appl Physiol.* 2007 Sep;103(3):1093–8.
97. Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics.* 2014 Feb;13(2):397–406.
98. Reza MM, Subramaniyam N, Sim CM, Ge X, Sathiakumar D, McFarlane C, et al. Irisin is a pro-myogenic factor that induces skeletal muscle hypertrophy and rescues denervation-induced atrophy. *Nat Commun.* 2017 Oct 24;8(1):1104.
99. Colaianni G, Mongelli T, Cuscito C, Pignataro P, Lippo L, Spiro G, et al. Irisin prevents and restores bone loss and muscle atrophy in hind-limb suspended mice. *Sci Rep.* 2017 Jun 6;7(1):2811.
100. Huang SH, Yang SM, Lo JJ, Wu SH, Tai MH. Irisin Gene Delivery Ameliorates Burn-Induced Sensory and Motor Neuropathy. *Int J Mol Sci* [Internet]. 2020 Oct 21;21(20). Available from: <http://dx.doi.org/10.3390/ijms21207798>
101. Reza MM, Sim CM, Subramaniyam N, Ge X, Sharma M, Kambadur R, et al. Irisin treatment improves healing of dystrophic skeletal muscle. *Oncotarget.* 2017 Nov 17;8(58):98553–66.

8. ARTIGOS

8.1 ARTIGO I

Title Page

Clinicogenetic characterization and Response to Disease-Modifying Therapies in Spinal Muscular Atrophy: Real-World Experience from a Reference Center in Southern Brazil

Não disponível, pois se trata de artigo submetido para publicação.

8.2 ARTIGO II

(Brain Commun. 2024 Feb28;6(2):fcae062. doi:10.1093/braincomms/fcae062. eCollection 2024) - QUALIS B3

Title Page

Serum Myostatin as a Candidate Disease Severity and Progression Biomarker of Spinal Muscular Atrophy

Ana Letícia Amorim de Albuquerque¹, Júlia Kersting Chadanowicz², Giovanna Câmara Giudicelli^{3,4}, Ana Lucia Portella Staub², Arthur Carpeggiani Weber², Jordana Miranda De Souza Silva¹, Michele Michelin Becker⁵, Thayne Woycinck Kowalski^{3,4,6}, Marina Siebert^{7,8}, Jonas Alex Morales Saute^{1,2,6,9*}.

¹Graduate Program in Medicine: Medical Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

²Clinical Neurogenetics research group, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

³Bioinformatics core, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

⁴Graduate Program in Genetics and Molecular Biology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

⁵Child Neurology Unit, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

⁶Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

⁷Unit of Laboratorial Research, Experimental Research Center, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil.

⁸Graduate Program in Gastroenterology and Hepatology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

⁹Department of Internal Medicine, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

*Corresponding author:

Jonas Alex Morales Saute

Hospital de Clínicas de Porto Alegre, Medical Genetics Service. Av. Ramiro Barcelos, 2350, Santa Cecília 90035903 - Porto Alegre, RS – Brazil. Phone +55 51 33598011. E-mail: jsaute@hcpa.edu.br

ABSTRACT

INTRODUCTION: The identification of biomarkers for spinal muscular atrophy (SMA) is crucial for predicting disease progression, severity, and response to new disease-modifying therapies. **OBJECTIVE:** This study aimed to investigate the role of serum levels of myostatin and follistatin as biomarkers for SMA, considering muscle atrophy secondary to denervation as the main clinical manifestation of the disease. **METHODOLOGY:** The study evaluated the differential gene expression of myostatin and follistatin in a lesional model of *gastrocnemius* denervation in mice, as well as in a meta-analysis of three datasets in transgenic mice models of SMA, and in two studies involving humans with SMA. Subsequently, a case-control study involving 27 SMA patients and 27 controls was conducted, followed by a 12-month cohort study with 25 SMA cases. Serum levels of myostatin and follistatin were analyzed using enzyme-linked immunosorbent assay at a single center in southern Brazil. **RESULTS:** Skeletal muscle gene expression of myostatin decreased and of follistatin increased following lesional muscle denervation in mice, consistent with findings in the SMA transgenic mice meta-analysis and in the *iliopsoas* muscle of 5 patients with SMA type 1. Median serum myostatin levels were significantly lower in SMA patients (98 pg/mL; 5-157) compared to controls (412 pg/mL; 299-730) ($p < 0.001$). Lower myostatin levels were associated with greater disease severity based on clinician-rated outcomes ($Rho = 0.493-0.812$; $p < 0.05$). After 12 months, there was a further reduction in myostatin levels among SMA cases ($p = 0.021$). Follistatin levels did not differ between cases and controls, and no significant changes were observed over time. The follistatin:myostatin ratio was significantly increased in SMA subjects and inversely correlated with motor severity. **CONCLUSION:** Serum myostatin levels show promise as a novel biomarker for evaluating the severity and progression of SMA. The decrease in myostatin levels and the subsequent favorable environment for muscle growth may be attributed to denervation caused by motor neuron dysfunction.

KEYWORDS: Spinal Muscular Atrophy, biomarker, myokine, myostatin, follistatin, differential gene expression.

1. INTRODUCTION

Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease characterized by progressive motor neuron degeneration^{1,2,3}. Its incidence is estimated at 1 in 6000-10,000 live births, with a prevalence of 1 in 40-60 individuals as mutation carriers in the general population^{4,5}. SMA is a leading cause of infant mortality and the second most common fatal autosomal recessive disease^{1,2,3}.

The disease is caused by bi-allelic pathogenic variants in the survival of motor neuron 1 gene (*SMN1*), resulting in the absence of SMN protein production^{1,2,3,6}. The complete absence of this protein would be lethal during the embryonic period, however, the presence of the *SMN2* gene, a paralogous gene which varies in copy number, allows for the production of a small amount of functional SMN protein^{1,2,3}. The severity of SMA exhibits significant variation, and its clinical manifestations can be classified into five subtypes. Type 0 is the most severe form, occurring in the neonatal period. Type 1 is characterized by flaccid paralysis and the inability to sit without support, with symptoms emerging before 6 months of age. Type 2 has an onset between 6 and 18 months, where children can achieve independent sitting but are unable to walk independently. Type 3 is a more heterogeneous form, affecting children or young individuals who can walk independently but gradually experience limb weakness progression. Type 4 is the mildest form, with disease onset occurring after 18 years of age^{2,3}. Advancements in understanding SMA pathophysiology have led to significant progress in disease-modifying therapies targeting SMN levels like nusinersen, onasemnogene abeparvovec and risdiplam^{3,7,8}. Early initiation of therapy has shown the greatest effectiveness, particularly in pre-symptomatic children with 2 to 3 copies of *SMN2*^{2,8}. However, individual patients' clinical courses and disease progression sometimes deviate from the typical natural history, suggesting variations in treatment response regardless of age at initiation^{9,10}.

Therefore, it is important to identify SMA biomarkers for assessing disease severity, progression, prognosis, and therapeutic response. Previous studies have examined blood markers such as *SMN* mRNA and protein levels¹¹, leukocyte DNA methylation profile^{11,12}, and plasma proteins identified through unbiased proteomic studies¹³. However, these markers lacked clear differentiation among SMA subtypes and showed limited association with disease severity, reducing their clinical utility¹³. The plasma levels of neurofilament heavy chain (pNF-H)^{14,15} and light chain (NfL)^{16,17,18,19} have demonstrated potential as biomarkers for axonal damage, reflecting disease severity and treatment response in infantile-onset SMA. However, these findings have not been reproducible in patients with SMA types 2 and 3, nor in chronic stages of SMA^{17,18,19}.

Considering SMA as a neuronal disease with clinical manifestations primarily occurring due to denervation-induced skeletal muscle alterations, we aimed to evaluate the potential of myostatin, a negative regulator of muscle growth, its antagonist, follistatin, and their ratio as biomarkers. We analyzed skeletal muscle differential gene expression (DGE) data from lesional denervation and transgenic mice models of SMA, as well as from humans with SMA in a bioinformatic study. Subsequently, we assessed the serum levels of myostatin and follistatin in a clinical study to investigate their potential as biomarkers for disease severity, progression, and treatment response to novel therapies.

2. METHODOLOGY

2.1 Study Design

The study consisted of two phases. The first phase involved a bioinformatic study in which the analysis of candidate myokine expression was performed using raw data from DGE studies in muscle or blood samples from humans and mice, obtained from public repositories. The second phase included a cross-sectional case-control study, followed by a cohort study conducted at a single center.

2.2 Bioinformatic study

To investigate the potential alteration of myostatin and follistatin in SMA due to muscle denervation, we initially manually selected a study that examined the temporal DGE of myokines in the *gastrocnemius* muscle of mice following tibial nerve denervation²⁰. After that, transcriptome datasets evaluating SMA disease were searched on publicly available repositories Gene Expression Omnibus (GEO)²¹ and ArrayExpress (AE)²². The following terms were used as keywords: “(SMA) OR (SMA AND muscle) OR (SMA AND blood) OR (Spinal muscular atrophy) OR (Spinal muscular atrophy AND muscle) OR (Spinal muscular atrophy AND blood)”. Filters were applied to meet the inclusion criteria of studies conducted on blood or muscle samples of humans and/or mice through microarray and/or RNA-Seq methodologies. Experiments performed in cell lines, with less than three samples per group, without control groups, or without raw data available in the databases were excluded.

2.3 Clinical study

The first phase of the clinical study involved a cross-sectional case-control study, where serum levels of myostatin and follistatin were measured in both groups. Additionally, in the case group, clinician-rated outcomes (ClinRO) were assessed: Children's Hospital of Philadelphia Infant-Test-of-Neuromuscular-Disorders (CHOP-INTEND); Hammersmith-Infant Neurological-Examination (HINE); Hammersmith-Functional-Motor-Scale-Expanded (HFMSE); and Revised-Upper-Limb-Module (RULM). SMA functional status was categorized as non-sitters; sitters (defined as able to sit without support, stand alone or stand with assistance); and walkers (defined as able to walk independently). In the second phase, a longitudinal cohort was conducted exclusively with the case group. This cohort involved a 12-month follow-up period during which the analysis of biomarkers was repeated.

Subjects in the study were recruited consecutively from May to October 2021 at the Neuromuscular Genetics outpatient clinic of Hospital de Clínicas de Porto Alegre and included individuals with a confirmed genetic diagnosis of SMA-5q type 1, 2, or 3 undergoing regular follow-up.

The control group consisted of healthy participants with a body mass index (BMI) appropriate for their age, selected based on sex and age matching with the case group. The control group was recruited from the hospital community through invitations extended to healthy relatives of patients consulting at the hospital due to other diseases or patients seeking consultation for other health issues that did not meet exclusion criteria (e.g., those on the waiting list for umbilical or inguinal hernia surgery, etc.). Individuals with other neurological or systemic conditions that could lead to additional motor impairments or negatively impact cardiopulmonary performance, such as stroke sequelae, chronic obstructive pulmonary disease, decompensated heart failure, severe asthma, were excluded from both groups.

The study was conducted in accordance with ethical guidelines and was approved by the institutional research ethics committee (GPPG-HCPA-2020-0731). Informed consent was obtained from all participants, adhering to the ethical principles outlined in the Declaration of Helsinki.

2.3.1 Serum analysis

Three mL of serum samples were obtained from peripheral venous blood of participants and controls and placed in serum separator tubes. The samples were left at room temperature (18-25°C) for 30-45 minutes to clot, then centrifuged at 1000xg for 10 minutes at a temperature of 23°C. Care was taken to avoid hemolysis and the separated serum was stored in a freezer at -80°C until analysis. Serum levels of myostatin and follistatin were measured in duplicate with a commercially available enzyme-linked immunosorbent-assay kit (R&D-Systems, DuoSet® GDF-8/Myostatin, cat. no. DY788-05, and DuoSet® human Follistatin, cat. no. DY669, Minneapolis, MN, USA), in accordance with the manufacturer's instructions.

2.4 Statistical analysis

2.4.1 Datasets processing and meta-analysis

The microarray studies were manually extracted through GEO/AE and later analyzed with the *affy* package²³ in R v4.2.2²⁴ and RStudio v2022.7.2.576 softwares applying a robust multiarray average (RMA) normalization. The raw data from the RNA-Seq datasets were processed on the useGalaxy server²⁵ as follows: .fastq quality was evaluated in the FastQC²⁶, alignment was performed using the Bowtie2²⁷, and quantification using the featurecounts²⁸ tools. DGE analysis was conducted for each dataset in R and RStudio using the *limma* package²⁹ for the microarray studies and *edgeR* package³⁰ for the RNA-Seq assays. Principal Component Analysis was performed for each dataset to evaluate the heterogeneity of the samples. The surrogate variable analysis normalization was performed to reduce batch effects. The resulting genes from the DGE analysis with $\log_{2}FC \geq 1$ and $P\text{-Value} \leq 0.05$ were considered upregulated, whilst genes with $\log_{2}FC \leq -1$ and $P\text{-Value} \leq 0.05$ were considered downregulated.

2.4.2 Analysis of the clinical study

Statistical tests were selected according to the distribution of data given by Shapiro-Wilk test and histograms. The variables in the study did not have normal distribution and were shown as median (interquartile ranges), except for BMI, CHOP-INTEND, HFMSE and RULM which are presented as mean (95% confidence intervals, CI). Comparisons between paired cases and controls were performed with Wilcoxon signed-rank test, with corrections for weight and BMI (covariates) performed using rank analysis of covariance (Quade's test), or Chi-Square. Comparisons among SMA subtypes, functional status and *SMN2* copy numbers were performed with Kruskal-Wallis test. Comparisons between SMA treated and untreated subjects at baseline were performed with Mann-Whitney U-test. Changes in myostatin and follistatin serum levels and its ratio from baseline to 12 months of follow-up were performed with Wilcoxon signed-rank test. Correlations between myokines levels at baseline with age, age at onset, reported disease duration and the ClinROs were performed with Spearman correlation-test. We also performed Spearman correlation test searching for the association of the difference in myokines levels from 12 months to baseline (DeltaMyokine) with disease severity variables at baseline and time since onset of treatment (TSOT). TSOT considered the number of days of treatment when baseline samples were collected, with positive values indicating subjects already taking the treatment at baseline, zero meaning that treatment was started at

baseline visit and negative values that treatment was started after baseline. Untreated patients were censored for this last analysis.

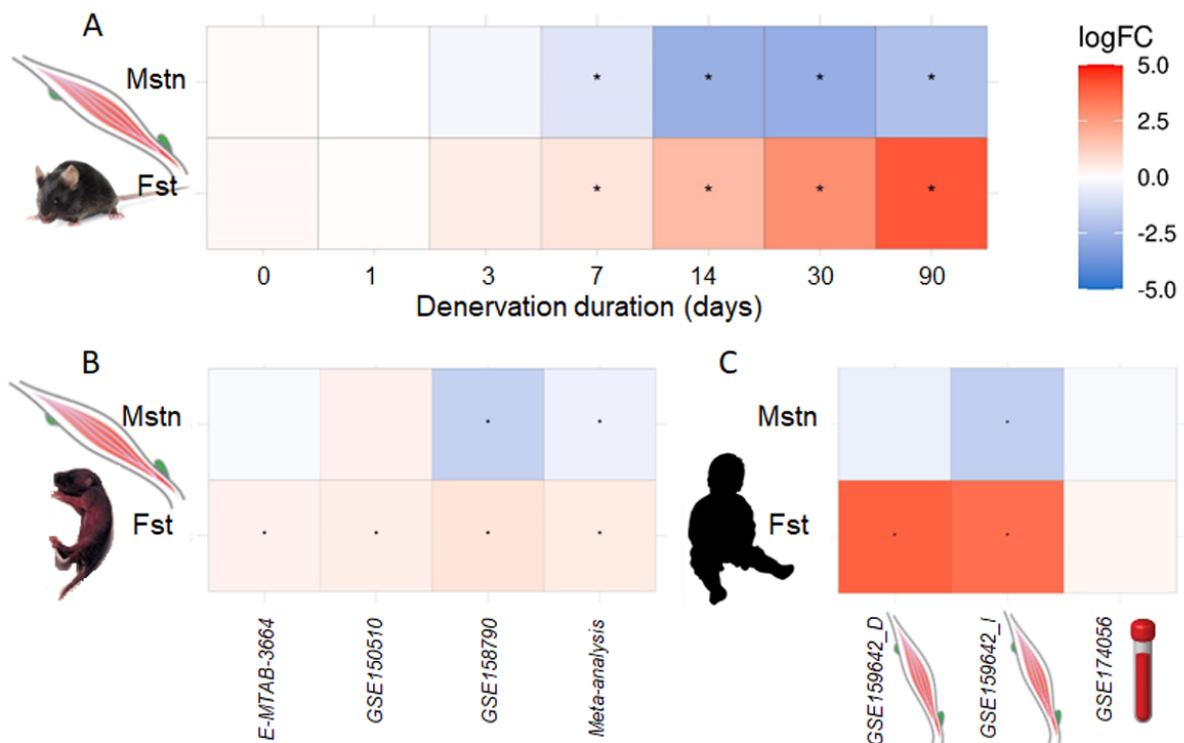
3. RESULTS

3.1 Bioinformatic Study: Transcriptome datasets

3.1.1 Temporal pattern of myostatin and follistatin DGE in muscle submitted to lesional denervation

To investigate the potential alterations in myokine regulation due to muscle denervation and atrophy in SMA, we examined the DGE of myostatin (*Mstn*) and follistatin (*Fst*) in a mouse model of neurogenic skeletal muscle atrophy²⁰. We analyzed RNA-seq data from the *gastrocnemius* muscle of male mice following lesional denervation of the tibial nerve. The time points evaluated included baseline, 1, 3, 7, 14, 30, and 90 days post-lesion, with four mice per group. Our analysis revealed a significant decrease in *Mstn* expression and an increase in *Fst* expression in the denervated muscle, starting at 7 days post-lesion and persisting until 90 days (Figure 1A, Supplementary Table 1).

Figure 1 – Heat-map of the differential expression of myokines in acute and chronic neurogenic skeletal muscle atrophy in mice.



3.1.2 Systematic review of public databases on DGE in SMA

The systematic review of GEO/AE databases identified three relevant studies with RNA-Seq in mice, two conducted with the Taiwanese model *Smn*^{-/-};*SMN2* SMA mice³¹⁻³³, one with the SMN Delta7 model²⁹. In two studies, DGE was assessed in the *tibialis anterior* muscle, with one study examining postnatal day 7 in three transgenic mice and three controls of both sexes (GSE150510)³³ and the other examining postnatal day 10 in four transgenic mice (sex not informed) and five controls (GSE158790)³². The third study (E-MTAB-3664)³¹ evaluated skeletal muscle in four affected and four control mice (similar proportion of male and females)

at postnatal days 1 and 4³¹, which were grouped together prior to analysis. A decrease in *Mstn* expression in SMA mice was found on the GSE158790 dataset and the meta-analysis of all three studies. Conversely, *Fst* expression was found to be increased in SMA mice across all three datasets and in the meta-analysis (**Figure 1B, Supplementary Table 1**).

We identified two human studies that assessed transcriptomes in muscle tissue or blood samples. In the study conducted by Ramos and colleagues³² (GSE159642), DGE was examined in post-mortem diaphragm and *iliopsoas* samples from five patients with SMA type 1 (aged 4-16 months, except for one case with unknown age) and one additional diaphragm sample from a patient with SMA type 2 (72 months old). These samples were compared to three control samples from individuals who died of unknown causes/cardiac arrest (aged 19-168 months). The findings of this study demonstrated a similar pattern of DGE to the mice studies, with decreased expression of *MSTN* and increased expression of *FST* in the *iliopsoas* (I) muscle of SMA patients, as well as increased expression of *FST* in the diaphragm (D) (**Figure 1C, Supplementary Table 1**). In the study GSE174056, DGE analysis was performed on whole-blood samples using RNA-seq. This study included five patients with SMA type 1 (all with 2 copies of *SMN2*) under 1 year of age (121-259 days) and five healthy age-matched controls under 1 year old³³. The results showed no significant differences in the expression of myostatin (LogFC = -0.1894; P-Value = 0.891) or follistatin (LogFC = 0.2449; P-Value = 0.757) (**Figure 1C**). Due to the limited number of eligible human studies, a meta-analysis could not be performed.

These results supported the hypothesis that myostatin and follistatin could serve as potential biomarkers for SMA, warranting further evaluation in a clinical study.

3.2 Clinical Study

A total of 54 participants were included in the study, comprising 27 homozygous cases with the common deletion in the *SMN1* and 27 healthy individuals. The detailed clinical, genetic, and demographic characteristics of the participants can be found in **Table 1**. At baseline, 11 out of 27 (40.7%) cases were receiving disease-modifying therapies related to SMN levels.. All of them received nusinersen, and four out of the eleven (27.3%) cases switched to onasemnogene abeparvovec. During the 12-month follow-up period, four subjects initiated treatment and one patient transitioned from nusinersen to onasemnogene abeparvovec.

Table 1. Main demographics, clinical and genetic characteristics of the study sample at baseline

Variable	Controls (n=27)	SMA subjects (n=27)
Male	14 (51.8%)	13 (48.2%)
Age - mo	143 (40 - 252)	132 (38 - 276)
Age at onset - mo	-	7 (2-12.5)
Disease duration - mo	-	110.5 (32.25 - 252.75)
Weight - kg	53.65 (15.87 - 75.75)	35 (14.54 - 60)
BMI - kg/m ²	20.7 (19.01 - 22.38)	19.93 (16.85 - 23.0)
SMA type	-	Type 1 - 12/27 (44.4%) Type 2- 9/27 (33.3%) Type 3 - 6/27 (22.2%)
<i>SMN2</i> copy number	-	2 copies - 13/27 (48.1%) 3 copies - 10/27 (37%) 4 copies - 3/27 (11.1%)
Functional status	Walkers 27/27 (100%)	Non-sitters 10/27 (37%) Sitters 15/27 (55.5%)

		Walkers 2/27 (7.4%)
<i>Ventilatory support</i>	0/27 (0%)	13 (48.1%)
CHOP-INTEND (N=17)	-	25.29 (16.16 - 34.43)
HINE (N=16)	-	4.25 (1.32 - 7.18)
HFMSE (N=8)	-	19.5 (8.57 - 30.43)
RULM (N=6)	-	22.83 (12.12 - 33.5)
On disease modifying therapies	-	11 (40.7%)
Use of nusinersen	-	11 (40.7%)
Use of Onasemnogene abeparvec	-	4 (14.8%)

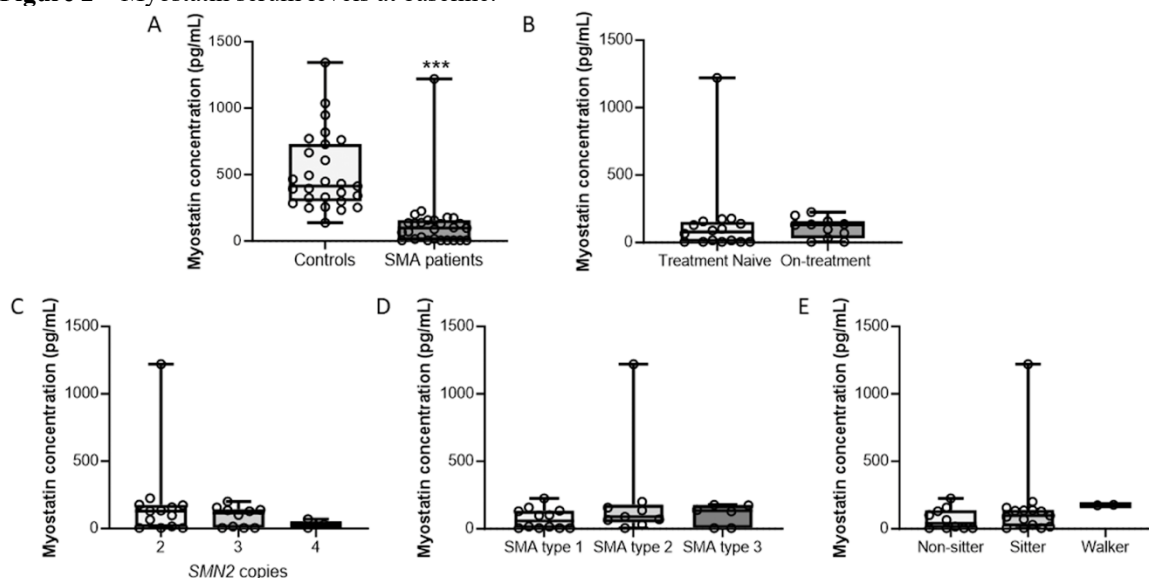
Data are shown as median (interquartile range) or frequencies (percentages), except for BMI which is shown as mean (95% confidence interval). BMI, body mass index; CHOP INTEND, Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; HINE, Hammersmith Infant Neurological Examination; HFMSE, Hammersmith Functional Motor Scale-Expanded; Mo, months; RULM, Revised Upper Limb Module; SMA, spinal muscular atrophy.

3.2.1 Serum levels of myokines

3.2.1.1 Myostatin

Serum myostatin levels were significantly lower in subjects with SMA, with a median of 98 pg/mL (5-157 pg/mL), compared to controls who had a median of 412 pg/mL (299-730 pg/mL; $p < 0.001$ corrected for weight or BMI; **Figure 2A**). Median myostatin levels were 40.92 pg/mL (5-137.52 pg/mL) in the non-sitters group, 97.85 pg/mL (17.42-139.8 pg/mL) in the sitters group, and 176.2 pg/mL (174.4-ND pg/mL) in the walkers group ($p = 0.138$, **Figure 2E**). There was no significant difference in myostatin levels among SMA subtypes ($p = 0.477$, **Figure 2B**), based on *SMN2* copy number ($p = 0.465$, **Figure 2C**), treatment status ($p = 0.422$, **Figure 2D**), or gender ($p = 0.830$).

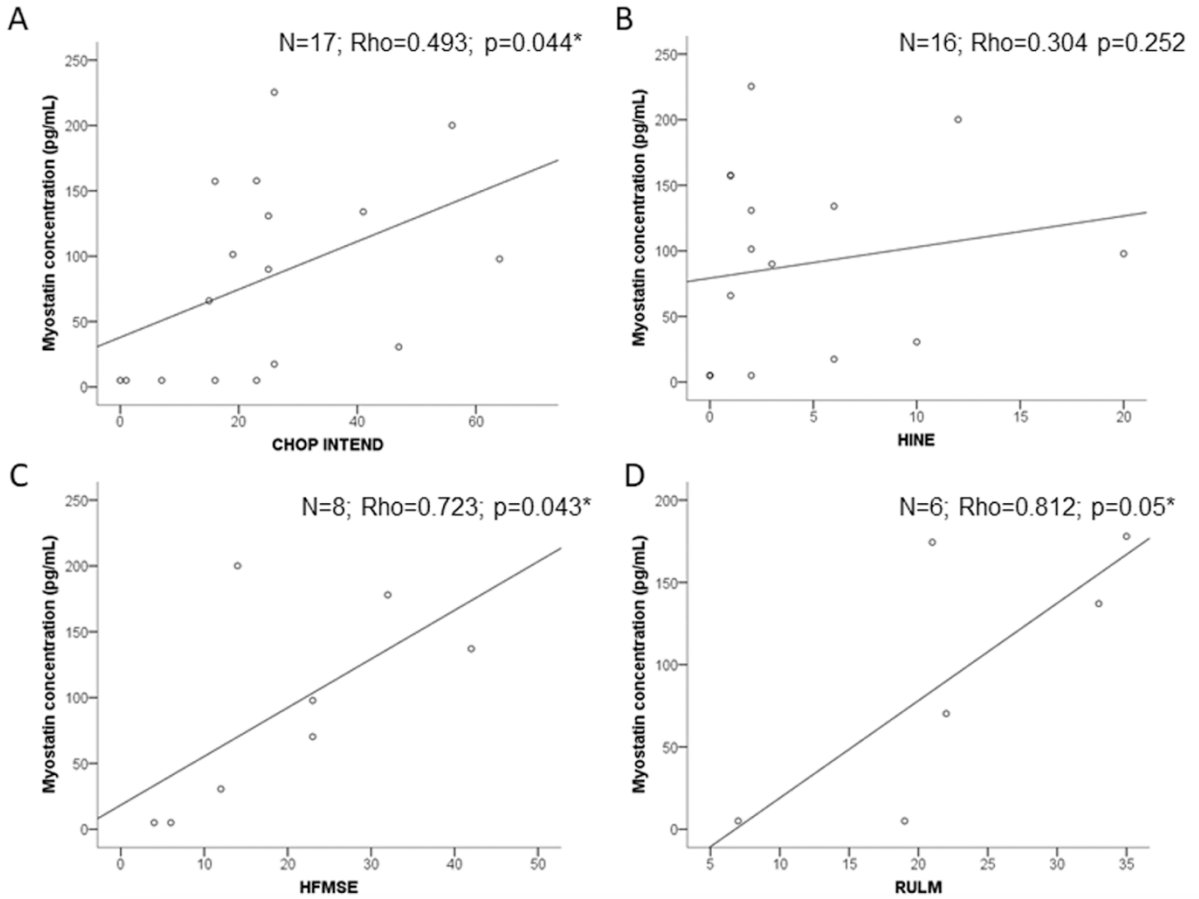
Figure 2 – Myostatin serum levels at baseline.



There was moderate to strong direct correlations between myostatin levels and CHOP-INTEND (N=17; $Rho = 0.493$, $p = 0.044$, **Figure 3A**), HFMSE (N=8; $Rho = 0.723$, $p = 0.043$, **Figure 3C**) and RULM scores (N=6; $Rho = 0.812$, $p = 0.05$, **Figure 3D**), in which lower myostatin levels were associated with worse motor performance (lower scale scores). However, there was no significant correlation between serum myostatin levels and HINE (N=16; $Rho = 0.304$,

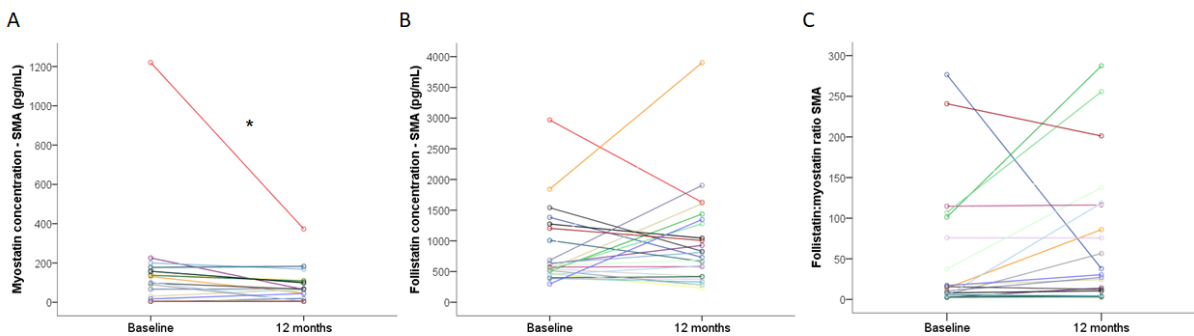
$p=0.252$, **Figure 3B**). No significant correlations were found between serum myostatin levels with age ($Rho=0.041$, $p=0.840$), weight ($Rho=0.023$, $p=0.910$), BMI ($Rho=0.167$, $p=0.414$), age at onset of symptoms ($Rho=0.185$, $p=0.365$), or disease duration ($Rho=-0.004$, $p=0.986$).

Figure 3 – Serum myostatin levels correlation with clinician-rated outcomes.



During the 12 months of follow-up, there was evidence of an additional reduction in myostatin levels to 55 (5-104.5) pg/mL ($p=0.021$, **Figure 4A**). However, there was no correlation between the change in myostatin levels over time and the severity of the disease at baseline ($p>0.05$ for all comparisons) or the TSOT variable ($Rho=0.022$, $p=0.940$).

Figure 4 – Myokines levels on longitudinal 12-months follow-up.

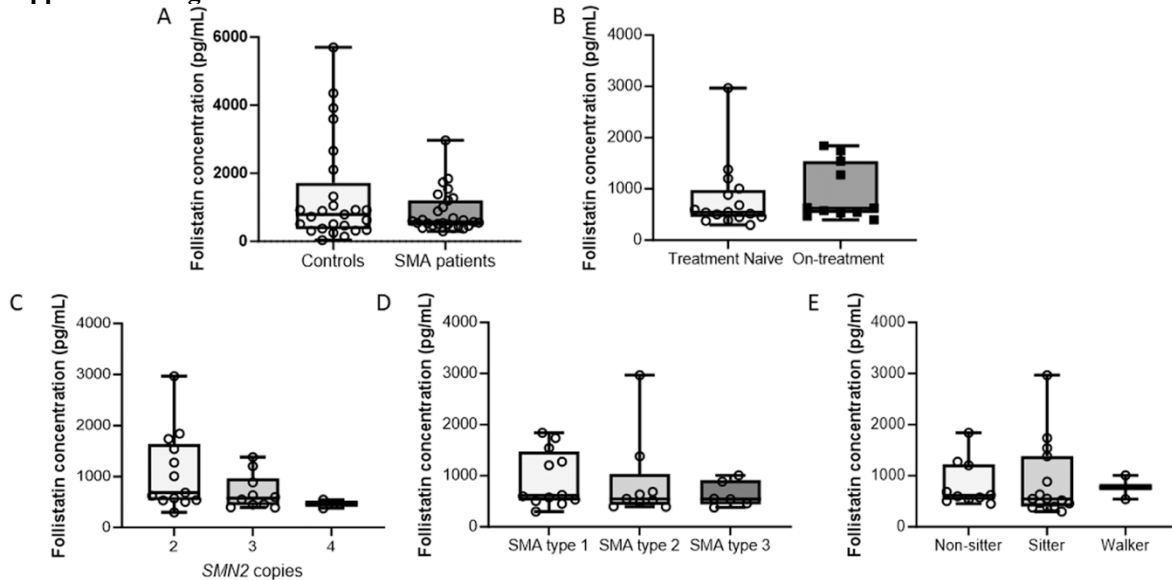


3.2.1.2 Follistatin

There was no significant difference in serum follistatin levels between SMA cases and controls (cases: 573.5 pg/mL, controls: 790.8 pg/mL, $p=0.288$ - $p=0.438$ corrected for weight and

$p=0.760$ corrected for BMI, **Supplementary-Figure 1A**). Similarly, no differences were observed in follistatin levels among SMA subtypes ($p=0.610$, **Supplementary-Figure 1B**), based on *SMN2* copy number ($p=0.109$, **Supplementary-Figure 1C**), between treated and untreated patients ($p=0.272$, **Supplementary-Figure 1D**), among functional status groups ($p=0.680$, **Supplementary-Figure 1E**) or between genders ($p=0.458$).

Supplemental Figure 1 - Follistatin serum levels at baseline.



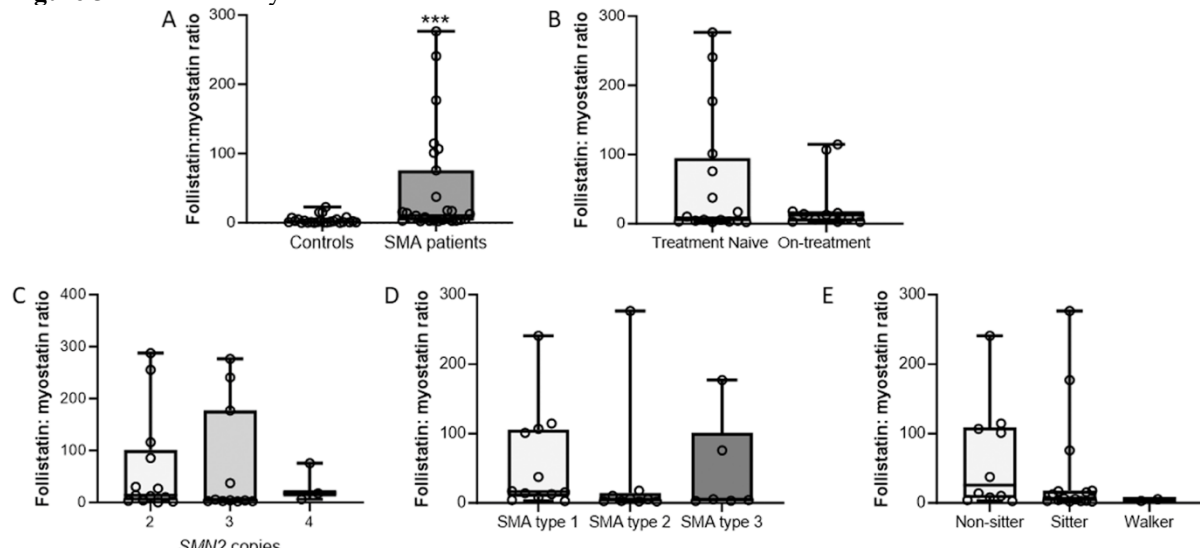
Follistatin levels only showed a correlation with age at symptoms onset ($Rho=-0.425$, $p=0.030$), while no significant correlations were found with age ($Rho=-0.208$, $p=0.297$), weight ($Rho=-0.244$, $p=0.219$), BMI ($Rho=-0.300$, $p=0.137$), disease duration ($Rho=-0.192$, $p=0.349$), HINE ($Rho=0.113$, $p=0.676$), CHOP-INTEND ($Rho=0.279$, $p=0.278$), HFMSE ($Rho=0.084$, $p=0.844$), or RULM ($Rho=-0.429$, $p=0.397$).

There was no significant change in follistatin levels over the 12-month follow-up period ($p=0.493$), with levels measuring 813.0 pg/mL (418.25-1392.0 pg/mL) at the end of the study (**Figure 4B**). Additionally, there was no correlation between the changes in follistatin levels during the study and the baseline disease severity variables or the TSOT variable ($p>0.05$ for all comparisons).

3.2.1.3 Follistatin:myostatin ratio

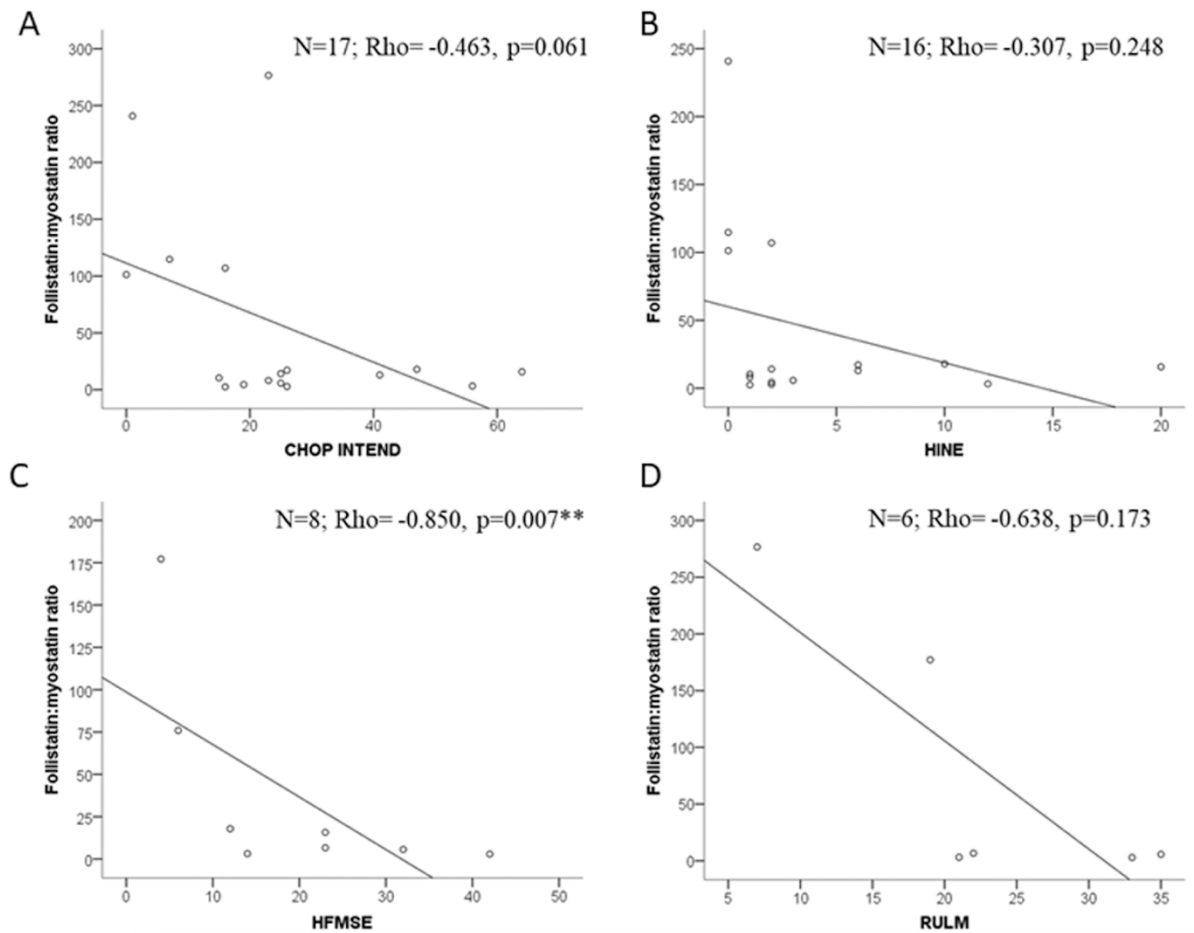
The follistatin:myostatin ratio was significantly increased in SMA subjects (10.39; 3.28-75.9) compared to controls (1.55; 0.73-5.15, $p<0.001$ corrected for weight or BMI, **Figure 5A**). However, there was no difference in the follistatin:myostatin ratio between different SMA subtypes ($p=0.160$, **Figure 5B**), based on the number of *SMN2* copies ($p=0.790$, **Figure 5C**), between treated and untreated patients ($p=0.942$, **Figure 5D**), among functional status groups ($p=0.215$, **Figure 5E**) or between genders ($p=0.905$, data not shown).

Figure 5 – Follistatin:myostatin ratio at baseline.



The follistatin:myostatin ratio showed a significant inverse correlation with HFMSE scores (N=8; $Rho=-0.850$, $p=0.007$, **Supplementary-Figure 2C**). Although not statistically significant, similar trends were observed for CHOP-INTEND (**Supplementary-Figure 2A**), HINE (**Supplementary-Figure 2B**), and RULM (**Supplementary-Figure 2D**), where higher ratios were associated with lower scores indicative of greater disease severity. The follistatin:myostatin ratio did not show significant correlations with age ($Rho=-0.188$, $p=0.348$), weight ($Rho=-0.170$, $p=0.396$), BMI ($Rho=-0.297$, $p=0.141$), age at onset of symptoms ($Rho=-0.295$, $p=0.144$), or disease duration ($Rho=-0.115$, $p=0.575$). There was a non-statistically significant trend towards an additional increase in the follistatin:myostatin ratio over the 12-month follow-up period ($p=0.083$), with a ratio of 27.13 (4.59-115.5) at the end of the study (**Figure 4C**). The difference in the follistatin:myostatin ratio during the study correlated only with disease duration at baseline ($Rho=-0.404$, $p=0.045$), while no significant correlations were found with other disease severity variables at baseline or with the TSOT variable ($p>0.05$ for all other comparisons).

Supplemental Figure 2 – Follistatin:myostatin correlation with clinician-rated outcomes.



4. DISCUSSION

Gene expression analysis in transgenic SMA models, as well as in chronically denervated mice muscle and muscle tissue samples from SMA patients in our bioinformatic study supported a potential role for myostatin and follistatin as biomarkers for SMA. The subsequent clinical study confirmed the significance of myostatin as a biomarker, as SMA patients exhibited a substantial reduction in serum levels. Notably, individuals with more severe motor impairments demonstrated even lower myostatin levels, which further declined after a 12-month follow-up period. Conversely, serum follistatin levels did not differ significantly between SMA cases and controls, resulting in an increased follistatin:myostatin ratio in SMA. The decreased gene expression of myostatin over time in acutely denervated skeletal muscle tissue along with an increased follistatin:myostatin ratio, mirrored the observations in SMA patients, suggesting that the denervation process resulting from motor neuron dysfunction/death in SMA may be a key component responsible for lower myostatin serum levels.

Myostatin

Myostatin, a member of the transforming growth-factor β (TGF- β) family, is primarily expressed and secreted by skeletal muscle and serves as a negative regulator of muscle growth. It exerts its effects by binding to plasma membrane-associated activin type IIB and type IIA (ActRIIB/IIA) receptors, leading to inhibition of muscle stem cell proliferation and fusion, as well as modulation of local protein synthesis^{36,37}. Previous studies have demonstrated reduced myostatin levels in various muscular diseases, including muscular dystrophies, myotonic disorders, and inclusion body myopathies^{38,39}. In a previous study, serum myostatin levels were

assessed in four patients with infantile-onset SMA, with a mean age of 11 years at assessment, demonstrating a significant reduction compared to controls and even lower levels than those in patients with primary myopathies⁴⁰. Our findings not only confirm this anecdotal report but also establish a correlation between lower myostatin levels and greater motor disease severity according to ClinROs, indicating its potential as a biomarker of disease severity. Additionally, we observed a progressive reduction in myostatin levels over time, suggesting its role as a candidate biomarker of disease progression. Of note, a potential floor effect was identified for myostatin serum levels in advanced disease stages. Seven patients with SMA had myostatin levels at the detection threshold of the method, with four out of the seven being patients with SMA type 1 aged between 3 and 8 years, all in advanced stages, and one out of the seven being a patient with SMA type 3 with a disease duration of 38 years. In all of these cases, myostatin levels at the 12-month follow-up remained at the detection threshold of the method, including in a SMA type 1 patient for whom treatment with nusinersen was initiated on the same day as baseline sample collection.

The results of the clinical study were reinforced by the DGE analyses, which revealed decreased myostatin expression in denervated *gastrocnemius* muscle in mice, skeletal muscle of SMA transgenic mice, and *iliopsoas* muscle (severely affected muscle) of SMA patients with advanced disease. The unaltered gene expression of myostatin in whole blood of SMA patients³⁵ was expected, considering its preferential expression in muscle tissue²⁷. Reduced *Mstn* expression started after 7 days of tibial nerve lesion, which is coincident with the onset of muscle atrophy in that model²⁰. However, taking into account the consistent findings of reduced gene expression of *Mstn* in muscle tissue when normalized by protein levels across the transgenic mice and human study in SMA, it is more likely that altered regulation of myostatin, rather than muscle loss alone, explains the reduced circulating myostatin levels in SMA, similar to what is considered for other neuromuscular diseases⁴¹. The lack of correlation between myostatin levels and weight and BMI further supports this interpretation; however, future studies correcting myostatin serum levels for lean mass based on dual energy X-ray absorptiometry or MRI scans and also assessing its muscle tissue expression in a larger sample of SMA subjects in different functional status and subtypes will be important to better characterize the source of reduced myostatin levels in SMA. The progressive reduction in myostatin expression following acute tibial nerve injury indicates that denervation processes play a crucial role in the downregulation of myostatin. Thus, it is plausible to suggest that in SMA denervation resulting from dysfunction or death of motor neurons in the anterior horn of the spinal cord is a major contributing factor to the altered regulation of myostatin.

No differences in myostatin levels were observed between patients treated with disease-modifying therapies and drug-naïve individuals, nor was there an association between the annual variation in myostatin levels and the variable TSOT. It is important to note that the present study was not designed or adequately powered to detect differences in candidate treatment response biomarkers. The inclusion of 11 patients undergoing treatment at the baseline assessment, as well as four patients who started treatment on the same day or after sample collection, may have introduced a confounding factor when assessing the differences in biomarkers compared to controls and in relation to disease severity and SMA subtypes. The sample of patients included in the treatment group was biased towards individuals with early and more severe forms of the disease, as 83.3% of SMA type 1 patients and 55.5% of SMA type 2 patients were receiving treatment. Conversely, the untreated group consisted mostly of individuals with late-onset forms of the disease, as no patients with SMA type 3 were under treatment. Considering the significant correlation between lower myostatin levels and more severe disease based on ClinROs, it is conceivable to speculate that treatment could have led

to increased myostatin levels in the early and severe forms of the disease, acting as a confounding factor in the comparative analysis among SMA subtypes and in relation to the number of *SMN2* copies. Additionally, studies conducted in the SMN Delta7 mouse model have demonstrated that neonatal treatment with antisense oligomers to restore SMN expression can increase myostatin expression in muscle tissue, approaching levels similar to those in controls, thus highlighting the potential of myostatin as a treatment response biomarker for novel disease-modifying therapies⁴². Similarly, an interesting study in the Golden Retriever muscular dystrophy model showed the role of myostatin as a treatment response biomarker in other neuromuscular diseases³⁸. Of note, the confounding factor related to access to disease-modifying treatments in different SMA subtypes would play a diminished role in comparing myokine levels between groups based on functionality. For instance, patients with SMA type 1 responding to treatment could exhibit a similar functional status to patients with SMA type 2 (sitters). We observed lower levels of myostatin in the non-sitters group, followed by the sitters group, with the highest values observed among cases in the walkers group. However, given that the magnitudes of the differences were smaller compared to those observed between cases and controls, the study lacked the statistical power to detect such distinctions. Future studies with larger sample sizes will be essential to assess differences in myostatin levels among SMA functional statuses. Additionally, investigations exclusively involving treatment-naïve patients will aid in evaluating differences among subtypes and their correlation with the number of *SMN2* copies. Furthermore, studies focusing primarily on evaluating myostatin as a treatment response biomarker for novel disease-modifying therapies, with larger sample sizes stratified by different SMA subtypes, and including patients ranging from early to advanced disease stages are warranted.

Follistatin

Follistatin, a single-chain glycoprotein, is expressed in most tissues where activin mRNAs are present. It functions by inhibiting the binding of myostatin and other members of the TGF- β family to ActRIIB/IIA receptors, playing a crucial role in muscle fiber formation, growth, and hypertrophy^{43,44}. In our study, serum levels of follistatin were slightly elevated compared to controls, which is consistent with the previous anecdotal study involving four SMA cases⁴⁰, but the difference was not statistically significant. We did not observe any correlation between follistatin levels and other clinical indicators of disease severity or disease progression. These results differ from gene expression studies that consistently showed increased follistatin expression in the skeletal muscle of transgenic SMA mice, SMA patients, and denervated mouse *gastrocnemius* muscle. Unlike myostatin, follistatin exhibits a broader expression profile, with liver expression being more than 8 times greater than skeletal muscle expression⁴⁵. Consequently, increased expression of follistatin in muscle may not necessarily result in elevated serum levels of this myokine. Furthermore, changes in gene expression do not necessarily lead to alterations in protein levels, as there are many post-transcriptional regulatory mechanisms at play. In this sense, the role of serum follistatin as a biomarker for neuromuscular diseases is less clear compared to myostatin, with some studies reporting normal levels^{46,47}, while others indicate increased levels^{40,37} compared to controls.

Follistatin:myostatin ratio

The increase in the follistatin:myostatin ratio appears to be a consequence of the reduction in myostatin levels in SMA and is associated with positive regulators of muscle growth in healthy individuals, promoting muscle hypertrophy⁴⁸. While not directly demonstrated, it can be inferred that the study involving four cases of SMA also showed an increase in the follistatin:myostatin ratio, considering the slightly elevated levels of follistatin and the reduced levels of myostatin observed⁴⁰. These findings differ from a study conducted in ALS, where a

decrease in the follistatin:myostatin ratio was observed in patients compared to controls⁴². Similarly, a recent study in a subtype of limb-girdle muscular dystrophy reported similar results to our study, with an increased follistatin:myostatin ratio that correlated with disease severity variables. In both studies, correlations were stronger when myostatin was evaluated independently rather than in the context of the follistatin:myostatin ratio⁴⁷.

Therapeutic implications

Several studies in animal models of SMA have investigated the inhibition of myostatin as a potential therapeutic target, often utilizing follistatin or follistatin analogues^{40,43,49-53}. Initial studies using recombinant follistatin administration in the SMN Delta7 mouse model demonstrated increased muscle mass, improved motor performance, and extended survival, regardless of SMN levels. However, more recent studies in the same model using transgenic inactivation of myostatin or transgenic overexpression of follistatin did not replicate these results⁴¹. More promising results, albeit with smaller effect sizes, have been observed in animal models of SMA with milder disease phenotypes⁴¹. Our study's findings, showing a marked reduction in myostatin levels in SMA and an increased follistatin:myostatin ratio indicating a favorable environment for muscle growth, raise questions regarding the biological plausibility of myostatin inhibition as a highly effective therapeutic strategy for the disease. However, this does not contradict the potential combined use of disease-modifying therapies targeting SMN alongside myostatin pathway inhibition. In this regard, a recent study demonstrated a synergistic effect of these interventions in SMN Delta7 mice^{41,42}. In that study, myostatin expression was restored to levels similar to controls using SMN-related therapy, and within this context, myostatin inhibition led to increased body weight, muscle mass, fiber size, motor function, and physical performance.

Study limitations

One of the limitations of our study is that it is a single-center study with a relatively small sample size. As we only found one study that measured myostatin levels in four patients with infantile-onset SMA and long-term illness, we conducted an exploratory study considering the differences between cases and controls in serum myostatin and follistatin levels and their ratio, all as main outcomes, without statistical corrections for multiple comparisons. Despite the limitations of our convenience sample, we were able to identify significant differences in myostatin levels and the follistatin:myostatin ratio. Regarding follistatin levels, we conducted a sample size calculation using the final data, considering an 80% power to detect a type II error and a 5% significance level. The calculation indicated that the sample size would need to be increased to 506 subjects, which is not feasible for a study on a rare disease. Therefore, it was considered that the study had sufficient power to identify relevant differences in the outcomes of interest. The similarity of our results with those of the anecdotal study⁴⁰ and the congruence with the findings of the DGE studies for myostatin indicate the robustness of the data. The heterogeneity of the SMA population, encompassing different SMA subtypes, and the recruitment during COVID-19 pandemic, resulted in a limited number of patients with complete clinical rating outcomes (ClinROs) available. It is crucial for future studies with larger sample sizes to assess the correlations between myostatin levels and ClinROs more comprehensively among SMA subtypes. Another limitation is the lack of power to assess the potential of serum myostatin levels as a treatment response biomarker. Future longitudinal studies are necessary to address this question and to compare the sensitivity of myostatin serum levels to changes in ClinROs and to determine if baseline myostatin levels and their changes over time can predict treatment response. Finally, it will be important to compare the biomarker properties (disease severity, progression and pharmacodynamic) of myostatin with that of other

simpler to evaluate and widely available muscle markers like serum creatinine and creatine kinase and of pNF-H and NfL levels in future studies.

5. CONCLUSION

In conclusion, our study highlights myostatin as a potential biomarker for disease severity and progression in SMA, with its reduction likely linked to the denervation process caused by dysfunction or loss of anterior horn neurons. The findings also suggest that targeting myostatin alone may not be an effective therapeutic strategy for SMA, given the already substantial inhibition of this pathway in the disease. Instead, combining myostatin inhibition with SMN-related therapies may hold promise. Future research should focus on investigating the role of myostatin as a treatment response biomarker in the context of disease-modifying therapies for SMA.

6. Data availability statement:

Data not provided in the article because of space limitations may be shared (anonymized) at the request of any qualified investigator for purposes of replicating procedures and results.

7. Acknowledgments:

The authors thank patients and their families for participating in this study. The English language revision was conducted with the assistance of an AI language model developed by OpenAI.

8. Funding:

The study was funded by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Grant Number: 22/2551-0000657-4), Fundo de Incentivo à Pesquisa e Eventos-Hospital de Clínicas de Porto Alegre (FIPE-HCPA) (Grant Number: 2020-0731), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Grant Number: PROEX: 0730/2020). Saute JA and Chadanowicz JK are supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

9. Competing interests:

We have no conflicts of interest to disclose.

10. REFERENCES

1. Kolb SJ, Kissel JT. Spinal Muscular Atrophy. *Neurologic Clinics*. 2015;33(4):831-846. doi:10.1016/j.ncl.2015.07.004
2. Prior TW, Snyder PJ, Rink BD, et al. Newborn and carrier screening for spinal muscular atrophy. *Am J Med Genet A*. 2010;152A(7):1608-1616.
3. Sugarman EA, Nagan N, Zhu H, et al. Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. *Eur J Hum Genet*. 2012;20(1):27-32.
4. D'Amico A, Mercuri E, Tiziano FD, Bertini E. Spinal muscular atrophy. *Orphanet J Rare Dis*. 2011;6:71.
5. Mercuri E, Sumner CJ, Muntoni F, Darras BT, Finkel RS. Spinal muscular atrophy. *Nat Rev Dis Primers*. 2022;8(1):52.
6. Melki J, Abdelhak S, Sheth P, et al. Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. *Nature*. 1990;344(6268):767-768.
7. Strauss KA, Farrar MA, Muntoni F, et al. Onasemnogene abeparvovec for presymptomatic infants with two copies of SMN2 at risk for spinal muscular atrophy type 1: the Phase III SPR1NT trial. *Nat Med*. 2022;28(7):1381-1389.
8. Strauss KA, Farrar MA, Muntoni F, et al. Onasemnogene abeparvovec for presymptomatic infants with three copies of SMN2 at risk for spinal muscular atrophy: the Phase III SPR1NT trial. *Nat Med*. 2022;28(7):1390-1397.
9. Schorling DC, Pechmann A, Kirschner J. Advances in Treatment of Spinal Muscular Atrophy - New Phenotypes, New Challenges, New Implications for Care. *J Neuromuscul Dis*. 2020;7(1):1-13.
10. Waldrop MA, Elsheikh BH. Spinal Muscular Atrophy in the Treatment Era. *Neurol Clin*. 2020;38(3):505-518.
11. Sumner CJ, Kolb SJ, Harmison GG, et al. SMN mRNA and protein levels in peripheral blood: biomarkers for SMA clinical trials. *Neurology*. 2006;66(7):1067-1073.
12. Zheleznyakova GY, Voisin S, Kiselev AV, et al. Genome-wide analysis shows association of epigenetic changes in regulators of Rab and Rho GTPases with spinal muscular atrophy severity. *Eur J Hum Genet*. 2013;21(9):988-993.
13. Finkel RS, Crawford TO, Swoboda KJ, et al. Candidate proteins, metabolites and transcripts in the Biomarkers for Spinal Muscular Atrophy (BforSMA) clinical study. *PLoS One*. 2012;7(4):e35462.
14. Navarrete-Opazo A, Garrison S, Waite M. Molecular Biomarkers for Spinal Muscular Atrophy: A Systematic Review. *Neurol Clin Pract*. 2021;11(4):e524-e536.

15. Darras BT, Crawford TO, Finkel RS, et al. Neurofilament as a potential biomarker for spinal muscular atrophy. *Ann Clin Transl Neurol.* 2019;6(5):932-944.
16. Olsson B, Alberg L, Cullen NC, et al. NFL is a marker of treatment response in children with SMA treated with nusinersen. *J Neurol.* 2019;266(9):2129-2136.
17. Wurster CD, Steinacker P, Günther R, et al. Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen. *J Neurol.* 2020;267(1):36-44.
18. Rich KA, Fox A, Yalvac M, et al. Neurofilament Levels in CSF and Serum in an Adult SMA Cohort Treated with Nusinersen. *J Neuromuscul Dis.* 2022;9(1):111-119.
19. Seo G, Kim S, Byun JC, Kwon S, Lee YJ. Evaluation of the neurofilament light chain as a biomarker in children with spinal muscular atrophy treated with nusinersen. *Brain Dev.* Published online August 2, 2023. doi:10.1016/j.braindev.2023.07.005
20. Ehmsen JT, Kawaguchi R, Mi R, Coppola G, Höke A. Longitudinal RNA-Seq analysis of acute and chronic neurogenic skeletal muscle atrophy. *Sci Data.* 2019;6(1):179.
21. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 2002;30(1):207-210.
22. Parkinson H, Kapushesky M, Shojatalab M, et al. ArrayExpress--a public database of microarray experiments and gene expression profiles. *Nucleic Acids Res.* 2007;35(Database issue):D747-D750.
23. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics.* 2004;20(3):307-315.
24. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. The R Project for Statistical Computing. Published 2022. <https://www.R-project.org/>
25. Galaxy Community. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. *Nucleic Acids Res.* 2022;50(W1):W345-W351.
26. Andrews S. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics. Published 2010. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
27. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 2012;9(4):357-359.
28. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics.* 2014;30(7):923-930.
29. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43(7):e47.
30. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010;26(1):139-140.

31. Doktor TK, Hua Y, Andersen HS, et al. RNA-sequencing of a mouse-model of spinal muscular atrophy reveals tissue-wide changes in splicing of U12-dependent introns. *Nucleic Acids Res.* 2017;45(1):395-416.
32. McCormack NM, Villalón E, Viollet C, et al. Survival motor neuron deficiency slows myoblast fusion through reduced myomaker and myomixer expression. *J Cachexia Sarcopenia Muscle.* 2021;12(4):1098-1116.
33. Meijboom KE, Volpato V, Monzón-Sandoval J, et al. Combining multiomics and drug perturbation profiles to identify muscle-specific treatments for spinal muscular atrophy. *JCI Insight.* 2021;6(13). doi:10.1172/jci.insight.149446
34. Ramos DM, d'Ydewalle C, Gabbeta V, et al. Age-dependent SMN expression in disease-relevant tissue and implications for SMA treatment. *J Clin Invest.* 2019;129(11):4817-4831.
35. Eichelberger EJ, Alves CRR, Zhang R, et al. Increased systemic HSP70B levels in spinal muscular atrophy infants. *Ann Clin Transl Neurol.* 2021;8(7):1495-1501.
36. Sartori R, Gregorevic P, Sandri M. TGF β and BMP signaling in skeletal muscle: potential significance for muscle-related disease. *Trends Endocrinol Metab.* 2014;25(9):464-471.
37. Lee JH, Jun HS. Role of Myokines in Regulating Skeletal Muscle Mass and Function. *Front Physiol.* 2019;10. doi:10.3389/fphys.2019.00042
38. Mariot V, Le Guiner C, Barthélémy I, et al. Myostatin Is a Quantifiable Biomarker for Monitoring Pharmaco-gene Therapy in Duchenne Muscular Dystrophy. *Mol Ther Methods Clin Dev.* 2020;18:415-421.
39. Burch PM, Pogoryelova O, Palandra J, et al. Reduced serum myostatin concentrations associated with genetic muscle disease progression. *J Neurol.* 2017;264(3):541-553.
40. Mariot V, Joubert R, Hourdé C, et al. Downregulation of myostatin pathway in neuromuscular diseases may explain challenges of anti-myostatin therapeutic approaches. *Nat Commun.* 2017;8(1):1859.
41. Abati E, Manini A, Comi GP, Corti S. Inhibition of myostatin and related signaling pathways for the treatment of muscle atrophy in motor neuron diseases. *Cell Mol Life Sci.* 2022;79(7):374.
42. Zhou H, Meng J, Malerba A, et al. Myostatin inhibition in combination with antisense oligonucleotide therapy improves outcomes in spinal muscular atrophy. *J Cachexia Sarcopenia Muscle.* 2020;11(3):768-782.
43. Kaneko H, ed. Follistatin. In: *Handbook of Hormones.* Academic Press; 2016:298-e33C - 2.
44. Wu C, Borné Y, Gao R, et al. Elevated circulating follistatin associates with an increased risk of type 2 diabetes. *Nat Commun.* 2021;12(1):6486.
45. Fagerberg L, Hallström BM, Oksvold P, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics.* 2014;13(2):397-406.

46. Marozzo R, Pegoraro V, Angelini C. MiRNAs, Myostatin, and Muscle MRI Imaging as Biomarkers of Clinical Features in Becker Muscular Dystrophy. *Diagnostics (Basel)*. 2020;10(9). doi:10.3390/diagnostics10090713
47. Moore U, Fernández-Simón E, Schiava M, et al. Myostatin and follistatin as monitoring and prognostic biomarkers in dysferlinopathy. *Neuromuscul Disord*. 2023;33(2):199-207.
48. Bijeh N, Mohammadnia-Ahmadi M, Hooshamnd-Moghadam B, Eskandari M, Golestani F. Effects of Soy Milk in Conjunction With Resistance Training on Physical Performance and Skeletal Muscle Regulatory Markers in Older Men. *Biol Res Nurs*. 2022;24(3):294-307.
49. Liu M, Hammers DW, Barton ER, Sweeney HL. Activin Receptor Type IIB Inhibition Improves Muscle Phenotype and Function in a Mouse Model of Spinal Muscular Atrophy. *PLoS One*. 2016;11(11):e0166803.
50. Long KK, O'Shea KM, Khairallah RJ, et al. Specific inhibition of myostatin activation is beneficial in mouse models of SMA therapy. *Hum Mol Genet*. 2019;28(7):1076-1089.
51. Chen TH. New and Developing Therapies in Spinal Muscular Atrophy: From Genotype to Phenotype to Treatment and Where Do We Stand? *Int J Mol Sci*. 2020;21(9). doi:10.3390/ijms21093297
52. Rose FF Jr, Mattis VB, Rindt H, Lorson CL. Delivery of recombinant follistatin lessens disease severity in a mouse model of spinal muscular atrophy. *Hum Mol Genet*. 2009;18(6):997-1005.
53. Harris AW, Butchbach MER. The effect of the DcpS inhibitor D156844 on the protective action of follistatin in mice with spinal muscular atrophy. *Neuromuscul Disord*. 2015;25(9):699-705.

Figures Legends

Figure 1 – Heat-map of the differential expression of myokines in acute and chronic neurogenic skeletal muscle atrophy in mice.

A - Differential gene expression (DGE) of *Mstn* and *Fst* RNA in *gastrocnemii* of C57BL/6J male mice after 0 (baseline), 1, 3, 7, 14, 30, or 90 days of tibial nerve denervation from Ehmsen et al, 2019 dataset. Significant gastrocnemius atrophy is induced within one week after denervation in this model, with atrophy becoming progressively more severe over time. B - DGE of *Mstn* and *Fst* RNA in two studies with the Taiwanese model *Smn*^{-/-};*SMN2* (E-MTAB-3664 and GSE150510, with similar proportion of male and female mice) and one with the SMN Delta7 model (GSE158790, sex of mice not informed) and the meta-analysis of the three studies. C - DGE of *MSTN* and *FST* RNA in post-mortem diaphragm and iliopsoas samples from SMA patients (GSE159642) and on whole-blood samples of SMA type 1 patients and controls (GSE174056). *indicates statistical significance with p-values. Heat colors indicate increased while cold colors indicate reduced expression of RNA when compared to control tissue. DGE analysis was conducted for each dataset in R and RStudio using the edgeR. DGE analysis with $\log_{2}FC \geq 1$ and $P\text{-Value} \leq 0.05$ were considered upregulated, whilst genes with $\log_{2}FC \leq -1$ and $P\text{-Value} \leq 0.05$ were considered downregulated. D, diaphragm; I, *iliopsoas*; LogFC, log-fold changes.

Figure 2 – Myostatin serum levels at baseline

SMA, spinal muscular atrophy. *** $p < 0.001$. Paired comparisons between cases and controls were performed with Wilcoxon signed-rank test, were $Z = -4.036$ and $p < 0.001$ for myostatin levels analysis. Comparisons among SMA subtypes, *SMN2* copy numbers and functional status were performed with Kruskal-Wallis test, comparisons between SMA treated, and untreated subjects at baseline were performed with Mann-Whitney U-test. Dots represent single data points.

Figure 3 – Serum myostatin levels correlation with clinician-rated outcomes

CHOP INTEND, Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; HINE, Hammersmith Infant Neurological Examination; HFMSE, Hammersmith Functional Motor Scale-Expanded; RULM, Revised Upper Limb Module. * $p < 0.05$.

Figure 4 – Myokines levels on longitudinal 12-months follow-up

SMA, spinal muscular atrophy. * $p < 0.05$. Paired comparisons were performed with Wilcoxon signed-rank test, were $Z = -2.427$ and $p = 0.015$ for myostatin levels analysis. Dots represent single data points.

Figure 5 – Follistatin:myostatin ratio at baseline

*** $p < 0.001$. Paired comparisons between cases and controls were performed with Wilcoxon signed-rank test, were $Z = -4.036$ and $p < 0.001$ for myostatin levels and $Z = 3.646$ and $p < 0.001$ for follistatin:myostatin ratio analysis. Comparisons among SMA subtypes, *SMN2* copy numbers and functional status were performed with Kruskal-Wallis test, comparisons between SMA treated, and untreated subjects at baseline were performed with Mann-Whitney U-test. Dots represent single data points.

Supplemental Figure 1 - Follistatin serum levels at baseline

SMA, spinal muscular atrophy. Paired comparisons between cases and controls were performed with Wilcoxon signed-rank test, comparisons among SMA subtypes, *SMN2* copy numbers and functional status were performed with Kruskal-Wallis test, comparisons between

SMA treated, and untreated subjects at baseline were performed with Mann-Whitney U-test. Dots represent single data points.

Supplemental Figure 2 – Follistatin:myostatin correlation with clinician-rated outcomes
CHOP INTEND, Children’s Hospital of Philadelphia Infant Test of Neuromuscular Disorders;
HINE, Hammersmith Infant Neurological Examination; HFMSE, Hammersmith Functional
Motor Scale–Expanded; RULM, Revised Upper Limb Module. ** $p < 0.01$.

9. CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

Avanços científicos têm sido feitos recentemente no entendimento da Atrofia Muscular Espinhal, bem como na busca por novas terapias modificadoras de doença, e de biomarcadores que possam ser utilizados na avaliação desta condição (gravidade, progressão) e de resposta às terapias instituídas.

Realizamos um estudo de casuística relatando a experiência de mundo real do nosso centro com esta condição, trazendo dados relevantes, reforçando a eficácia de terapias modificadoras de doença para AME no Brasil e no contexto de países com perfil socioeconômico similar, a qual é maior quanto mais precoce e quanto melhor o status funcional do paciente

Utilizamos modelos de expressão gênica diferencial, analisados através de estudo de bioinformática, para propor novos biomarcadores para a AME com foco no acometimento do tecido muscular. Após, realizamos um estudo clínico de centro único com duas fases (transversal caso-controle e coorte prospectiva) que se propôs a avaliar os biomarcadores candidatos do estudo anterior em pacientes com AME, tendo sido o primeiro estudo a avaliar de forma consistente e sistematizada o papel de biomarcador de diferentes miocinas em indivíduos com essa doença. Nosso trabalho trouxe relevantes achados em relação ao papel de miocinas musculares como potenciais marcadores na Atrofia Muscular Espinhal, sugerindo que a miostatina é uma molécula promissora como biomarcadora de doença, de gravidade e de progressão na AME. Já folistatina, antagonista da miostatina, e a irisina, um hormônio induzido pelo exercício, não se mostraram bons marcadores na AME, limitando seu potencial uso em pesquisas futuras.

Espera-se que outros estudos possam ser conduzidos com uma amostra maior para confirmar a validade externa dos nossos achados, e com um desenho que permita avaliar se tais moléculas poderiam também ser potenciais marcadores de resposta ao tratamento, o que seria um dado extremamente relevante para personalização do uso das novas terapias modificadoras de doença na AME.

10. ANEXOS

10.1 Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO Grupo Controle

Nº do projeto GPPG ou CAAE _____

Título do Projeto: **MIOCINAS COMO BIOMARCADORES DA ATROFIA MUSCULAR ESPINHAL**

Você está sendo convidado(a) a participar de uma pesquisa cujo objetivo é avaliar marcadores biológicos de doença e de progressão da Atrofia Muscular Espinhal. Para a realização do estudo é necessário comparar os resultados obtidos com o grupo de pacientes que apresentam a Atrofia Muscular Espinhal com um grupo de pessoas que não apresenta esta doença. Você está sendo convidado para participar do grupo controle do estudo, ou seja, que não possui a doença.

Esta pesquisa está sendo realizada pelos serviços de Neurologia e Genética Médica do Hospital de Clínicas de Porto Alegre (HCPA).

Se você (ou responsável) concordar com a participação na pesquisa, os procedimentos envolvidos são os seguintes:

1) Coleta de sangue periférico para dosagem dos marcadores musculares: miostatina, folistatina e irisina. O material biológico coletado será armazenado de forma codificada. A amostra de sangue será utilizada apenas para fins desta pesquisa.

A coleta de sangue será realizada no Centro de Pesquisa Clínica do HCPA. O tempo total previsto para esta avaliação é de 15 minutos.

Você poderá apresentar desconfortos relacionados à coleta de sangue como desconforto no local da punção, com risco de hematoma e eritema (mancha vermelha/ roxa) no local da coleta. O desconforto será mínimo, pois se trata de uma coleta de sangue geralmente da veia do braço que será realizada por profissional treinado e devidamente habilitado para realizar esse procedimento.

A participação no estudo não trará benefício direto ao participante. O estudo poderá possibilitar a definição de novos biomarcadores para a Atrofia Muscular Espinhal, ampliando o entendimento sobre esta doença, e fornecendo evidências que possam contribuir para o desenvolvimento de novas terapias para o tratamento de indivíduos com essa condição.

Sua participação na pesquisa é totalmente voluntária, ou seja, não é obrigatória. Caso você decida não autorizar, ou ainda, desistir da participação e retirar sua autorização, não haverá nenhum prejuízo ao atendimento que a pessoa recebe ou possa vir a receber na instituição.

Não está previsto nenhum tipo de pagamento pela participação na pesquisa e você não terá nenhum custo com respeito aos procedimentos envolvidos.

Caso ocorra alguma intercorrência ou dano, resultante da participação na pesquisa, o participante receberá todo o atendimento necessário, sem nenhum custo pessoal.

Os dados coletados durante a pesquisa serão sempre tratados confidencialmente. Os resultados serão apresentados de forma conjunta, sem a identificação dos participantes, ou seja, o seu nome não aparecerá na publicação dos resultados.

Caso você tenha dúvidas, poderá entrar em contato com o pesquisador responsável Dr. Jonas Alex Morales Saute pelo telefone (51) 3359-8011, com a pesquisadora Ana Letícia Amorim de Albuquerque, pelo telefone (82) 99963-3213, ou com o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (HCPA), pelo telefone (51) 3359-7640, email cep@hcpa.edu.br ou no 2º andar do HCPA, sala 2229, de segunda à sexta, das 8h às 17h.

Esse Termo é assinado em duas vias, sendo uma para o participante e outra para os pesquisadores.

Nome do participante da pesquisa

Assinatura (Participante ou Responsável)

Nome do pesquisador que aplicou o Termo

Assinatura

Local e Data: _____

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Grupo Casos

Nº do projeto GPPG ou CAAE _____

Título do Projeto: **MIOCINAS COMO BIOMARCADORES DA ATROFIA MUSCULAR ESPINHAL**

Você está sendo convidado(a) a participar de uma pesquisa cujo objetivo é avaliar marcadores biológicos de doença e de progressão da Atrofia Muscular Espinhal. Esta pesquisa está sendo realizada pelos serviços de Neurologia e Genética Médica do Hospital de Clínicas de Porto Alegre (HCPA).

Se você (ou responsável) concordar com a participação na pesquisa, os procedimentos envolvidos são os seguintes:

1) Exame clínico: será realizado através de escalas padronizadas do exame neurológico que já é realizado em suas consultas;

2) Coleta de sangue periférico para dosagem dos marcadores musculares: miostatina, folistatina e irisina. O material biológico coletado será armazenado de forma codificada. A amostra de sangue será utilizada apenas para fins desta pesquisa.

A avaliação clínica e a coleta de sangue serão realizadas no Centro de Pesquisa Clínica do HCPA. As avaliações serão realizadas no início do estudo e repetidas após 1 e 2 anos da consulta inicial.

Você poderá apresentar desconforto e/ou cansaço relacionados à realização do exame físico neurológico (com duração de cerca de 1 hora) e os desconfortos relacionados à coleta de sangue como desconforto no local da punção, com risco de hematoma e eritema (mancha vermelha/ roxa) no local da coleta. O desconforto será mínimo, pois se trata de uma coleta de sangue geralmente da veia do braço que será realizada por profissional treinado e devidamente habilitado para realizar esse procedimento.

A participação no estudo não trará benefício direto ao participante. O estudo poderá possibilitar a definição de novos biomarcadores para a Atrofia Muscular Espinhal, ampliando o entendimento sobre esta doença, e fornecendo evidências que possam contribuir para o desenvolvimento de novas terapias para o tratamento de indivíduos com essa condição.

Sua participação na pesquisa é totalmente voluntária, ou seja, não é obrigatória. Caso você decida não participar, ou ainda, desistir de participar e retirar seu consentimento, não haverá nenhum prejuízo ao atendimento que você recebe ou possa vir a receber na instituição.

Não está previsto nenhum tipo de pagamento pela sua participação na pesquisa e você não terá nenhum custo com respeito aos procedimentos envolvidos.

Caso ocorra alguma intercorrência ou dano, resultante de sua participação na pesquisa, você receberá todo o atendimento necessário, sem nenhum custo pessoal.

Os dados coletados durante a pesquisa serão sempre tratados confidencialmente. Os resultados serão apresentados de forma conjunta, sem a identificação dos participantes, ou seja, o seu nome não aparecerá na publicação dos resultados.

Caso você tenha dúvidas, poderá entrar em contato com o pesquisador responsável Dr. Jonas Alex Morales Saute pelo telefone (51) 3359-8011, com a pesquisadora Ana Letícia Amorim de Albuquerque, pelo telefone (82) 99963-3213, ou com o Comitê de Ética em

Pesquisa do Hospital de Clínicas de Porto Alegre (HCPA), pelo telefone (51) 3359-7640, email cep@hcpa.edu.br ou no 2º andar do HCPA, sala 2229, de segunda à sexta, das 8h às 17h.

Esse Termo é assinado em duas vias, sendo uma para o participante e outra para os pesquisadores.

Nome do participante da pesquisa

Assinatura (Participante ou Responsável)

Nome do pesquisador que aplicou o Termo

Assinatura

Local e Data: _____

10.2 STROBE Checklist

Artigo I: Clinicogenetic characterization and Response to Disease-Modifying Therapies in Spinal Muscular Atrophy: Real-World Experience from a Reference Center in Southern Brazil.

	Item No	Recommendation	Page
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	35
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	36
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	37
Objectives	3	State specific objectives, including any prespecified hypotheses	37
Methods			
Study design	4	Present key elements of study design early in the paper	37
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	37-38
Participants	6	(a) <i>Cohort study</i> —Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up <i>Case-control study</i> —Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls <i>Cross-sectional study</i> —Give the eligibility criteria, and the sources and methods of selection of participants	37-38
		(b) <i>Cohort study</i> —For matched studies, give matching criteria and number of exposed and unexposed <i>Case-control study</i> —For matched studies, give matching criteria and the number of controls per case	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	37-38
Data sources/measurement	8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	37-38
Bias	9	Describe any efforts to address potential sources of bias	43
Study size	10	Explain how the study size was arrived at	37-38
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	37-38
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	38
		(b) Describe any methods used to examine subgroups and interactions	38
		(c) Explain how missing data were addressed	39

		(d) <i>Cohort study</i> —If applicable, explain how loss to follow-up was addressed <i>Case-control study</i> —If applicable, explain how matching of cases and controls was addressed <i>Cross-sectional study</i> —If applicable, describe analytical methods taking account of sampling strategy	N/A
		(e) Describe any sensitivity analyses	38

Results			
Participants	13	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	37-38
		(b) Give reasons for non-participation at each stage	37-38
		(c) Consider use of a flow diagram	NA
Descriptive data	14	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	38-39
		(b) Indicate number of participants with missing data for each variable of interest	38-39
		(c) <i>Cohort study</i> —Summarise follow-up time (eg, average and total amount)	NA
Outcome data	15	<i>Cohort study</i> —Report numbers of outcome events or summary measures over time	NA
		<i>Case-control study</i> —Report numbers in each exposure category, or summary measures of exposure	NA
		<i>Cross-sectional study</i> —Report numbers of outcome events or summary measures	38-40
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	NA
		(b) Report category boundaries when continuous variables were categorized	38-40
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	38-40
Discussion			
Key results	18	Summarise key results with reference to study objectives	38-40
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	43
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	43
Generalisability	21	Discuss the generalisability (external validity) of the study results	43
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	NA

Artigo II: Serum myostatin as a candidate disease severity and progression biomarker of spinal muscular atrophy.

	Item No	Recommendation	Page
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	47
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	48
Introduction			
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	49
Objectives	3	State specific objectives, including any prespecified hypotheses	49
Methods			
Study design	4	Present key elements of study design early in the paper	50-51
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	50-51
Participants	6	(a) <i>Cohort study</i> —Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up <i>Case-control study</i> —Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls <i>Cross-sectional study</i> —Give the eligibility criteria, and the sources and methods of selection of participants	50-51
		(b) <i>Cohort study</i> —For matched studies, give matching criteria and number of exposed and unexposed <i>Case-control study</i> —For matched studies, give matching criteria and the number of controls per case	50-51
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	50-51
Data sources/ measurement	8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	50-51
Bias	9	Describe any efforts to address potential sources of bias	50-51
Study size	10	Explain how the study size was arrived at	50-51, 61
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	50-51
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	51
		(b) Describe any methods used to examine subgroups and interactions	51
		(c) Explain how missing data were addressed	51-52
		(d) <i>Cohort study</i> —If applicable, explain how loss to follow-up was addressed <i>Case-control study</i> —If applicable, explain how matching of cases and controls was addressed <i>Cross-sectional study</i> —If applicable, describe analytical methods taking account of sampling strategy	50-51

		(e) Describe any sensitivity analyses	51
Results			
Participants	13	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	52-54
		(b) Give reasons for non-participation at each stage	NA
		(c) Consider use of a flow diagram	NA
Descriptive data	14	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	52-54
		(b) Indicate number of participants with missing data for each variable of interest	52-54
		(c) <i>Cohort study</i> —Summarise follow-up time (eg, average and total amount)	54-58
Outcome data	15	<i>Cohort study</i> —Report numbers of outcome events or summary measures over time	53-58
		<i>Case-control study</i> —Report numbers in each exposure category, or summary measures of exposure	53-58
		<i>Cross-sectional study</i> —Report numbers of outcome events or summary measures	NA
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	54-58
		(b) Report category boundaries when continuous variables were categorized	54-58
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	54-58
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	54-58
Discussion			
Key results	18	Summarise key results with reference to study objectives	58-61
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	61
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	62
Generalisability	21	Discuss the generalisability (external validity) of the study results	61
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	62

10.3 Destaques

10.3.1 Destaque no Congresso Brasileiro de Neurogenética (2023)

Figura 6: Prêmio no CBGM em 2023.



Certificamos que,

O Prêmio de 2º lugar

foi conferido ao trabalho intitulado

MIOSTATINA SERICA COMO BIOMARCADOR DE GRAVIDADE E PROGRESSAO DA ATROFIA MUSCULAR ESPINHAL

de autoria: **Ana Letícia Amorim de Albuquerque, Júlia Kersting Chadanowicz, Giovanna Giudicelli, Arthur Carpeggiani Weber, Ana Lucia Portella Staub, Michele Michelin Becker, Jordana Miranda de Souza Silva, Thayne Woycinck Kowalski, Marina Siebert, Jonas Alex Morales Saute**

apresentado na categoria **Trabalho Científico Original Infantil - Apresentação Oral** durante o **III CONGRESSO BRASILEIRO DE NEUROGENÉTICA**, organizado pela Academia Brasileira de Neurologia – ABN, realizado nos dias 9, 10 e 11 de março de 2023, na Cidade de São Paulo/SP – Centro de Convenções Frei Caneca.


JONAS A. MORALES SAUTE
PRESIDENTE E RESPONSÁVEL TÉCNICO
CIENTÍFICO DO EVENTO
CRM-RS: 33039


FERNANDO KOK
COMISSÃO ORGANIZADORA
DEPARTAMENTO CIENTÍFICO
DE NEUROGENÉTICA DA ABN


JOSÉ LUIZ PEDROSO
COMISSÃO ORGANIZADORA
DEPARTAMENTO CIENTÍFICO
DE NEUROGENÉTICA DA ABN


MARCONDES C. FRANÇA JÚNIOR
COMISSÃO ORGANIZADORA
DEPARTAMENTO CIENTÍFICO
DE NEUROGENÉTICA DA ABN


SARAH TEIXEIRA CAMARGOS
COMISSÃO ORGANIZADORA
DEPARTAMENTO CIENTÍFICO
DE NEUROGENÉTICA


CARLOS ROBERTO M. RIEDER
PRESIDENTE DA ACADEMIA
BRASILEIRA DE NEUROLOGIA

10.3.2 Destaque no I Encontro Científico do PPGCM (2023)

Figura 7: Prêmio Destaque no I Encontro Científico do PPGCM (2023).



10.3.3 Publicação no Jornal da UFRGS em janeiro de 2024

Figura 8: Jornal da Universidade (2024).



Link: <https://www.ufrgs.br/jornal/um-novo-marcador-da-atrofia-muscular-espinhal/>