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Molecular Analysis of Skeletal Muscle in *Negr1*-Deficient Mice

Bachelor's thesis (12 EAP)

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ABSTRACT

Molecular Analysis of Skeletal Muscle in *Negr1*-Deficient Mice

NEGR1 (neuronal growth regulator 1) is a cell adhesion molecule belonging to the IgLON family. NEGR1 has been mainly studied in the central nervous system. However, previous studies have also linked *Negr1* to skeletal muscle regulation. The aim of this thesis was to quantify and compare genotype-dependent and sex-specific differences in the mRNA expression of *Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*, *Cox1*, *Cox2*, and *Pvalb* in the skeletal muscle of *Negr1*^{-/-} and wild-type mice. Genotype-dependent differences were observed mainly in male *Negr1*^{-/-} mice, where *Cox1* and *Myh7* were downregulated, and *Tnnt3* was upregulated compared with wild-type mice. In female *Negr1*^{-/-} mice, *Myh7* was upregulated. These findings suggest that *Negr1* deficiency may be associated with altered expression of skeletal muscle-related genes, with males appearing more susceptible to those changes.

Keywords: IgLON, NEGR1, skeletal muscle, sex-specificity, mRNA expression

CRECS code: B580 Skeletal and muscular system diseases, rheumatological diseases; B640 Neurology, neuropsychiatry, neurophysiology

Skeletilihaste molekulaarne analüüs *Negr1*-puudulikkusega hiirtes

NEGR1 (neuronaalne kasvuregulaator 1) on rakuadhesiooni molekul, mis kuulub IgLON valkude perekonda. NEGR1-t on peamiselt uuritud kesknärviüsteemis, kuid varasemad uuringud on *Negr1* sidunud ka skeletilihaste regulatsiooniga. Käesoleva bakalaureusetöö eesmärgiks oli kvantifitseerida ja võrrelda genotüübist sõltuvaid ning soospetsiifilisi erinevusi *Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*, *Cox1*, *Cox2* ja *Pvalb* mRNA ekspressioonis *Negr1*^{-/-} ja metsiktüüpi hiirte skeletilihases. Genotüübist sõltuvad erinevused ilmnesisid peamiselt isastel *Negr1*^{-/-} hiirtel, kellel *Cox1* ja *Myh7* olid alareguleeritud ning *Tnnt3* ülereguleeritud võrreldes metsiktüüpi hiirtega. Emastel *Negr1*^{-/-} hiirtel oli ülereguleeritud vaid *Myh7*. Need tulemused viitavad sellele, et *Negr1*-puudulikkus võib olla seotud skeletilihaste geenide muutunud ekspressiooniga ning et isased *Negr1*^{-/-} hiired võivad olla nende muutuste suhtes tundlikumad.

Märksõnad: IgLON, NEGR1, skeletilihased, soospetsiifilisus, mRNA ekspressioon

CRECS kood: B580 Skeleti ja lihassüsteemi haigused, reumatoloogilised haigused; B640 Neuroloogia, neuropsühhiaatria, neurofüsioloogia

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ABBREVIATIONS

ADAM10	A Disintegrin and Metalloproteinase 10
ASD	Autism Spectrum Disorder
CAMs	Cell Adhesion Molecules
COX	Cytochrome c Oxidase
CT	Cycle Threshold
ERK1/2	Extracellular Signal-Regulated Kinases 1 and 2
FGFR2	Fibroblast Growth Factor Receptor 2
GPI	Glycosylphosphatidylinositol
Ig	Immuglobulin
Mettl3	Methyltransferase-like 3
m6A	N6-methyladenosine
NEGR1	Neuronal Growth Regulator 1
<i>Negr1</i> ^{-/-}	<i>Negr1</i> -deficient organism

INTRODUCTION

Skeletal muscle is one of the three major muscle types in the human body (Dave et al., 2023). It is a highly dynamic tissue that plays an essential role in locomotion, posture, and metabolic regulation (Frontera & Ochala, 2015; McCuller et al., 2023). Additionally, skeletal muscle serves as a major reservoir of body protein (Frontera & Ochala, 2015). Skeletal muscle mass is maintained through the balance between protein synthesis and degradation, which is influenced by factors such as physical activity, nutrition, hormonal regulation, and pathological conditions (Frontera & Ochala, 2015).

Given the physiological importance of skeletal muscle, it is essential to investigate the factors that regulate its function. One such factor could be neuronal growth regulator 1 (NEGR1), a member of the IgLON family of cell adhesion molecules (Funatsu et al., 1999). NEGR1 has been studied predominantly in the context of the nervous system, where it has been associated with several aspects of neuronal development, such as neuronal outgrowth (Marg et al., 1999) and synaptic regulation (Hashimoto et al., 2008). More recently, NEGR1 has been linked to body weight regulation and metabolic processes, suggesting that its functions may extend beyond the brain (Joo et al., 2019).

Although the consequences of *NEGR1* deficiency have likewise been examined mainly in relation to nervous system function, several findings indicate that it also affects peripheral tissues. Previous studies have reported alterations in body composition (Lee et al., 2012), reduced muscle mass, and decreased exercise capacity in *Negr1*^{-/-} mice (Joo et al., 2019), suggesting that NEGR1 may also play a role in skeletal muscle. A recent study found that 2,512 genes are differentially expressed in skeletal muscle of *Negr1*^{-/-} mice compared to wild-type mice (Maigoro et al., 2025). However, these findings do not yet provide a clear picture of the molecular mechanisms through which *NEGR1* deficiency may influence skeletal muscle, and analysis of targeted skeletal muscle-related genes remains limited. Furthermore, the potential sex-specific variations in these expression changes have not been addressed.

The literature review of the bachelor's thesis provides a more detailed overview of NEGR1 gene expression and its role in both the brain and skeletal muscle. It further provides an overview of skeletal muscle molecular structure, including the structural and functional proteins involved, whose expression is analyzed in the present study. The objective of this

thesis was to quantify and compare the relative mRNA expression levels of *Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*, *Cox1*, *Cox2*, and *Pvalb* in the tibialis anterior of *Negr1*^{-/-} mice and wild-type mice. Additionally, sex-specific differences in mRNA expression were examined between male and female knockout mice.

This thesis was conducted at the Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu.

1. LITERATURE REVIEW

1.1. Cell Adhesion Molecules

Cell adhesion molecules (CAMs) are cell-surface proteins that play a central role in immune responses, embryogenesis, and nervous system development (Petruzzelli et al., 1999). Their primary function is to mediate cell-cell and cell-extracellular matrix attachment (Petruzzelli et al., 1999). Four distinguished CAM families have been identified: cadherins, selectins, integrins, and the immunoglobulin (Ig) superfamily (Alberts et al., 2002). These families differ in their molecular structure, binding mechanisms, functional roles, and tissue distribution (Alberts et al., 2002).

Cadherins facilitate homophilic, Ca^{2+} -dependent adhesion and are essential for both tissue morphogenesis during embryogenesis and for maintaining tissue structure in the adult organism (Weis, 1995). Selectins and integrins mediate heterophilic, Ca^{2+} -dependent adhesion (Alberts et al., 2002). They enable leukocytes to adhere to vascular endothelial cells and migrate from the bloodstream into surrounding tissues (Alberts et al., 2002). Proteins of the Ig superfamily generally mediate Ca^{2+} -independent cell adhesion (Alberts et al., 2002; Miyata et al., 2000), and are broadly involved in neural development, including axon and synapse regulation, and the establishment of neuronal connectivity (Hawke et al., 1999; Lee et al., 2012).

1.2. The IgLON Family

The IgLON family, a member of the Ig superfamily of cell adhesion molecules, is associated with neural development and sensory processing (Hashimoto et al., 2008; Singh et al., 2024). The family comprises five proteins: IgLON1 (OPCML), IgLON2 (NTM), IgLON3 (LSAMP), IgLON4 (NEGR1), and IgLON5 (Singh et al., 2024).

OPCML encodes a protein implicated in opioid binding and has been characterized as a potential tumor suppressor (Simovic et al., 2019). As it is expressed in multiple tissues, OPCML participates in several cellular processes, including cell cycle regulation, apoptosis, and cell migration (Simovic et al., 2019). NTM is predominantly expressed in the brain and is linked to cognitive development and intellectual functioning (Venkannagari et al., 2020). The LSAMP function is strongly influenced by interactions with other IgLON proteins (Bregin et

al., 2020). Depending on the type of binding, LSAMP can either promote or inhibit neurite outgrowth and synaptic development (Bregin et al., 2020). LSAMP has also been identified as a negative regulator of myelination (Sharma et al., 2015). Although the precise biological role of IgLON5 remains unclear, autoimmune responses targeting this protein have been linked to sleep disturbances, motor dysfunction, and cognitive decline (Campetella et al., 2025). NEGR1 is mainly expressed in the brain, where it contributes to neuronal development (Joo et al., 2019). Genome-wide association studies have implicated NEGR1 as a susceptibility factor for several neuropsychiatric and physiological conditions (Maigoro et al., 2025; Singh et al., 2019). These conditions are further elaborated in the following sections.

1.3. Neuronal Growth Regulator 1 (NEGR1)

NEGR1 is a protein-coding gene located in the human chromosomal region 1p31.1 (Genovese et al., 2015). The gene has been discussed as a clinically relevant locus because partial deletions of 1p31.1 affecting only *NEGR1* have been reported in patients with neurodevelopmental and behavioral symptoms (Genovese et al., 2015). In the literature, *NEGR1* has been described under other names, including Kilon, Neurotractin, and IgLON4 (Funatsu et al., 1999; Marg et al., 1999; Schäfer et al., 2005; Singh et al., 2018). The name Kilon was used in an early study that identified the protein as a novel GPI-anchored protein in rat brain, specifically on dendrites and pyramidal neurons (Funatsu et al., 1999). The name Neurotractin was later used in a study showing that the protein promotes neurite outgrowth (Marg et al., 1999).

The protein encoded by *NEGR1* is anchored to the outer surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Funatsu et al., 1999; Miyata et al., 2003; Pischedda et al., 2014). This means that the functional part of the protein is exposed to the extracellular space, where it can interact with neighboring cells and proteins (Funatsu et al., 1999; Pischedda et al., 2014). *NEGR1* contains three extracellular Ig-like C2-type domains, three disulfide bridges, and six putative glycosylation sites (Figure 1), which are structural motifs commonly found in cell adhesion molecules and allow proteins to recognize and bind to other cell-surface proteins (Funatsu et al., 1999; Miyata et al., 2000; Ranaivoson et al., 2019). Through these extracellular domains, *NEGR1* can take part in homophilic interactions with *NEGR1* molecules on other cells and heterophilic interactions with other IgLON family proteins (Ranaivoson et al., 2019). These interactions help organize contacts between

neuronal cells and contribute to neurite growth, synaptic organization, and cell-surface signaling (Pischedda & Piccoli, 2016; Ranaivoson et al., 2019; Schäfer et al., 2005). *NEGR1* can also be released from the neuronal membrane as a soluble extracellular factor through a disintegrin and metalloproteinase 10 (ADAM10)-mediated shedding (Pischedda & Piccoli, 2016). Soluble *NEGR1* has been shown to promote neurite arborization via fibroblast growth factor receptor 2 (FGFR2) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling (Pischedda & Piccoli, 2016). These findings suggest that NEGR1 acts as both a membrane-associated adhesion molecule and a soluble factor that affects neuronal morphology.

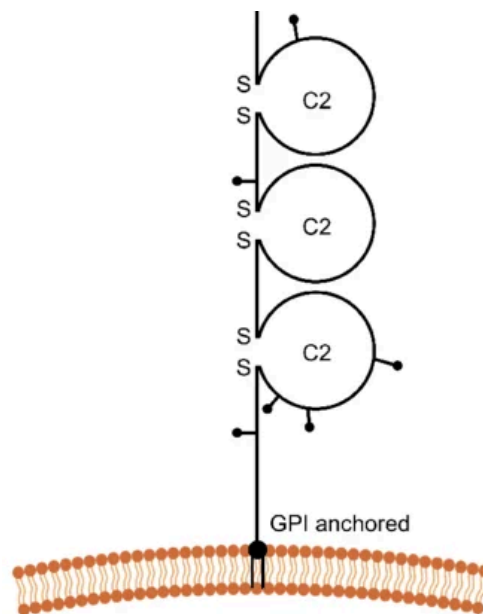


Figure 1. Structure of Negr1. Negr1 is anchored at the plasma membrane via the GPI anchor. Loops represent C2-type immunoglobulin-like domains, lines ending with dots are putative glycosylation sites, and SS mark disulfide bridges. Adapted from Noh et al. (2020).

Although NEGR1 is mainly known for its role in neuronal development, it has also been connected to broader physiological phenotypes (Lee et al., 2012; the GIANT Consortium, 2009). Genome-wide association studies identified variants near *NEGR1* among loci associated with body mass index, which suggested a possible link between neuronal genes and body weight regulation (the GIANT Consortium, 2009). Experimental inactivation of *Negr1* in mice later showed that loss of *NEGR1* can alter body mass and body composition,

suggesting the functional relevance of this gene beyond its original description as a neuronal protein (Lee et al., 2012).

1.3.1. Expression of NEGR1 in the Brain

The expression of NEGR1 has been mainly studied in the central nervous system, where it is linked to the development of the brain, specifically to neuronal growth, circuit formation, and behavioral regulation (Funatsu et al., 1999; Hashimoto et al., 2008; Miyata et al., 2000; Noh et al., 2020; Schäfer et al., 2005; Singh et al., 2018; Szczurkowska et al., 2018).

Early studies showed that *Negr1* expression is detectable in the brain during embryonic stages and increases during postnatal development in rat models (Funatsu et al., 1999), suggesting that NEGR1 is involved in neuronal development and maturation (Lee et al., 2012). *Negr1* has been reported in several brain regions, such as the entorhinal cortex, brain stem, olfactory bulb, and hypothalamus, with high expression in the cerebral cortex and the hippocampus (Funatsu et al., 1999; Lee et al., 2012; Noh et al., 2020; Singh et al., 2018; Szczurkowska et al., 2018). This distribution suggests that NEGR1 is relevant to several neuronal processes, including learning, memory, emotional and sensory processing, stress modulation, social behaviors, and energy-balance regulation (Lee et al., 2012; Singh et al., 2018).

Singh et al. (2018) showed experimental evidence that NEGR1 is involved in neuronal growth and brain connectivity. The authors showed that *Negr1* promoted neurite growth and is permissive for axon growth in cortical neurons. Additionally, impaired ingrowth and abnormal projections of entorhinal axons were found in the hippocampus of *Negr1*^{-/-} mice. Singh et al. (2018; 2019) also demonstrated that *Negr1*-deficient mice exhibit behavioral alterations in learning and social behavior, including hyperactivity during social interactions, impaired social dominance, and increased seizure susceptibility.

Noh et al. (2019) further linked NEGR1 to adult hippocampal neurogenesis and affective behavior. In *Negr1*-deficient mice, anxiety- and depression-like behaviors were increased compared with wild-type mice. These behavioral changes were accompanied by reduced adult hippocampal neurogenesis and impaired synaptic function in the dentate gyrus. These findings further support the role of NEGR1 in hippocampal circuit function and psychiatric disorder phenotypes.

Thus far, NEGR1 has been linked to various neuropsychiatric and neurodevelopmental disorders. Human genetic association studies have connected NEGR1 with autism spectrum disorder (ASD) (Autism Spectrum Disorder Working Group of the Psychiatric Genomics Consortium et al., 2019) and schizophrenia (Deng et al., 2025). Additionally, experimental mouse studies have shown that *Negr1* downregulation or knockout produces ASD-related behavioral phenotypes (Szczurkowska et al., 2018). Proteomic analyses in humans have shown altered NEGR1 expression in major depressive disorder and bipolar disorder (Maccarrone et al., 2013). Genetic and model-based evidence also suggests a link between NEGR1 and Alzheimer's disease (Ni et al., 2018).

1.3.2. Expression of NEGR1 in Skeletal Muscle

There is increasing evidence suggesting that NEGR1 is relevant beyond the brain. This is especially important because *Negr1*-deficient mice have shown functional and compositional alterations in skeletal muscle, suggesting that NEGR1 is also involved in whole-body physiological regulation (Joo et al., 2019; Lee et al., 2012). However, compared with its neural functions, the expression and role of NEGR1 in peripheral tissues remain poorly understood.

Lee et al. (2012) showed that inactivation of *Negr1* in mice reduced body mass and altered body composition. In the *Negr1*-I87N mouse model, lower body mass was associated with reduced lean mass, suggesting that *NEGR1* deficiency may affect tissues that contribute to lean body composition (Lee et al., 2012). Since skeletal muscle is a major component of lean mass, this finding indirectly points to skeletal muscle as a relevant tissue in *Negr1*-deficient phenotypes. It should be noted that Lee et al. (2012) did not directly analyze skeletal muscle, and the observed changes in body composition were thought to act through the central nervous system.

The clearest evidence for skeletal muscle involvement was provided by Joo et al. (2019), who reported that *Negr1*-deficient mice showed reduced skeletal muscle mass and impaired muscle performance. In this study, the tibialis anterior muscle weight was reduced by 1.2-fold, and the fibers showed a 1.7-fold decrease in cross-sectional area in *Negr1*-deficient mice compared to wild-type mice. The same mice also showed significantly reduced exercise capacity in the treadmill endurance test. Kaare et al. (2021) also reported reduced muscle fiber

size in the quadriceps femoris of male *Negr1*^{-/-} mice compared with wild-type males. Interestingly, no significant changes were observed in females.

Recent evidence further supports the importance of examining skeletal muscle in *Negr1*-deficient models. Maigoro et al. (2025) analyzed peripheral gene expression in several tissues of *Negr1*-deficient mice. In the gastrocnemius muscle, they identified 2,512 differentially expressed genes, of which 1,195 were upregulated, and 1,317 were downregulated compared with wild-type mice.

1.4. Skeletal Muscle Structure

1.4.1. Structural Organization of Skeletal Muscle

Skeletal muscle is a highly organized tissue with a hierarchical arrangement (Figure 2) (McCuller et al., 2023; Visone et al., 2016). It is composed of fascicles, which in turn contain multiple individual muscle fibers (Dave et al., 2023; Frontera & Ochala, 2015). These fibers can be divided into two groups based on contractile speed – slow-twitch and fast-twitch fibers (McCuller et al., 2023). These muscle fibers are long, multinucleated cells that contain a large number of myofibrils arranged in parallel throughout the sarcoplasm (Figure 2) (McCuller et al., 2023; Mukund & Subramaniam, 2020).

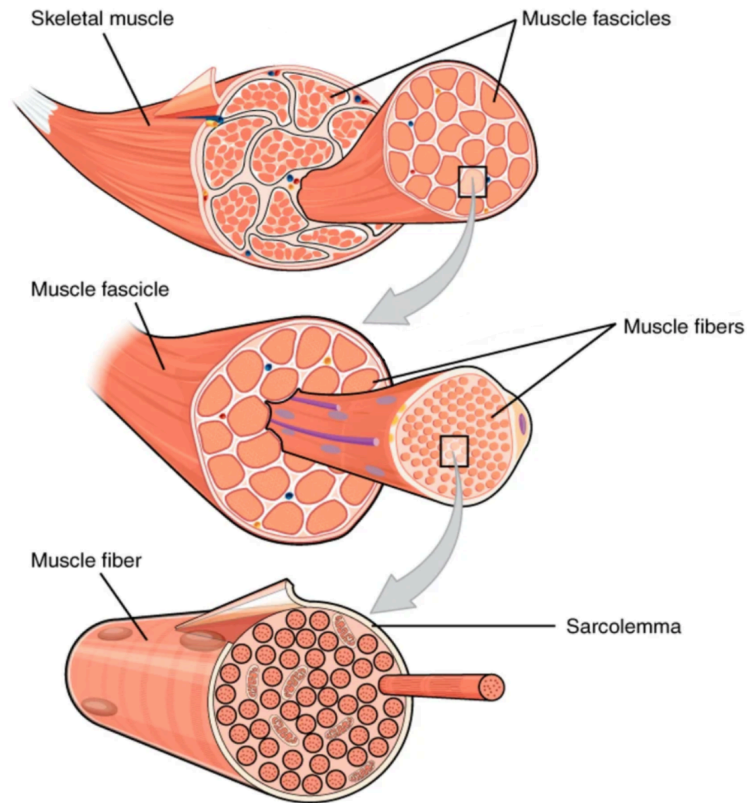


Figure 2. The Hierarchical Structure of the Skeletal Muscle. Skeletal muscle is organized into fascicles, which contain multiple individual muscle fibers. Muscle fibers are long cells surrounded by the sarcolemma and contain myofibrils. Adapted from Visone et al. (2016), where the image is reproduced from an earlier Creative Commons source (unavailable).

Myofibrils account for much of the internal organization of the muscle fiber. They are composed of repeating contractile units known as sarcomeres (Zhao et al., 2025), which are aligned along the length of the myofibril (McCuller et al., 2023; Mukund & Subramaniam, 2020). Sarcomeres are further composed of thin-, thick-, and elastic-filaments (Figure 3) (McCuller et al., 2023; Mukund & Subramaniam, 2020). This organization enables force generated at the molecular level to be coordinated within myofibrils, integrated across the muscle fiber, and transmitted to the whole muscle (McCuller et al., 2023; Mukund & Subramaniam, 2020).

Table 1 provides a more detailed overview of the structural (*Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*), mitochondrial (*Cox1* and *Cox2*), and calcium-binding (*Pvalb*) genes selected for the present experiment, including their encoded proteins, cellular or sarcomeric localization, and association with skeletal muscle fiber-types.

Table 1. Overview of the selected target genes and their skeletal muscle context.

Target Gene	Encoded Protein	Localization	Fiber-Type Association
<i>Myh7</i>	Beta-myosin heavy chain	Thick-filament	Slow-twitch
<i>Tpm1</i>	Alpha-tropomyosin	Thin-filament	Fast-twitch
<i>Tnnc2</i>	Troponin C	Thin-filament	Fast-twitch
<i>Tnnt3</i>	Troponin T	Thin-filament	Fast-twitch
<i>Acta1</i>	Alpha-actin	Thin-filament	Slow- and fast-twitch
<i>Ttn</i>	Titin	Elastic filament	Slow- and fast-twitch
<i>Cox1</i>	Cytochrome c oxidase subunit 1	Mitochondria	Slow- and fast-twitch
<i>Cox2</i>	Cytochrome c oxidase subunit 2	Mitochondria	Slow- and fast-twitch
<i>Pvalb</i>	Parvalbumin	Sarcoplasm	Fast-twitch

1.4.2. The Molecular Structure of the Sarcomere

The sarcomere is the basic contractile unit of skeletal muscle (Mukund & Subramaniam, 2020). It extends from one Z-disk (also known as Z-line) to the next. It contains a highly organized arrangement of thin filaments (actin and associated proteins) and thick filaments (myosin and associated proteins) (Figure 3) (McCuller et al., 2023; Mukund & Subramaniam, 2020). Thin and thick filaments form the main structural components of the sarcomere and, together, the contractile apparatus of skeletal muscle (Frontera & Ochala, 2015; Mukund & Subramaniam, 2020; Zhao et al., 2025).

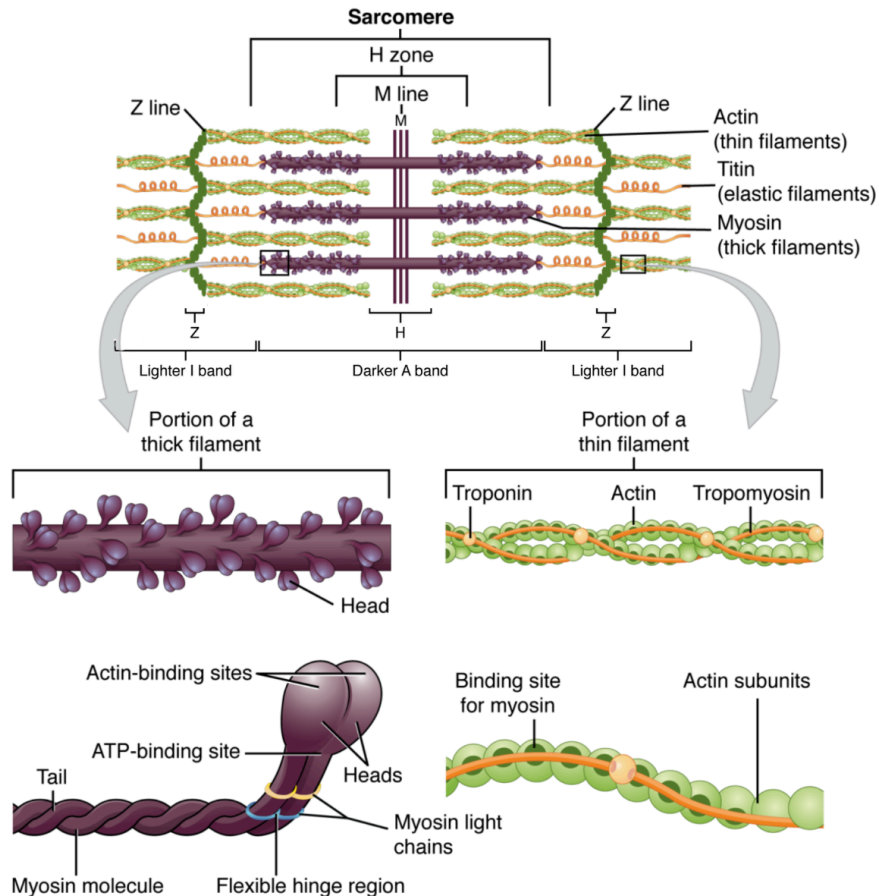


Figure 3. The Sarcomere. The region from one Z-line to the next Z-line. The Z-line forms the boundary of the sarcomere and anchors the thin filaments. The main filament systems include thin actin filaments, thick myosin filaments, and elastic titin filaments. Their organization includes the lighter I-bands, the darker A-band, the central H-zone, and the M-line. The enlarged sections illustrate the thick filament structure formed by myosin molecules (on the left) and the thin filament structure composed of actin, tropomyosin, and the troponin complex (on the right). Adapted from *Anatomy & Physiology* (chap. 10), by L. M. Biga et al., 2019, under CC BY-SA 4.0, and from *Anatomy and Physiology 2e* by OpenStax, licensed under CC BY 4.0 (Betts et al., 2026a; 2026b).

Thin filaments are primarily built from actin and are anchored to the Z-disk, from where they extend toward the center of the sarcomere, whereas thick filaments are mainly composed of myosin and occupy the central region of the sarcomere (McCuller et al., 2023; Mukund & Subramaniam, 2020) – this arrangement defines structural regions such as the I-band, A-band, H-zone, and M-line (Figure 3) (McCuller et al., 2023).

The I-band is the region containing only thin filaments. In contrast, the A-band corresponds to the full length of the thick filaments and includes areas of overlap between thin and thick filaments (Squire, 1997, as cited in McCuller et al., 2023). The H-zone is the central part of

the A-band where only thick filaments are present, and the M-line lies at the center of the sarcomere, where thick filaments are aligned and anchored (Squire, 1997, as cited in McCuller et al., 2023; Pham & Puckett, 2023). During contraction, the I-band and H-zone shorten, while the A-band remains constant in length – that happens because the filaments slide past one another (Squire, 1997, as cited in McCuller et al., 2023).

In addition to actin and myosin, both filament systems are associated with structural and regulatory proteins that contribute to filament stability and contractile control (Dave et al., 2023; McCuller et al., 2023; Pham & Puckett, 2023). These proteins help maintain the organization of the sarcomere and myofibrils, subsequently supporting the proper function of the contractile machinery (Dave et al., 2023; McCuller et al., 2023; Pham & Puckett, 2023).

Table 2 summarizes the functions of the selected target genes in skeletal muscle, along with the human disorders associated with pathogenic variants and the animal model findings of altered gene regulation.

Table 2. Functional and disease-related relevance of selected target genes.

Target Gene	Function in Skeletal Muscle	Reported Effects of Altered Expression, or Pathogenic Variants
<i>MYH7</i>	Myosin assembly; supporting muscle contraction (Bloemink et al., 2007; Gao et al., 2024; Geeves & Holmes, 1999)	Pathogenic variants – Laing early-onset distal myopathy (Laing et al., 1995; Meredith et al., 2004); scapuloperoneal myopathy (Pegoraro et al., 2007); myosin storage myopathy (Tajsharghi et al., 2003) Downregulation – slow-to-fast fiber-type transition (Sharlo et al., 2020; McCarthy et al., 2009) Upregulation – fast-to-slow fiber-type transition (Yan et al., 2025; Li et al., 2022)
<i>TPM1</i>	Regulating actin-myosin interaction and muscle contraction (Ebashi,	Pathogenic variants – cardiomyopathies (Redwood & Robinson, 2013)

	1963, as cited in Lehman et al., 2020); stabilizing the thin filament (Gordon et al., 2001)	
<i>TNNC2</i>	Ca ²⁺ sensing (Chaudhuri et al., 2005); triggering contraction-related structural changes (Van De Loch et al., 2021; Vinogradova et al., 2005)	Pathogenic variants – congenital myopathy (Van De Loch et al., 2021)
<i>TNNT3</i>	Anchoring the troponin complex to tropomyosin (Ohtsuki et al., 1967, as cited in Wei & Jin, 2016); supporting thin-filament regulation (Jin et al., 2008, as cited in Wei & Jin, 2016)	Pathogenic variants – distal arthrogryposis (Lu et al., 2021; Sandaradura et al., 2018)
<i>ACTA1</i>	Core thin-filament structure (Craig & Padron, 2004); supporting muscle contraction (Huxley & Niedergerke, 1954, as cited in Szikora et al., 2022)	Pathogenic variants – actin myopathy; nemaline myopathy (Nowak et al., 1999); cardiomyopathy; arthrogryposis (Clayton et al., 2024)
<i>TTN</i>	Providing sarcomere elasticity and passive stiffness (Hidalgo & Granzier, 2013; Squarci et al., 2023)	Muscle disuse – muscle atrophy (Udaka et al., 2008)
<i>COX1</i>	Enabling mitochondrial ATP production (Fontanesi et al., 2008)	Pathogenic variants – encephalomyopathy (Debray et al., 2014); rhabdomyolysis (Kollberg et al., 2005); hypotonia (Brischigliaro & Zeviani, 2021) Downregulation – mitochondrial dysfunction; oxidative stress (Holvoet et al. 2017; Bonnard et al., 2008)

<i>COX2</i>	Enabling mitochondrial ATP production (Fontanesi et al., 2008)	Pathogenic variants – myopathy (Rahman et al., 1999); rhabdomyolysis (McFarland et al., 2004)
<i>PVALB</i>	Supporting muscle relaxation; Ca ²⁺ regulation (Schwaller et al., 1999)	Downregulation – hypertrophy; upregulation – atrophy (Butera et al., 2021)

1.5. Altered Expression and Pathogenic Conditions: *MYH7*, *COX1*, and *TNNT3*

Although the present study analyzed a broader panel of skeletal muscle-related genes, this section focuses in more detail on genes that are most relevant for interpreting the experimental findings. The section summarizes altered expression, pathogenic conditions, and their consequences caused by alterations in *MYH7/Myh7*, *COX1/Cox1*, and *TNNT3/Tnnt3* in skeletal muscle.

1.5.1. *MYH7*

Pathogenic variants of *MYH7* can cause clinical disorders characterized by muscle weakness that often follows a selective anatomical pattern rather than affecting all skeletal muscles equally (Bahout et al., 2025). Laing early-onset distal myopathy often presents with early distal lower-limb weakness affecting ankle dorsiflexion and gait, and may later extend to finger extensors and selected proximal muscle groups in some patients (Laing et al., 1995; Mastaglia et al., 2002; Meredith et al., 2004). Scapuloperoneal myopathy is characterized by weakness of the scapular region and lower leg muscles, often presenting with scapular winging, foot dorsiflexion weakness, or foot drop (Bahout et al., 2025; Pegoraro et al., 2007). These symptoms are accompanied by difficulties with movements such as walking, stair climbing, rising from a chair, or lifting objects with the upper limbs (Pegoraro et al., 2007). Myosin storage myopathy is a distinct *MYH7*-related myopathy characterized by abnormal accumulation of beta-myosin heavy chain in slow-twitch muscle fibers, and it may clinically present with hypotonia and slowly progressive muscle weakness (Tajsharghi et al., 2003; Tajsharghi & Oldfors, 2013).

Increased *Myh7* expression has been linked to increased slow-twitch fiber proportion and fast-to-slow muscle fiber conversion in animal and cellular models (Yan et al., 2025; Li et al., 2022). For example, in a murine model, Yan et al. (2025) reported that methyltransferase-like 3 (*Mettl3*) -dependent N6-methyladenosine (m6A) methylation enhanced *Myh7* expression and increased the proportion of slow-twitch fibers while decreasing fast-twitch fiber proportion. Similarly, in C2C12 myogenic cells, Li et al. (2022) showed that overexpression of miR-208b increased *Myh7* gene expression while reducing *Myh4* expression. miR-208b is a microRNA encoded within the intron of the *Myh7* gene, which is essential for converting fast-twitch fibers to slow-twitch fibers (Van Rooij et al., 2009). That supported *Myh7* in fast-to-slow fiber-type conversion. In contrast, unloading studies in rats, such as hindlimb suspension, where reduced mechanical loading decreases *Myh7* transcription and triggers a slow-to-fast fiber-type transition (Sharlo et al., 2020). Furthermore, McCarthy et al. (2009) reported that a reduction in miR-208b causes a loss of the regulatory mechanism that represses fast-fiber gene expression (McCarthy et al., 2009).

1.5.2. *COX1*

Pathogenic mutations in *COX1* that affect the activity of the COX complex have been associated with several pathological skeletal muscle conditions. These include mitochondrial encephalomyopathy, rhabdomyolysis, and hypotonia, all of which reflect impaired mitochondrial oxidative phosphorylation and reduced COX function (Debray et al., 2014; Kollberg et al., 2005; Madhok & Shabbir, 2022; Peredo & Hannibal, 2009). Mitochondrial encephalomyopathy is characterized by impaired COX activity and may present with neurological symptoms and skeletal muscle manifestations, including exercise intolerance, muscle weakness, muscle atrophy, cerebellar ataxia, and seizures (Debray et al., 2014). COX deficiency has also been linked to rhabdomyolysis, a condition characterized by the breakdown of skeletal muscle fibers and the release of their components into the bloodstream (Kollberg et al., 2005). It is often accompanied by myoglobinuria (Kollberg et al., 2005). Associated muscle phenotypes include muscle weakness, pain, stiffness, and exercise intolerance (Kollberg et al., 2005). Additionally, COX deficiency has been associated with hypotonia, characterized by reduced resting muscle tone and decreased resistance to passive movement (Madhok & Shabbir, 2022; Peredo & Hannibal, 2009). Clinically, this may appear as limb floppiness or poor postural control (Madhok & Shabbir, 2022; Peredo & Hannibal, 2009).

Holvoet et al. (2017) demonstrated that low *CoxI* expression in plaque macrophages is a feature of mitochondrial dysfunction linked to insulin resistance and oxidative stress in both murine and porcine models. Bonnard et al. (2008) also observed a reduction in *CoxI* expression in skeletal muscle of diet-induced diabetic mice. In their model, metabolic stress led to a significant downregulation of *CoxI* mRNA, which was associated with altered mitochondrial biogenesis, structure, and function. While Bonnard et al. (2008) demonstrated that these changes were driven by increased oxidative stress. Additionally, mitochondrial dysfunction has been hypothesized to link oxidative stress with reduced fitness (Wisløff et al., 2005)

1.5.3. *TNNT3*

Pathogenic mutations in *TNNT3* have been associated with distal arthrogryposis. It is characterized by contractures of the distal limbs, with features involving both the upper and lower limbs, including overriding fingers, ulnar deviation at the wrist, and foot deformities (Bamshad et al., 2009; Lu et al., 2021). The p.R63C variant in *TNNT3* increases the stability of the encoded protein, leading to its accumulation (Lu et al., 2021).

2. EXPERIMENTAL PART

2.1. Objective

This thesis is part of a study conducted in Eero Vasar's research group at the Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu.

Within this research group, *Negr1*^{-/-} mice have so far been studied primarily at the level of neuropsychiatric disorders (Muthuraman et al., 2026; Reinsberg et al., 2025; Singh et al., 2018, 2019, 2024). Their recent publication also indicated abnormal regulation of the actin-associated motor protein Myosin ID and the actin-binding filament regulator Tropomyosin 1 in the hippocampus, suggesting a potential brain-muscle interaction axis in *Negr1*-deficient mice (Muthuraman et al., 2026). However, these mice have also shown unusual locomotor patterns that may suggest altered skeletal muscle function. Previous studies within the research group (unpublished) showed poor muscle strength in *Negr1*^{-/-} mice. A combination of structural (*Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*), mitochondrial (*Cox1* and *Cox2*), and calcium-binding (*Pvalb*) genes was selected for qPCR analysis to understand different biological pathways that may contribute to the altered muscle strength and locomotor phenotype in *Negr1*^{-/-} mice.

The objectives of this thesis were to:

1. quantify the relative mRNA expression levels of *Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*, *Cox1*, *Cox2*, and *Pvalb* in *Negr1*^{-/-} mice and wild-type mice;
2. compare the relative mRNA expression levels between *Negr1*^{-/-} mice and wild-type mice;
3. analyze sex-specific mRNA expression differences between male and female *Negr1*^{-/-} mice.

2.2. Materials and Methods

2.2.1. Animals

Male and female wild-type mice and their homozygous *Negr1*^{-/-} littermates, previously described by Lee et al. (2012), were used for skeletal muscle mRNA expression analysis by

RT-qPCR. The mice were bred on an F2 hybrid genetic background derived from crosses between 129S5/SvEvBrd and C57BL/6N strains ((129S5/SvEvBrd × C57BL/6N) × (129S5/SvEvBrd × C57BL/6N)). The estrous cycle stages were not monitored in female mice.

Male and female mice of both genotypes (WT: wild-type and *Negr1*^{-/-}) were housed separately by sex and genotype in standard laboratory cages (42.5 (L) × 26.6 (W) × 15.5 (H) cm), with 8–10 animals per cage. Male cages were placed on different racks within the same room from weaning until the end of the experimental period. Animals were maintained under controlled conditions at 22 ± 1 °C on a 12 h light/12 h dark cycle, with lights off at 19:00. Each cage contained a 2 cm layer of aspen bedding (Tapvei, Estonia) and 0.5 l of aspen nesting material (Tapvei, Estonia), both of which were renewed weekly. Food pellets (R70, Lactamin AB, Sweden) and reverse osmosis-purified water were available at all times.

The mice were bred and maintained at the Institute of Biomedicine and Translational Medicine animal facility at the University of Tartu, Estonia. All animal procedures followed the regulations and guidelines approved by the Laboratory Animal Center of the same institute. Experimental protocols were approved by the Estonian National Board of Animal Experiments, Põllumajandus- ja Toiduamet (No. 150, 27 September 2019), and were performed in accordance with the European Communities Directive (2010/63/EU). The study is reported in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

All mice were euthanized by decapitation at 7 months old, and skeletal muscle (tibialis anterior) was collected, snap-frozen in liquid nitrogen, and stored at -80 °C until RNA extraction for RT-qPCR analysis. In the present thesis, the experimental cohort consisted of 45 animals divided into four groups according to genotype and sex: male wild-type mice (13), male *Negr1*^{-/-} (11), female wild-type mice (10), and female *Negr1*^{-/-} mice (11).

The author of the present thesis was not involved in the handling, breeding, euthanization, or experimental manipulation of live animals. These procedures were performed by qualified and licensed personnel.

2.2.2. RNA Extraction

Total RNA was extracted using the TRIzol reagent (Invitrogen, Waltham, MA, USA) and the chloroform (Fisher Chemical) extraction method, following the manufacturer's guidelines.

First, a total of 500 μ l of TRIzol was added to the muscle sample (~45 mg) in portions of 100 μ l, 100 μ l, and 300 μ l. Between TRIzol additions, the skeletal muscle was homogenized using a manual homogenizer until a semi-liquid consistency was achieved. After that, 100 μ l of chloroform was added to the sample. The sample was incubated at room temperature for 10 minutes to allow proper interaction between chloroform and the lysate. Following the incubation, the sample was centrifuged at 4 °C and 12,000 g for 15 minutes to induce phase separation. The RNA-containing, upper, transparent aqueous layer was carefully transferred to a new 1.5 ml microcentrifuge tube without disturbing the interphase. 250 μ l of isopropanol was added, and the mixture was gently mixed for 10 to 15 seconds. The resulting mixture was incubated on ice for 10 minutes, then placed in the fridge at -20 °C overnight to facilitate RNA precipitation. Then the following day, the RNA was pelleted by centrifugation for 10 minutes at 4 °C and 12,000 g. Following centrifugation, the supernatant was discarded, while the whitish layer or pellet was clearly seen. Then, the pellet was washed with 750 μ l of ethanol and centrifuged at 4 °C and 7,500 g for 5 minutes. The supernatant was discarded, and the RNA pellet was dissolved in 20 μ l of nuclease-free water.

The RNA concentration was measured by absorbance at 260 nm using a NANODROP 1000 Spectrophotometer (Thermo Fisher Scientific, USA), and the purity was evaluated using the OD260/OD280 ratio as an indicator of protein contamination and the OD260/OD230 ratio as an indicator of contamination by organic compounds. Pure RNA is determined by an OD260/OD280 ratio of 2.

2.2.3. cDNA Synthesis

cDNA was synthesized from 2 μ g of total RNA using the FIREScript RT cDNA Synthesis Kit with Oligo(dT) random primer mix (Solis BioDyne, Tartu, Estonia) according to the manufacturer's instructions. cDNA synthesis is a two-step process that begins with primer annealing and reverse transcription at 55 °C, followed by enzyme deactivation at 85 °C. After enzyme deactivation, the cDNA is kept on ice for 5 minutes to cool and then stored at -20 °C for further use. For quantitative polymerase chain reaction (qPCR) analysis, cDNA working stocks were prepared by diluting 10 μ l of each cDNA sample with 20 μ l of nuclease-free water.

2.2.4. Primer Designing

The primers were designed using *Mus musculus* mRNA sequences obtained from the National Center for Biotechnology Information (NCBI) database. Primers for the target genes and the housekeeping gene were selected using the PrimerBLAST service provided by the NCBI. The PCR product size was set to 80–120 base pairs, and the primer melting temperature was chosen to be a maximum of 61°C. Additionally, the primer had to span an exon-exon junction, with a maximum target amplicon size of 120 base pairs. Other PrimerBLAST settings were kept at the program's default values. Based on the sequences provided by the site, an order for primers was placed. Later, the primer stocks were dissolved in nuclease-free water to a final concentration of 100 µM. The primer working solutions were prepared by combining 5 µl of the gene-specific forward primer and 5 µl of the gene-specific reverse primer, then diluting the mix with 90 µl of nuclease-free water, resulting in a 1:20 dilution of each primer.

Primer sequences reported in previous literature were also included as alternative candidates. Both PrimerBLAST-designed primers and previously reported primers were tested by qPCR. The final primer pairs were selected based on qPCR performance, assessed by amplification plot and melting curve analysis.

The primers used, along with their sequences and sources, are listed in Table 3. The housekeeping gene selected as a reference was *Actb*.

Table 3. List of primers used for mRNA expression measurements in the current study.

Target Genes	Primer Sequence	Source
<i>Myh7</i>	F: AGATGAATGCCGAGCTCACT R: CTCATCCAAACCAGCCATCT	(Tong et al., 2025)
<i>Tpm1</i>	F: GAAGTCACTGGAGGCTCAGG R: CCGAGTTTCAGCCTCCTTCA	NCBI PrimerBLAST
<i>Tnnc2</i>	F: AACCATGACGGACCAACAGG R: TTAACGCTGATGTCCCCACC	(Lixin et al., 2019)
<i>Tnnt3</i>	F: CCCTCATTGACAGCCACTTT R: CCTCCTCTCTTCTGGCCTTC	(Ju et al., 2013)

<i>Acta1</i>	F: CTGAGCGTGGCTATTCCTTC R: GGCATACAGGTCCTTCCTG	(Mizuno et al., 2009)
<i>Ttn</i>	F: ACCAGAGCAAAGTGTAACGG R: GAACTACCACACAAGGCAGG	(Garvey et al., 2002)
<i>Cox1</i>	F: GCCTTTCAGGAATACCACGA R: AGGTTGGTTCCTCGAATGTG	(Litwiniuk et al., 2016)
<i>Cox2</i>	F: AATTAGCTCCTTAGTCCTCT R: CTTGGTCGGTTTGATGTTAC	(Tan, 2018)
<i>Pvalb</i>	F: GATAGGAGCCTTTGCTGCTG R: GCCAGAAGCGTCTTTGTTTC	(Butera et al., 2021)
<i>Actb</i>	F: TCCCTGGAGAAGAGCTATGAGC R: GAGGTCTTTACGGATGTCAACG	(Kim et al., 2021)

2.2.5. qPCR Procedure

Gene-specific qPCR reaction mixes were prepared separately for each cDNA sample and each target or housekeeping gene. Each reaction mix contained 8.8 µl of 5× HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia), 28.7 µl of nuclease-free water, 1.5 µl of diluted cDNA, and 4 µl of the corresponding gene-specific primer mix, resulting in a final volume of 43 µl. For each sample-gene combination, the prepared reaction mix was loaded onto a 384-well qPCR plate in technical quadruplicates, with 10 µl per well. The plate was sealed with optical film, centrifuged at 1,500 rpm for 1 min, and subjected to qPCR amplification for 50 cycles on a QuantStudio 12KFlex instrument (Thermo Fisher Scientific) according to the manufacturer's instructions. The qPCR thermal cycling conditions are presented in Table 4.

Table 4. qPCR Cycle Description Table

Cycle Step	Temperature	Time	Cycles
Initial hold	50 °C	2 min	1x
Initial denaturation	95 °C	10 min	
Denaturation	95 °C	15 s	50x
Annealing/extension	60 °C	1 min	
Melting curve	95 °C	15 s	1x
	60 °C	1 min	
	95 °C	15 s	

qPCR combines PCR amplification with fluorescent dyes, allowing the accumulation of PCR products to be monitored during each amplification cycle (Harshitha & Arunraj, 2021). The relative mRNA expression levels, determined from fluorescence signals generated during template amplification, were noted for further analysis. RT-qPCR data were analyzed for their relative mRNA expression ($2^{-\Delta CT}$) as described in previous literature (Livak & Schmittgen, 2001). For each sample, the cycle threshold (CT) value was used for further analysis. The expression of each target gene was normalized to the reference gene *Actb* by calculating ΔCT as follows: $\Delta CT = CT(\text{target gene}) - CT(\text{Actb})$. Relative mRNA expression was then calculated using the $2^{-\Delta CT}$ method.

2.2.6. Statistics

Statistical analysis was performed with GraphPad Prism 10.6.1. The data were transformed to $\log_2(Y)$. The outliers were identified using Grubbs' method, and a two-way ANOVA (Factors: Sex and Genotype) was performed, followed by a Bonferroni post hoc test. For the post hoc analysis, the normality and lognormality were assessed using the Shapiro-Wilk test. Based on the results from the Shapiro-Wilk test, parametric (Welch's t test) or non-parametric (Mann-Whitney test) analysis was performed. Results were presented as mean $\pm$ SEM. A $p < 0.05$ was considered statistically significant. Significance levels in figures are indicated as

follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) for genotype-dependent differences in post hoc analysis, and & denotes the main effect of Sex.

Across the analyses, group sizes ranged from 9 to 13 for male wild-type mice, 8 to 11 for male *Negr1*^{-/-} mice, 8 to 10 for female wild-type mice, and 8 to 11 for female *Negr1*^{-/-} mice.

2.3. Results

The results revealed significant genotype x sex interactions for *Cox1* and *Myh7* (F (1, 38) = 5.981, $p = 0.0192$; F (1, 33) = 6.718, $p = 0.0141$, respectively) (Table 5). Multiple comparisons showed that in male *Negr1*^{-/-} mice, *Cox1* ($P = 0.0316$) and *Myh7* ($P = 0.0316$) were significantly downregulated compared with male wild-type mice. In contrast, *Myh7* was significantly upregulated in female *Negr1*^{-/-} mice than in female wild-type mice ($P = 0.0411$). *Tnnt3* expression was also significantly upregulated in male *Negr1*^{-/-} mice compared with male wild-type mice ($P = 0.0482$). No significant genotype-dependent differences were detected in female mice for *Cox1* and *Tnnt3*. (Figure 4)

Several genes showed significant sex-specific differences within the *Negr1*^{-/-} group. Results showed a significant main effect of sex for *Pvalb* (F (1, 40) = 46.30, $p < 0.0001$), *Cox1* (F (1, 38) = 20.85, $p < 0.0001$), *Myh7* (F (1, 33) = 13.23, $p = 0.0009$), *Cox2* (F (1, 36) = 10.15, $p = 0.0030$), *Tnnt3* (F (1, 34) = 8.278, $p = 0.0069$), and *Ttn* (F (1, 37) = 4.700, $p = 0.0367$) (Table 5). *Pvalb*, *Myh7*, and *Tnnt3* expression were higher in male *Negr1*^{-/-} mice than in female *Negr1*^{-/-} mice, while *Cox1* and *Cox2* expression were higher in female *Negr1*^{-/-} mice than in male *Negr1*^{-/-} mice. (Figure 4)

For *Tnnc2*, *Tpm1*, and *Acta1*, no statistically significant genotype- nor sex-specific differences were observed (Figure 4). Their expression levels appeared broadly similar between wild-type and *Negr1*^{-/-} mice in both males and females.

Table 5. Log2-Transformed Relative mRNA Expression of Selected Target Genes in the Tibialis Anterior Muscle of Wild-Type and *Negr1*^{-/-} Mice by Sex, and with Two-Way ANOVA results. Statistically significant p values are marked in bold.

	Male		Female		Two-Way ANOVA		
	WT Mean ± SEM	<i>Negr1</i> ^{-/-} Mean ± SEM	WT Mean ± SEM	<i>Negr1</i> ^{-/-} Mean ± SEM		F	P
<i>Myh7</i>	4.109	1.157	-0.3324	0.4113	genotype sex interaction	F (1, 33) = 2.399 F (1, 33) = 13.23 F (1, 33) = 6.718	0.1310 0.0009 0.0141
<i>Tpm1</i>	5.142	4.925	3.785	3.617	genotype sex interaction	F (1, 34) = 0.06849 F (1, 34) = 3.270 F (1, 34) = 0.001121	0.7951 0.0794 0.9735
<i>Tnnc2</i>	2.000	3.454	2.401	2.110	genotype sex interaction	F (1, 35) = 0.7111 F (1, 35) = 0.4674 F (1, 35) = 1.600	0.4048 0.4987 0.2143
<i>Tnnt3</i>	5.528	6.899	4.854	4.143	genotype sex interaction	F (1, 34) = 0.3070 F (1, 34) = 8.278 F (1, 34) = 3.052	0.5832 0.0069 0.0897
<i>Acta1</i>	2.293	4.217	3.303	2.313	genotype sex interaction	F (1, 34) = 0.2581 F (1, 34) = 0.2366 F (1, 34) = 2.512	0.6147 0.6298 0.1223
<i>Ttn</i>	1.643	1.945	0.9101	0.6380	genotype sex interaction	F (1, 37) = 0.001033 F (1, 37) = 4.700 F (1, 37) = 0.3728	0.9745 0.0367 0.5452
<i>Cox1</i>	12.37	10.12	13.58	14.13	genotype sex interaction	F (1, 38) = 2.210 F (1, 38) = 20.85 F (1, 38) = 5.981	0.1454 < 0.0001 0.0192
<i>Cox2</i>	12.55	11.63	13.39	14.46	genotype sex interaction	F (1, 36) = 0.01646 F (1, 36) = 10.15 F (1, 36) = 2.980	0.8986 0.0030 0.0929
<i>Pvalb</i>	5.108	6.238	1.136	0.4124	genotype sex interaction	F (1, 40) = 0.07983 F (1, 40) = 46.30 F (1, 40) = 1.657	0.7790 < 0.0001 0.2054

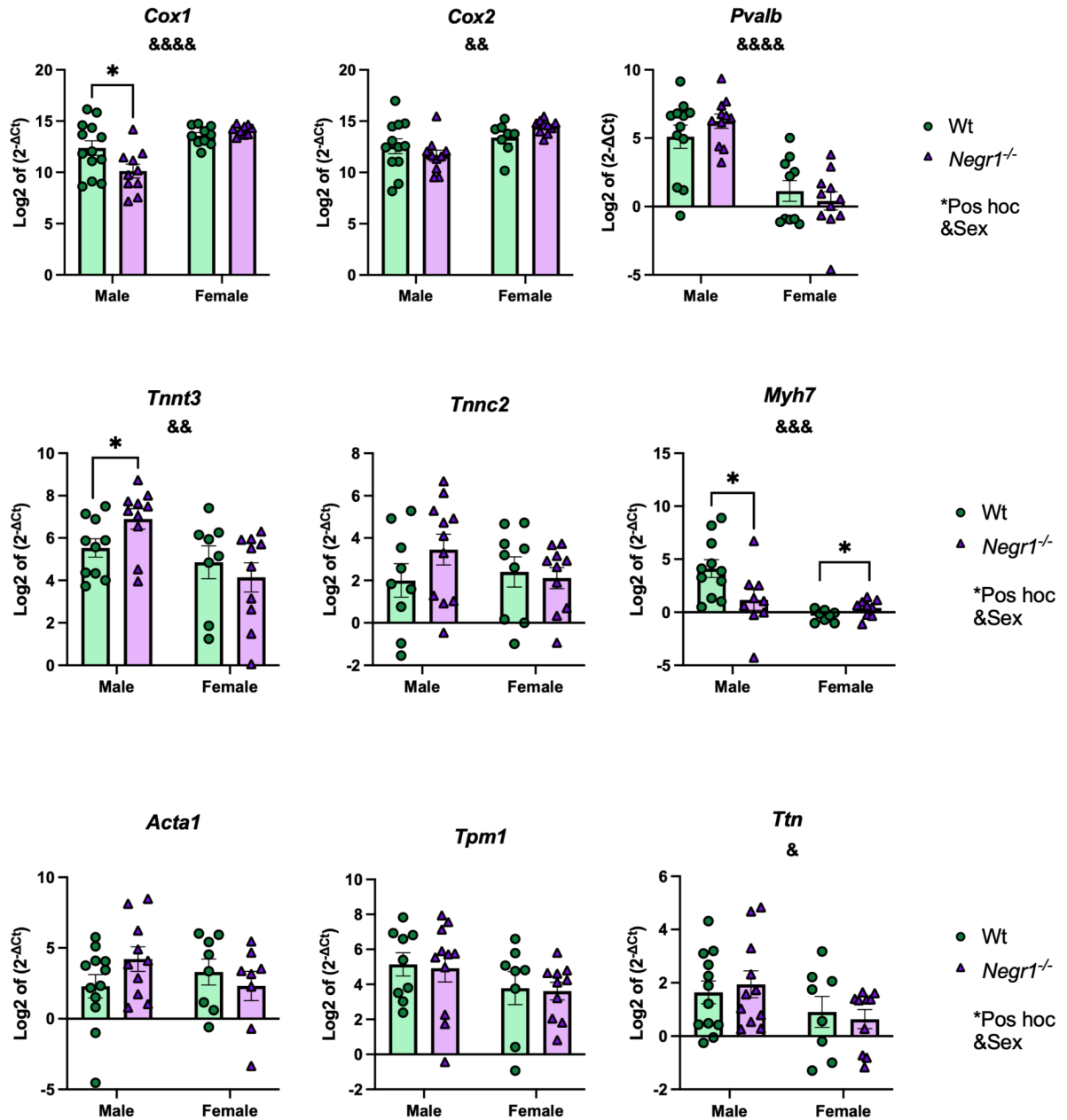


Figure 4. Expression Levels of Selected Skeletal Muscle-Related Genes in Wild-Type and *Negr1*^{-/-} Mice. The relative mRNA expression levels of (from left to right) *Cox1*, *Cox2*, *Pvalb*, *Tnnt3*, *Tnnc2*, *Myh7*, *Acta1*, *Tpm1*, and *Ttn* were analyzed in skeletal muscle tissue from male and female wild-type and *Negr1*^{-/-} mice. Individual points represent biological replicates, and bars represent group SEMs. Asterisks indicate statistically significant genotype-dependent differences between wild-type and *Negr1*^{-/-} mice within the same sex ($p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)), while ampersands indicate statistically significant sex-dependent differences between male and female *Negr1*^{-/-} mice ($p < 0.05$ (&), $p < 0.01$ (&&), $p < 0.001$ (&&&), $p < 0.0001$ (&&&&)).

2.4. Discussion

The genotype-dependent changes observed in *Negr1*^{-/-} mice suggest that *Negr1* deficiency may be associated with altered skeletal muscle mRNA expression, particularly in genes related to oxidative metabolism and contractile machinery.

In the present study, *Cox1* was significantly downregulated in male *Negr1*^{-/-} mice (Figure 4), suggesting altered mitochondrial regulation in skeletal muscle. Although this finding cannot be directly linked to pathogenic *COX1* mutations, it remains relevant because both reduced *Cox1* expression and structural COX1 defects may, in theory, affect the function of the COX complex in a similar manner. As previous studies have shown that mutations in *COX1* can be associated with COX deficiency and skeletal muscle phenotypes, including exercise intolerance, muscle weakness, muscle atrophy, and muscle pain (see Section 1.5.2.) (Debray et al., 2014; Kollberg et al., 2005), the downregulation *Cox1* observed here may be interpreted as a potential indication that COX-related mitochondrial function could be affected in male *Negr1*^{-/-} mice. This is further supported by previous literature, which describes reduced exercise capacity and skeletal muscle atrophy in *Negr1*^{-/-} mice (Joo et al., 2019; Kaare et al., 2021). Furthermore, expression-based studies have shown that reduced *Cox1* mRNA levels are associated with mitochondrial dysfunction in diabetic mice (Bonnard et al., 2008; Holvoet et al., 2017), suggesting that the observed downregulation of *Cox1* in male *Negr1*^{-/-} mice may reflect a mitochondrial regulatory disturbance. These findings are relevant to the present study because *Negr1*^{-/-} mouse models have previously been associated with increased susceptibility to obesity- and insulin-resistance-related changes, with some effects being more pronounced in males (Kaare et al., 2021; Tarmunt et al., 2019). Additionally, mitochondrial dysfunction has been hypothesized to link oxidative stress with reduced fitness (Wisloff et al., 2005), a hypothesis already supported by observations in *Negr1*^{-/-} mice using endurance tests (Joo et al., 2019). Therefore, if reduced transcript levels are accompanied by lower Cox1 protein abundance or impaired COX activity, this could contribute to altered oxidative phosphorylation and thereby provide one possible explanation for the skeletal muscle-related phenotypes observed in *Negr1*^{-/-} mice.

The sex-dependent regulation of *Myh7* is one of the more notable findings of the present thesis, as it was downregulated in male *Negr1*^{-/-} mice but upregulated in female *Negr1*^{-/-} mice (Figure 4). This suggests that *Negr1* deficiency does not affect skeletal muscle mRNA expression alone but may interact with sex-specific regulatory mechanisms, such as sex

hormones. In males, *Myh7* downregulation may reflect a weakening or loss of slow-twitch muscle fibers. Since slow-twitch muscle fibers are closely associated with fatigue resistance and endurance capacity (Plotkin et al., 2021), the *Myh7* downregulation observed in male *Negr1*^{-/-} mice may be relevant, especially because reduced endurance has already been reported in *Negr1*^{-/-} mice (Joo et al., 2019). Furthermore, this could be relevant, as *MYH7*-related myopathies show that disruption of beta-myosin heavy chain function is associated with lower-limb muscle weakness (see Section 1.5.1) (Bahout et al., 2025; Meredith et al., 2004; Laing et al., 1995; Pegoraro et al., 2007; Tajsharghi et al., 2003; Tajsharghi & Oldfors, 2013). This association may be relevant because muscle weakness has also been observed in *Negr1*^{-/-} mouse models (unpublished). However, the mechanism in male *Negr1*^{-/-} mice is likely different, since no *Myh7* mutation or protein levels were examined. Therefore, it can not be stated that male *Negr1*^{-/-} mice will develop *MYH7*-related diseases, but the downregulation of *Myh7* could result in similar consequences in skeletal muscles. Another possible explanation is that *Myh7* downregulation triggers a slow-to-fast fiber-type transition, as reported in various studies (see Section 1.5.1). This interpretation is further supported by *Cox1* downregulation in male *Negr1*^{-/-} mice, as COX complex proteins are predominantly expressed in slow-twitch muscle fibers (Edman et al., 2024), likely due to higher mitochondrial volume. Together, the downregulation of *Myh7* and *Cox1* supports the possibility that the slow-twitch fiber-related gene expression is altered in male *Negr1*^{-/-} mice. In females, the *Myh7* upregulation is more difficult to connect to disease pathology, although myosin storage myopathy is a possibility. Even so, the current mRNA data are insufficient for this evaluation, and beta-myosin heavy chain protein levels must be measured to confirm this correlation. A more plausible interpretation of the upregulation of *Myh7* in female *Negr1*^{-/-} mice is muscle fiber-type transition. *Myh7* upregulation in female *Negr1*^{-/-} mice may indicate a shift toward slow-twitch muscle fibers, which is potentially a compensatory response to *Negr1*-deficiency. This interpretation is consistent with studies showing that *Myh7* upregulation is associated with a higher proportion of slow-twitch muscle fibers (see Section 1.5.1) (Li et al., 2022; Yan et al., 2025). However, in that case, it would be expected that fast-twitch muscle fiber-related genes are downregulated in female *Negr1*^{-/-} mice, although such a change was not observed.

The upregulation of *Tnnt3* in male *Negr1*^{-/-} mice (Figure 4) may indicate a shift toward fast-twitch fibers. This interpretation is strengthened by the fact that *Myh7* was downregulated. Together, the pattern of *Myh7* downregulation and *Tnnt3* upregulation

suggests that *Negr1* deficiency in male skeletal muscle could reflect a broader change in fiber-type regulation. Specifically, the upregulation of *Tnnt3* may indicate enhanced fast-fiber-associated thin-filament regulation, a compensatory mechanism caused by *Negr1* deficiency, or even the downregulation of *Myh7* and/or *Cox1*. Based on current data, it is not clear whether downregulation of *Myh7* causes upregulation of *Tnnt3*, upregulation of *Myh7* causes downregulation of *Tnnt3*, or whether they occur simultaneously. Additionally, a variant in *TNNT3* has been associated with distal arthrogryposis, where *TNNT3* stability is increased, leading to its accumulation (see Section 1.5.3) (Lu et al., 2021). To associate *Tnnt3* upregulation with distal arthrogryposis, further analyses would be needed, including phenotypic assessments and protein level measurements. However, this interpretation appears less likely than other proposed associations, as it would not explain the altered expression of *Myh7* and *Cox1*.

The selected gene panel limits the current genotype-dependent conclusions. The unaltered expression of *Tpm1* does not exclude the possibility that its homologs *Tpm2* and *Tpm3* may be altered in *Negr1*^{-/-} skeletal muscle. Similarly, although altered *Myh7* expression was observed, the present study did not assess other myosin heavy chain isoforms, such as *Myh2*, *Myh4*, and *Myh8*, which are associated with fast-twitch muscle fibers. If there truly is a fiber-type change occurring in *Negr1*^{-/-}, then it would be expected if fast-twitch myosin heavy chain isoforms were downregulated in female *Negr1*^{-/-} mice, and upregulated in male *Negr1*^{-/-} mice. Therefore, further analysis of related tropomyosin and myosin isoforms would be necessary to determine whether these changes are isolated or part of a broader disturbance in contractile gene regulation. An additional limitation of the present experiment is the relatively small sample size. Because mRNA expression may vary between individual mice, the limited number of samples may have reduced the statistical power to detect more genotype-dependent differences. That increases the possibility of false-negative results.

In addition to the genotype-dependent changes observed in *Negr1*^{-/-} mice, the results also show significant sex-specific differences in the expression of several skeletal muscle-associated genes, including *Pvalb*, *Cox1*, *Cox2*, *Myh7*, *Tnnt3*, and *Ttn* (Figure 4). These results indicate that the transcriptional profile of the tibialis anterior differed significantly between male and female *Negr1*^{-/-} mice. The skeletal muscle mRNA expression profile observed in this study appears to be shaped by the biological sex of the *Negr1*^{-/-} mice. Notably, most of the genotype-dependent differences were observed in males, as females

showed altered expression in only *Myh7* and males in addition to *Myh7*, in *Cox1* and *Tnnt3*. Interestingly, Kaare et al (2021) also reported sex-specific skeletal muscle alterations in *Negr1*^{-/-} mice (see Section 1.3.2). Kaare et al. (2021) suggested that such sex-specific differences may have resulted from other signaling molecules, such as sex hormones, but the exact mechanism remains elusive. When considered together with the present results, these findings suggest that the skeletal muscle of male *Negr1*^{-/-} mice may be more susceptible to the effects of *Negr1* deficiency. However, the reasons behind these sex-related differences remain unclear. Possible explanations include differences in muscle fiber composition, mitochondrial metabolism, and hormonal regulation that contribute to susceptibility to *Negr1* deficiency. Further studies, such as phenotypic assessments, would be needed to determine whether these sex-specific transcriptional differences cause any of the previously described muscle disorders.

As NEGR1 has been associated with neuropsychiatric conditions that also involve motor and neuromuscular impairments (Bologna et al., 2020; Hepple & Rice, 2016; Ogawa et al., 2018; Vancampfort et al., 2013; Viertiö et al., 2009), it is relevant to consider whether the previously observed skeletal muscle impairment in *Negr1*^{-/-} mice may be linked to broader disease-related mechanisms. In this context, the altered expression of genes related to oxidative metabolism and the contractile machinery observed in the present thesis may indicate that the transcriptional disturbances in the skeletal muscle of *Negr1*^{-/-} mice may also be influenced by the central nervous system. However, because the present data cannot confirm these associations, further assessments are necessary to determine whether *Negr1*^{-/-} mice exhibit motor impairments similar to those observed in patients with these neurological conditions. Complete transcriptomic profiling by RNA sequencing could also provide valuable insights into the hypothesis.

CONCLUSION

The aim of the present thesis was to quantify and compare genotype-dependent and sex-specific differences in the relative mRNA expression of skeletal muscle-related genes in the tibialis anterior muscle of *Negr1*^{-/-} and wild-type mice. The genes selected for this experiment were *Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*, *Cox1*, *Cox2*, and *Pvalb*, and their expression levels were measured using qPCR.

Genotype-dependent changes were observed in *Cox1*, *Myh7*, and *Tnnt3*. In male *Negr1*^{-/-} mice, *Myh7* and *Cox1* were significantly downregulated, while *Tnnt3* was upregulated. In female *Negr1*^{-/-} mice, *Myh7* was significantly upregulated, whereas the expression of *Cox1* and *Tnnt3* remained unchanged. Sex-specific differences within the *Negr1*^{-/-} group were observed in *Pvalb*, *Myh7*, *Tnnt3*, and *Ttn*, which were expressed higher in male *Negr1*^{-/-} mice than in female *Negr1*^{-/-} mice. *Cox1* and *Cox2* expressions were higher in female *Negr1*^{-/-} mice than in male *Negr1*^{-/-} mice. For *Tnnc2*, *Tpm1*, and *Acta1*, no statistically significant genotype- nor sex-specific differences were observed.

These findings suggest that *Negr1* deficiency may affect skeletal muscle by selectively altering mRNA expression. The downregulation of *Cox1* in male *Negr1*^{-/-} mice may indicate altered mitochondrial regulation and possibly a connection to pathogenic COX complex deficiency-related diseases. The *Myh7* being downregulated in male *Negr1*^{-/-} mice, and upregulated in female *Negr1*^{-/-} mice, may reflect a fiber-type transition – slow-to-fast fiber-type in males, and fast-to-slow fiber-type in females. The upregulation of *Tnnt3* in male *Negr1*^{-/-} mice may further suggest altered fast-twitch fiber and thin-filament regulation. As male *Negr1*^{-/-} mice showed altered mRNA regulation in relation to more genes, compared with female *Negr1*^{-/-} mice, this gives grounds to think that males may be more susceptible to the deficiency of *Negr1*.

Taken together with previous literature on skeletal muscle gene regulation and phenotypes, the present results indicate that *Negr1* deficiency is associated with altered expression of genes involved in oxidative metabolism and the contractile machinery in murine skeletal muscle. These findings support the possibility that NEGR1 plays a role in skeletal muscle biology. However, further studies are needed to determine whether the observed transcriptional changes are associated with functional muscle phenotypes.

RESÜMEE

Skeletilihaste molekulaarne analüüs *Negr1*-puudulikkusega hiirtes

Birgit Pilve

Resümee

NEGR1 (neuronaalne kasvuregulaator 1) on rakuadhesiooni molekul, mis kuulub IgLON valkude perekonda. NEGR1 mängib olulist rolli kesknärvisüsteemi kujunemisel, sealhulgas ajuosadevaheliste ühenduste loomisel ning käitumis regulatsioonis. Lisaks on NEGR1-t seostatud ka skeletilihaste regulatsiooniga, mõjutades nii lihasfunktsiooni kui ka lihaskoe kujunemist. *Negr1*^{-/-} hiirtes on täheldatud ka häiritud liikumisvõimet ja lihasnõrkust. Seni on NEGR1-t seostatud mitmete erinevate psühhiaatriliste ja neuroarenguliste häiretega, sealhulgas raskekujulise depressiooni, autismispektri häirete ja skisofreeniaga. Samas pole NEGR1-t veel selgelt seostatud ühegi liikumist raskendava diagnoosiga.

Varasemad uuringud on *Negr1*^{-/-} hiirtel kirjeldanud vähenenud skeletilihaste massi ja lihaskiudude ristlõike pindala. Lisaks on neil hiirtel täheldatud vähenenud füüsilise koormuse taluvust. *Negr1*^{-/-} hiirte skeletilihastes on võrreldes metsiktüüpi hiirtega tuvastatud 1195 ülereguleeritud ning 1317 alareguleeritud geeni. Käesoleva bakalaureusetöö eesmärk oli kvantifitseerida ja võrrelda genotüübist sõltuvaid ning soospetsiifilisi erinevusi skeletilihastega seotud geenide suhtelist mRNA ekspressiooni *Negr1*^{-/-} ja metsiktüüpi hiirte *tibialis anterior*'i lihastes. Katsesse valitud geenid olid *Myh7*, *Tpm1*, *Tnnc2*, *Tnnt3*, *Acta1*, *Ttn*, *Cox1*, *Cox2*, ja *Pvalb*, mille mRNA ekspressioonitasemeid mõõdeti qPCR meetodiga.

Genotüübist sõltuvad muutuseid täheldati *Cox1*, *Myh7* ja *Tnnt3* regulatsioonis. Isastel *Negr1*^{-/-} hiirtel olid *Myh7* ja *Cox1* alareguleeritud ning *Tnnt3* oli ülereguleeritud. Emastel *Negr1*^{-/-} hiirtel oli *Myh7* ülereguleeritud, kuid *Cox1* ja *Tnnt3* regulatsioonis muutuseid ei ilmnenud. Soospetsiifilisi erinevusi täheldati *Pvalb*, *Myh7*, *Tnnt3* ja *Ttn* ekspressioonis, mille tase oli isastel *Negr1*^{-/-} hiirtel kõrgem kui emastel *Negr1*^{-/-} hiirtel. *Cox1* ja *Cox2* ekspressioon oli aga kõrgem emastel *Negr1*^{-/-} hiirtel kui isastel *Negr1*^{-/-} hiirtel. *Tnnc2*, *Tpm1* ja *Acta* puhul statistiliselt olulisi genotüübi- ega soospetsiifilisi erinevusi ei täheldatud.

Tulemused viitavad sellele, et *Negr1*-puudulikkus võib mõjutada skeletilihaseid valikuliste muutuste kaudu mRNA ekspressioonis. *Cox1* alareguleeritus isastes *Negr1*^{-/-} hiirtes võib viidata muutunud mitokondriaalsele regulatsioonile ning võimalikele COX kompleksi

puudulikkusega seotud patoloogilistele seisunditele. *Myh7* alaregulatsioon isastes *Negr1*^{-/-} hiirtes ja üleregulatsioon emastes *Negr1*^{-/-} hiirtes võib peegeldada muutusi lihaskiutüüpide regulatsioonis: isastel võimalikku nihet aeglase kontraktsiooniga lihaskiududelt kiire kontraktsiooniga lihaskiudude suunas ning emastel vastupidiselt kiire kontraktsiooniga lihaskiududelt aeglase kontraktsiooniga lihaskiudude suunas. *Tnnt3* üleregulatsioon isastel *Negr1*^{-/-} hiirtel võib omakorda viidata kiirete lihaskiudude ja õhukese filamendi regulatsiooni muutustele. Kuna isastel *Negr1*^{-/-} hiirtel täheldati muutusi rohkemate geenide mRNA ekspressioonis kui emastel *Negr1*^{-/-} hiirtel, annab see alust oletada, et isased võivad olla *Negr1* puudulikkuse suhtes tundlikumad.

Koos varasema kirjandusega seoses skeletilihaste geeniregulatsiooni ja fenotüüpidega viitavad käesoleva töö tulemused sellele, et *Negr1* puudulikkus on seotud oksüdatiivses metabolismis ja kontraktilses aparatuuris osalevate geenide muutunud ekspressiooniga hiire skeletilihases. Need leiud toetavad *NEGR1* võimalikku rolli skeletilihaste regulatsioonis, kuid edasised uuringud on vajalikud, et teha kindlaks, kas täheldatud transkriptsioonilised muutused seostuvad ka patogeensete lihasfenotüüpidega.

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