Targeting versican as a therapeutic strategy in Duchenne muscular dystrophy

by

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BSc, BHlthSc (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University
March, 2018
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Acknowledgments

This thesis is the culmination of many years of persistence, dedication and determination that resulted in a rewarding and emotional research journey. This thesis would not have been possible if not for the following people to whom I now extend my sincere and heartfelt gratitude.

I would like to firstly thank my supervisors. Nicole, thank you for introducing me to the wonderful, yet wacky, world of the matrix. I am thankful for your help in perfecting my communication, presentation and written skills, allowing for self-confidence and personal growth. Dan, I’m grateful to you and Nicole for the conception of the study, and for your extensive knowledge of versican and ADAMTS proteases. Bryony, thank you for your encouragement, advice and motivation. I sincerely appreciate that you took time to read my drafts, and for giving me constructive feedback. Yann, I appreciate your continuous support and guidance, and I am especially thankful for your cheerful banter and for making everything better.

I would also like to thank Muscular Dystrophy Australia (MDA) and Boris Struk. Research is an integrated undertaking, and through the MDA program of supporting PhD student scholarships, funding was made available at Deakin University to purchase the muscle function testing equipment which was essential for the completion of this thesis.

To my ‘lab-brother’ Alex. I am immensely thankful for our comradery: all our chats, pep talks, complaining, bragging, understanding, and helping each other out.

To Sarah, a mentor who placed me on the right path. To Nicky and Kelly, who mentored me after Honours. To Brianna, who taught me my excellent experimental and aseptic techniques, and honed my attention to detail. To Kirstie, Hadi, Prusoth, Taryan, Hafsa, Caroline, and many others for being the loveliest friends anyone could ever have. To Helen and Liz for keeping the lab under control and allowing for the smooth running of experiments. A big thank
you to everyone within the School of Medicine, particularly the McGee Lab, and to those from the School of Exercise and Nutrition. I’m forever grateful for everyone’s kindness and willingness to help whenever required. I wish to thank Len for helping set up the muscle function equipment used in this thesis. Furthermore, I’m extremely grateful that my PhD was supported by an Australian Postgraduate Scholarship, funded by the Australian Government.

I also wish to thank my family and friends who have waited so impatiently over the past few years. I can now happily say I can unshackle myself from my computer desk, emerge from the lab and come back to the land of the living! A special shout-out to mum, dad, my cousin Sharna, and my grandma for being so patient during this time. Also to my greats Aunty Ann (Dec.) and Uncle Roy, who always had faith and believed I could do anything.

To my beloved James. Thank you for putting up with all the emotions that a PhD brings. Thank you for being supportive of a wife who brought a PhD home, who often neglected you to write this behemoth of a thesis. Thank you for taking care of me and telling me everything will be ok. Thank you for the many cups of tea, chocolate and cuddles, and for drying the many thesis tears. This glory is for you as much as it is for me! ♥ To our much-loved furkids, 3 angora and minilop terrors and 4 little piggles, thank you for your unwavering love, fluffiness and cuteness that evaporated all stress and frustration of the PhD and thesis.

Finally, my sincerest gratitude to my examiners and my dear readers. I greatly appreciate you taking the time to read my thesis. Thank you.
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Abstract

Duchenne muscular dystrophy (DMD) is a fatal disease arising from a mutation in the dystrophin gene, resulting in the loss of the functional protein. This causes the muscle fibres to become extremely prone to contraction induced muscle damage, resulting in excessive inflammation and ineffective regeneration. These processes ultimately lead to the loss of muscle fibres and expansion of the extracellular matrix (ECM). Fibrosis is not just a consequence of the disease, but also actively contributes to muscle degeneration.

In healthy muscles after injury, a transitional matrix is deposited into the area which regulates inflammatory and myogenic cells. For repair to be effective the synthesis and remodelling of this transitional matrix is crucial. An important transitional matrix protein is the chondroitin sulphate proteoglycan, versican, which is known to modulate myogenesis, inflammation and fibrosis. Versican is highly upregulated in dystrophic muscles and it is hypothesised that this persistent upregulation of versican contributes to DMD disease pathology.

In DMD, muscle function is improved through glucocorticoid treatment. In other diseases with a heightened pro-inflammatory and fibrotic state, the synthesis and remodelling of the transitional matrix can be regulated by glucocorticoids. However, the role of versican in DMD disease progression is unknown. Therefore, this thesis explores:

2. The physiological relevance of versican in dystrophy using the dystrophic mdx mouse model with genetically reduced versican and subsequent characterisation of contractile, histological and metabolic properties of the diaphragm and hindlimb muscles.

Glucocorticoids have pleiotropic effects on dystrophic muscles; including dampening inflammation, improving regeneration and reducing fibrosis, leading to prolonged ambulation in DMD patients. However, the underlying cellular
mechanisms are not fully characterised. Results from Chapter 3 confirmed an excess of versican in \textit{mdx} mice, especially in the severely affected diaphragm muscles. \textit{In-vitro} dexamethasone treated C2C12 cells had decreased versican gene and protein expression with enhanced myoblast fusion and myotube formation, even in the presence of excess versican.

In Chapter 4, to investigate whether the genetic reduction of versican improves \textit{mdx} diaphragm function, female \textit{mdx} mice were bred with male heterozygous hdf mice, which are haploinsufficient for the versican allele, to generate male F1 \textit{mdx} and \textit{mdx}-hdf pups. The genetic reduction of versican was found to have beneficial effects upon the pathology of 21 week \textit{mdx}-hdf mice, as it increased spontaneous physical activity and also the strength, endurance and force recovery of isolated diaphragm muscle strips ex vivo.

As DMD is a paediatric disease which renders fast twitch muscle fibres especially susceptible to degeneration, Chapter 5 describes the effects of versican reduction properties of the fast twitch EDL and the slow twitch soleus hindlimb muscles in male \textit{mdx} and \textit{mdx}-hdf mice during periods of growth and maturity. Versican synthesis and remodelling is highly upregulated in dystrophic muscles during postnatal growth. In growing, 6 week old mice, the genetic reduction of versican improved the strength and endurance of soleus muscles, and the endurance of EDL muscles. These functional benefits were greatly reduced in hindlimb muscles from adult 26 week old mice. Chapter 5 highlights the importance of considering fibre type and growth when targeting versican in dystrophic muscles.

Results obtained in this thesis further solidify versican as a potential therapeutic target, and is overall beneficial to understanding the processes and consequences of a dysregulated matrix in DMD pathology. Together, the highly novel findings within this thesis make a significant contribution to the fields of fibrosis and DMD.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>½ RT</td>
<td>½ relaxation time</td>
</tr>
<tr>
<td>αSMa</td>
<td>Smooth muscle α-actin</td>
</tr>
<tr>
<td>Acta2</td>
<td>Smooth muscle α-actin2</td>
</tr>
<tr>
<td>ADAM</td>
<td>A Disintegrin And Metalloproteinase</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A Disintegrin And Metalloproteinase with ThromboSpondin motifs</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>CKm</td>
<td>Creatine Kinase muscle</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross sectional area</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated molecular patterns</td>
</tr>
<tr>
<td>DAPC</td>
<td>Dystropin-associated protein complex</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DKO</td>
<td>Double knockout</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>Hdf</td>
<td>Heart defect</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecules</td>
</tr>
<tr>
<td>Lo</td>
<td>Optimum length</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>LVIDs</td>
<td>Left ventricular internal diameter (systole)</td>
</tr>
<tr>
<td>Max dx/dt</td>
<td>Maximal rate of force production</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Macrophage chemoattractant protein-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NTC</td>
<td>Non template control</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet-endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>REE</td>
<td>Resting energy expenditure</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange rate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>sPo</td>
<td>Specific force</td>
</tr>
<tr>
<td>sPt</td>
<td>Specific</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>TA</td>
<td><em>Tibialis anterior</em></td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Tumor growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TPT</td>
<td>Time to peak tension</td>
</tr>
<tr>
<td>Vcan</td>
<td>Versican</td>
</tr>
<tr>
<td>VO₂</td>
<td>Volume oxygen</td>
</tr>
<tr>
<td>VCO₂</td>
<td>Volume carbon dioxide</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Chapter 1: A review of the literature

1.1 Overview of skeletal muscle and surrounding matrices

1.1.1 Skeletal muscle fibre type and organisation

Striated skeletal muscle is a highly complex structure comprised of numerous bundles of individual muscle fibres, satellite cells, tendons, blood vessels, capillaries and nerves, held together by a specialised extracellular matrix (ECM) (1). There are three layers of skeletal muscle ECM: the epimysium which surrounds the entire muscle, the perimysium which surrounds muscle fibre bundles, and the endomysium which surrounds the individual muscle fibres.

Skeletal muscle is structurally and functionally diverse, but broadly all muscle fibres can be classified into type I and type II muscle fibres (2). Type I, slow twitch fibres are fatigue resistant due to their high concentration of mitochondria and capacity for oxidative metabolism (3, 4); however, their force output is relatively low (5). Type II, fast twitch fibres are required for strength and sprinting, and have a larger cross-sectional area than type I fibres (6). The type II fibres can be further classified into type IIa, IIx and IIb. Type IIa have the highest oxidative capacity and best endurance (7). Whilst, type IIb have the highest force producing capacity, they are very susceptible to fatigue as they rely on anaerobic glycolysis (5). Finally, type IIx fibres are an intermediate form of muscle fibre compared to type IIa and IIb fibres (5, 7). In human skeletal muscles, fast twitch fibres are a combination of type IIa and IIx (8, 9), whereas in skeletal muscle of mice, these fibres are primarily IIb (10). Type I and II muscle fibres not only have unique metabolic and contractile properties, but the composition of their endomysial matrix also differs (11, 12) (see section 1.2.4.2).

The focus of this literature review and thesis will be upon the endomysium. The endomysium lies just above the sarcolemma, enveloping the individual muscle fibre and adjoining satellite cells (13). It has an important role in protecting the integrity of the muscle fibre during contractile activity, and following muscle damage, the remodelling of the endomysium is critical for a carefully regulated
and effective inflammatory and regenerative response (13). Given that inflammation and regenerative processes contribute to pathology of dystrophy, the endomysial matrix is a particularly important focus for research on dystrophic pathology.

1.2 The endomysial matrix of skeletal muscle

The endomysial matrix of skeletal muscle is a highly complex environment, whose structure and function is influenced by age, muscle type, cellular processes, and disease. It is therefore important to first understand each phase of the matrix before reviewing those implicated during disease.

1.2.1 The developmental matrix during morphogenesis

The developmental matrix is crucial for cellular processes and growth to occur. Embryonic cells are able to produce a specialised developmental matrix during morphogenesis (14). The process of morphogenesis will not be described in great detail here as it is beyond the scope of this thesis, however it has previously been extensively reviewed (14, 15). Even though matrix components have been previously mapped and characterised, the exact role of the muscle matrix during morphogenesis is still not fully understood (15). It is important, however, to acknowledge that a developmental matrix is one that is very different to a mature matrix. This is because there are many processes that are occurring during this highly complex transitional period of growth.

Briefly, this developmental matrix is a loose hydrated matrix that is comprised of proteins such as laminins, fibronectin, tenasin (15), as well as hyaluronan (16) and versican (17). These proteins are extensively produced and remodelled throughout embryonic muscle development. This may be because the developmental matrix acts as a scaffold, both nurturing and directing cells such that correct proliferation, migration and differentiation can occur. For instance, versican can act as a barrier for neural crest cells, preventing them from migrating to the wrong area during development (18, 19).
Relevant to this thesis, versican, shown to be present within the skeletal muscle of the developing murine embryo (17), is also known to increase the proliferation of young turkey satellite cells (20), suggesting it plays a crucial role in muscle development. It is important to note that some proteins, such as versican, are largely upregulated during development, yet are scarce in the mature matrix (17).

### 1.2.2 Structure and function of the mature skeletal muscle endomysium

The mature endomysium (Figure 1.1) is a highly complex and specialised matrix essential to the integrity and function of muscle fibres and the adjacent satellite cells. Skeletal muscle contraction requires the transmission of lateral force from the contractile apparatus outwards (21), which relies on a fully functional and stabilised endomysial matrix, to prevent muscle fibre damage.

To appreciate how force is dissipated from the contractile apparatus through the sarcolemma and into the muscle fibre ECM, it is important to understand the structure of the surrounding endomysium. The endomysium contains two specialised layers, the basement membrane (comprising the basal and reticular laminas) and the interstitial matrix (see Figure 1.1). The basement membrane is the immediate matrix that attaches the muscle fibre, and therefore the intracellular contractile apparatus, to the surrounding connective tissue. One way this link is formed is through an important intracellular protein called dystrophin which binds and stabilises the large, multiprotein transmembrane dystrophin-associated protein complex (DAPC) on the sarcolemma. Dystrophin is needed to stabilise the muscle membrane during contractile activity. Without dystrophin the entire DAPC dissociates and the structural integrity of the muscle fibre and sarcolemma is severely compromised (22). The DAPC complex is comprised of numerous proteins, including dystroglycans, sarcoglycans, sarcospan, dystrobrevins and syntrophin (23, 24). This complex plays a major role in the transmission of contractile forces within the endomysium, and is also important for cell signalling (25). Laminin is present throughout the basal lamina (26) and is able to bind to the α subunit of dystroglycan within the DAPC.
complex, thus providing the critical linkage of the contractile apparatus to the surrounding ECM. Laminin is also able to bind to major membrane receptors known as integrins, which are equally as important as the DAPC complex, not only for their ability to transmit forces, but also for their cell signalling properties (27). Integrins can also bind collagen IV, which binds to itself to form a flexible ECM network (28-30).

Laminin and collagen IV are also the major constituents of the basal lamina (31, 32). Laminin is able to bind and form clustered networks of itself, and through additional binding of proteins, such as nidogen (entactin) which binds collagen IV, can enable the formation of more complex matrix networks (33). Collagen IV networks are also found within the adjacent reticular lamina, along with networks of collagen VI (26). Both the basal and reticular laminas, which together comprise the basement membrane, contain these non-fibrillar collagen networks (26) which are flexible enough to allow for the movement of blood through capillaries and migration of inflammatory cells (30), yet are strong enough to provide stability to the adjacent sarcolemma during muscle contraction. The basement membrane also provides the vital link between the sarcolemma and the interstitial matrix (30) through collagen VI rich networks (34), which are able to co-localise with fibrillar collagens I and III (21) in the outer edge of the reticular lamina (32).

The collagen isoforms found in the interstitial matrix differ from those found in the basement membrane as they do not form networks, instead assembling themselves into strong fibrils (26), which run longitudinally above the sarcolemma. Therefore, collagen I and III provide additional strength and support to the surrounding matrix and muscle fibres. This is important as the role of the interstitial matrix is not only bind all muscle fibres, nerves and blood vessels to within close proximity to each other, providing structure and support, but also helps preserve muscle integrity through the dissipation of force during muscle contraction (35).
Figure 1.1 A simple overview of the endomysial matrix
Inside the muscle cell, dystrophin, an integral structural protein in muscle, can be seen binding to the DAPC complex, and associates with nNOS below the sarcolemma. The sarcolemma expresses receptors such as integrins and CD44, from which they bind and link the layers of the endomysium. These endomysial layers are highly specialised matrices that encompass an individual muscle fibre. The basal and reticular laminas together form the basement membrane. They are comprised of laminins, nidogen, hyaluronan and versican and fibrillar collagens IV and VI, which provide a flexible yet structured environment, and allows the dissipation of muscle produced force. Note the quiescent satellite cell nestled against the sarcolemma, yet underneath the basal lamina. Lying above the basement membrane is the interstitial matrix, which is comprised of non-fibrillar collagens I and III. These collagens can interact and bind with collagens and receptors in the basement membrane, and provides structure and support to the cell and surrounding environment. Furthermore, versican, a chondroitin sulphate proteoglycan, can be found throughout the endomysium and is highly upregulated during development or in response to injury. Versican, bound by hyaluronan or by itself, can be found interacting with CD44 at the cell membrane. Figure has been modified from (36) using (21, 26, 37).
1.2.3 Proteoglycans associated with developmental and mature matrices

Proteoglycans are another important class of proteins found within the interstitial matrix and the basal lamina. Key proteoglycans in skeletal muscle are biglycan, decorin, hyaluronan and versican. The expression of these proteins are specific and are differentially regulated during development, growth and in adult muscle, as well as during regeneration and fibrosis (38-41). Biglycan contains chondroitin sulphate (CS) glycosaminoglycans (GAGs) and is known to bind to α-dystroglycan on the DAPC complex within the basal lamina (42). Decorin contains CS and dermatan sulphate (DS) GAGs, and is found within the interstitial matrix where it binds collagen (43). Decorin and biglycan are both able to bind transforming growth factor beta (TGFβ) (44), a cytokine associated with fibrosis and the regulation of muscle precursor cell proliferation and differentiation (45, 46).

Another much larger and more complex proteoglycan found in the basal lamina and the interstitial matrix is versican (37) (see Figure 1.1). Versican mostly contains CS and some DS GAGs (47, 48). These allow for interactions with a number of ECM proteins, including hyaluronan (49) which forms a link to the cell membrane by binding to CD44 (50). Like decorin and biglycan, versican is able to bind various chemokines and cytokines through its GAG side chains (51), however TGFβ is bound through its attachment to the core protein (52). When the CS GAG chains become oversulphated, they are able to strongly bind L- and P-selectin (53) found on inflammatory cells which raises the possibility of versican being able to regulate the migration of leukocytes within the interstitial matrix (54, 55).

Regulation of proteoglycan expression within skeletal muscle is highly complex, with versican and biglycan being highly upregulated during embryogenesis and development, whilst being very scarce in the ECM of healthy adult muscles (17, 39). Unlike versican and biglycan, decorin is expressed highly towards the end of development before spreading throughout the ECM in adult muscle (56).
1.2.4 Regions of specialised endomysium

It is important to note that the endomysium of muscle fibres is a highly complex environment, and is not consistent across the entirety of a muscle. There are specific niches and regions of function, such as the neuromuscular junction, fibre types and even satellite cells. This further highlights the complexity and function of the endomysium.

1.2.4.1 Neuromuscular junction

For muscle contraction to occur, a motor neuron must be connected to muscle fibres via a neuromuscular junction (57). The neuromuscular junction has a specialised basal lamina that is continuous between the nerve terminal and the muscle endplate. It attaches the nerve to the muscle fibre (58), providing specialised support for action potentials to reach muscle fibres in a quick and uninhibited manner (57). The protein composition of basal lamina at the neuromuscular junction is similar to the mature muscle basal lamina, although different matrix proteins are expressed. A well characterised example of this is utrophin, a functional homologue of dystrophin (59). Utrophin is highly expressed in developing and regenerating muscle fibres (60), however in adult muscle, utrophin is found predominately at the neuromuscular junction (61) where it is stabilised by biglycan (62). Like dystrophin, utrophin also links the intracellular actin to the ECM via the DAPC (59, 60) and is therefore thought to be beneficial in stabilising the muscle sarcolemma (63).

1.2.4.2 Fibre type

There are also differences in the structure of the endomysial ECM between slow and fast twitch muscle. This has implications for force transmission and membrane stabilisation during muscle contractile activity. Slow type I muscle fibres express more dystrophin (64) and utrophin (65) than fast type II fibres, leading to a more stabilized membrane during muscle contractions. Findings from rat soleus muscles indicate that type I fibres also contain more collagen IV (11), which may lead to a more flexible basement membrane (30). This is important as type I fibres are recruited in all muscle contractions, so matrix
flexibility is crucial (66, 67). Type II fibres produce the strongest forces within muscle (66) and are therefore thought to be more susceptible to contraction induced damage (68). Type II fibres from rat \textit{rectus femoris} muscle have been found to contain more laminin than type I fibres (11). This would allow for stronger adhesion to the ECM by binding proteins such as nidogen which provides a link through the matrix via collagen IV networks, thus helping to dissipate contractile forces.

\textbf{1.2.4.3 Satellite cells}

Satellite cells reside above the muscle fibre sarcolemma in a specialised niche within the basal lamina (69, 70). The satellite cell niche is highly complex and is not yet fully understood. Briefly, the niche and its associated matrix proteins provide a protective and nurturing environment for the satellite cells, keeping them in a quiescent state (71, 72). Satellite cells express various receptors, such as integrin \( \alpha 7\beta 1 \) which binds laminin to provide a stable attachment to the basal lamina, and M-cadherin which anchors the satellite cells to the adjoining muscle fibre (69). In this manner, satellite cells are kept securely in the niche, in a position that nurtures and supports, until they become activated. Satellite cells undergo activation through exposure to various stimuli, ranging from contractile forces to cytokines and growth factors, produced from the adjacent muscle fibres and surrounding matrices upon injury.

\textbf{1.2.5 The transitional endomysial matrix during regeneration}

The expression of various developmental proteins is greatly decreased upon the formation of a mature matrix, and a similar expression pattern may be seen in response to an injury that causes tissue damage. This may happen as part of normal muscle adaptation, where the muscle undergoes cycles of degeneration and regeneration in order to increase and improve muscle strength, or as a consequence of musculoskeletal diseases, such as DMD. When muscle damage occurs, as part of the inflammatory and regenerative response, the mature matrix is remodelled and a specialised, transitional matrix, similar to that seen during development, is synthesised in its place (73). This transitional matrix is
comprised of fibronectin, tenascin C, hyaluronan (73), and other proteins, such as versican (74). This loose matrix directs the infiltration and behaviour of cells such as leukocytes, fibroblasts and muscle progenitor cells, by acting as a scaffold and allowing them to embed themselves throughout the injured space before beginning to secrete and lay down mature proteins (75-77). In this manner, the transitional matrix is able to regulate crucial processes for effective tissue repair.

1.2.6 Dysregulation of the endomysial matrix leads to fibrosis

The endomysium is a highly specialised matrix whose composition can vary depending on the subcellular location of the muscle fibre, on muscle fibre type and the stage of muscle development. Collectively, this highlights the importance of the endomysium for healthy muscle function as it regulates almost all aspects of cellular behaviour. Any cellular signalling event that involves receptor-mediated responses or membrane soluble molecules must negotiate the matrix. As such, an abnormal endomysial matrix will inevitably result in abnormal cellular responses.

The endomysium regulates cellular behaviour through proteins and their cleaved bioactive fragments, formed through extracellular protease activity. These include matrix metalloproteinase (MMP) (78), A Disintegrin And Metalloprotease (ADAMS) (79), and A Disintegrin And Metalloproteinase with ThromboSpondin motifs (ADAMTS) proteases (80). However, to maintain matrix integrity, this remodelling needs to be carefully regulated and thus protease inhibitors are also very important. These include the tissue inhibitor of metalloproteinases (TIMP) 1-4 which can inhibit MMP, ADAM and ADAMTS proteases (81, 82). The remodelling of the endomysial matrix is functionally significant as it can store and regulate the bioavailability of cytokines and growth factors via proteoglycans containing heparan (83), dermatan or chondroitin sulphate GAG chains (84). Endomysial remodelling, including the synthesis of a transitional matrix, is observed during a range of physiological and pathological processes. These include embryogenesis and development (14), muscle injury, and in neuromuscular diseases such as DMD (85).
In a healthy muscle, protein synthesis and degradation are carefully regulated to drive appropriate cell behaviour (86). However, if matrix synthesis and degradation becomes dysregulated, then fibrosis occurs (87). Fibrosis is the permanent expansion of the endomysial matrix, occurring due to the unregulated and increased expression of matrix proteases (88), collagens and proteoglycans located in the basement membrane and interstitial matrix (40, 89, 90). Fibrosis is driven by increased inflammation, fibroblast proliferation and myofibroblast differentiation, as well as from muscle fibres themselves (87, 91, 92). Fibrosis not only alters cell behaviour, but also negatively impacts the transmission of force during muscle contraction (93).

Healthy muscle fibres are arranged in neat rows which aids contractile function, however when fibrosis is present, muscle fibres become highly disorganised and muscle strength deteriorates (94, 95). Increased fibrosis and disorganisation of muscle architecture is most prominently seen in muscular dystrophies, of which DMD is the most prevalent.

**1.3 Muscle pathology and fibrosis in DMD**

DMD is the most common and severe form of muscular dystrophy, affecting approximately 1 in 5000 boys worldwide (96). It is a recessive, X-linked hereditary disease caused by a mutation in the dystrophin gene, leading to the loss of expression of the functional protein (97). Dystrophin stabilises the sarcolemma during contractile activity (see section 1.2.2) (98). Dystrophin binds to f-actin in the cytoplasm and to the transmembrane protein β-dystroglycan (99), which is part of the DAPC. In DMD, the loss of dystrophin renders dystrophic muscles highly susceptible to contraction induced injury through the lost connection to the ECM. Aside from a structural role, dystrophin is also involved in cell signalling. The best characterised example is the integration of dystrophin with neuronal nitric oxide synthase (nNOS), which anchors nNOS to the sarcolemma (100). Through the synthesis of nitric oxide (NO), nNOS is able to regulate the blood flow to the muscle during contractile activity (101) and thereby regulate oxygenation and metabolite transfer (102). When dystrophin is
lost and the DAPC disintegrates, nNOS is observed to be localised and act
differently in dystrophic muscles (103). This change in NOS may cause further
muscle damage, such as by ischemia (104), leading to oxidative stress and
further muscle damage (105, 106).

Aberrant ECM synthesis and fibrosis is seen early in DMD. Muscle biopsies from
a 2.5 week old newborn infant show a modest expansion of the endomysium
and minor variations in myofibre size, although the linear arrangement of
myofibres remains relatively organised (94). Thus, endomysial fibrosis precedes
any overt signs of muscle degeneration, and increases in volume over time (94,
107). As a result, by 3 years of age when most boys are diagnosed, significant
fibrosis and damage, in the form of disorganised necrotic and regenerating
fibres, is seen in dystrophic muscles (94). Over time, this damage and
degeneration progresses to severely compromise limb and trunk muscle function
(108, 109), resulting in muscle weakness, and the loss of ambulation (110).

Not all muscles are equally affected in DMD. For example, the muscles in the
fingers remain undamaged, enabling patients to use a joystick to control a
motorised wheelchair (111). Muscle degeneration also occurs in the diaphragm
and the heart, compromising cardiorespiratory function and leading to death by
approximately 30 years of age (109-111). Following contraction induced injury,
such as from exercise, muscle from a healthy person will undergo effective ECM
remodelling and successful repair, leading to adaptation and improved function
(112). In DMD, muscle does not repair efficiently, which leads to muscle loss and
weakness (Figure 1.2).

It remains unclear as to why some muscles are more affected than others,
although it is likely to be a combination of factors rather than a singular cause,
especially as muscles have different functions and uses. One such cause may be
due to the defective satellite cells within dystrophic muscles, leading to impaired
regeneration and weakened muscles (113, 114). Furthermore, as discussed in
section 1.2.4.2 of this thesis, muscle fibres are known to have differing matrices,
and this specificity may also extend to the ECM surrounding individual muscles.
As such, a dysregulated matrix may also contribute to muscle specific damage. This is likely due to the increase in endomysial fibrosis which is present from very early in life (94), which is greatly exacerbated by the chronic damage and inflammation occurring in dystrophic muscles (115).

Figure 1.2 Comparison of healthy and dystrophic muscle obtained from human biopsies. These muscle sections were stained with H&E in order to observe muscle structure. Upon comparison with A) healthy muscle, B) dystrophic muscle has a severe pathology. As the disease progresses, the characteristics of dystrophy, such as fibrosis, loss of fibres, changes in fibre size and infiltration of adipose tissue, all become more aggressive and apparent in the severely affected dystrophic muscle. Figure has been modified from (116).
It is important to note however, that fibrosis is present in other muscle diseases that contain a normal functional dystrophin protein. One such disease is the limb-girdle muscle dystrophy type 2Q variant, caused by a mutation in a protein called plectin, which has characteristics such as a disorganised muscle structure and matrix, and endomysial fibrosis (117). Another muscular disease is systemic sclerosis, where increased fibrosis is one of the main pathological features affecting the skin, internal organs and skeletal muscle (118). These myopathy studies thereby provide evidence of increased fibrosis while having an intact dystrophin protein. Indeed, fibrosis itself, and not just dystrophin deficiency, actively contributes to the muscle degeneration seen in DMD, thereby making fibrosis an important therapeutic target (119).

There is no cure for DMD and despite significant side-effects, glucocorticoids are currently the only treatment with clinical efficacy (120). Briefly, glucocorticoids are of particular interest because they lower inflammation (121) and inhibit the synthesis of ECM proteins (122, 123). A more detailed review of glucocorticoids can be found in section 1.9.2. Any potential therapy for DMD will need to take into account the fibrotic state of patient muscles. Therefore, to be effective it is imperative that novel treatment strategies commence at a young age due to the early onset of endomysial fibrosis (124). DMD is a paediatric disease, and as pathology and fibrosis progressively become worse over time, it would be in best interests to treat boys with DMD as early as possible to help keep the endomysial connective tissue at a more normal level as seen within healthy tissues.

### 1.3.1 Mouse models used to study DMD

The DMD<sup>mdx</sup> (<em/mdx>) mouse is the most common animal model used to study DMD. Like patients with DMD, these mice have a mutation in the dystrophin gene and do not express the functional protein. However, these mice have a less severe muscle pathology than humans, with the diaphragm exhibiting a similar level of pathology to muscles of DMD patients (125).
In hindlimb muscles of mdx mice, there is a severe bout of degeneration and inflammation at approximately 3 week of age, which reduces to a steady level by 8 weeks and persists throughout life (126), with a functional decline in strength (127, 128). Hindlimb muscles from adult mdx mice are hypertrophic, have ongoing regeneration and degeneration, and reduced tolerance to exercise, likely due to increased muscle damage and inflammation, and perhaps because of cardiac pathology in older mdx mice (126, 129).

Like human patients with DMD, differences in susceptibility to damage and degeneration is observed in hindlimb muscles of mdx mice (127). In young 3 week old mdx mice, the slow twitch soleus is the most necrotic muscle (128). With age, the soleus is again the most damaged hindlimb muscle, being more prone to fibre branching (91), increased necrosis, and increased deposition of fibrotic and adipose tissues (130). The soleus is a postural muscle that is highly recruited to help to keep the ankle stable during movements such as walking (131), so it may be that the damage occurs from the continuous movements that it endures through hindlimb locomotion (128, 132).

Hearts from mdx mice have also been found to have elevated fibrosis present in the ventricle walls, along with both hypertrophic and necrotic cardiomyocytes (133). Although elevated collagen can be observed in left ventricles of mdx mice from as early as 6 months of age (134), they don’t appear to develop a dilated cardiac myopathy until 21 months (135), which impairs cardiac function and ultimately leads to heart failure (133). This may be why male mdx mice display a 19 % reduced lifespan when compared to wild type mice (130).

A second mouse model of DMD called the DMD$^{dko}$ (DKO or double knockout) is also available (136). These mice lack utrophin and dystrophin, and display more of a true dystrophic pathology to human DMD as they have a very severe disease pathology and a greatly reduced lifespan (137, 138). The DMD$^{dko}$ display scoliosis, short stature, difficulty breathing and have a waddling gait (136), dying when they are just a few weeks old. These mice are less commonly used for dystrophic studies as they do not express utrophin, whereas DMD patients and
mdx mice express this protein (139). Due to difficulties with breeding, these mice were therefore not used in the studies contained within this thesis.

1.3.2 Loss of dystrophin and skeletal muscle damage

The loss of the dystrophin protein in DMD causes the sarcolemma to become fragile and vulnerable to contraction induced muscle damage. When a dystrophic muscle fibre is damaged by contractile activity, the integrity of the sarcolemma becomes compromised and leaky. This triggers a series of adverse events including an increase in intracellular calcium (Ca\(^{2+}\)) which floods into the cell through the damaged membrane, as well as an increase in reactive oxygen species (ROS) (140, 141). The excessive influx of Ca\(^{2+}\) results in the disruption of calcium homeostasis within the cell and leads to calcium-dependent proteolysis by activating calpains which degrade muscle protein into peptides (142). These peptides can be further degraded to form amino acids by the ubiquitin-proteasome pathway (143). Excess ROS can damage dystrophic muscle fibres by disrupting redox sensitive signalling pathways, and by altering proteins, lipid and DNA structure, thereby having detrimental effects on muscle fibre function (105, 106).

It is important to note that the whole muscle fibre does not undergo necrosis when injured, instead the damaged part can be sealed off and regenerated (144). However if muscle damage happens frequently, as it does in DMD, the fibres will be inefficiently repaired, leading to an accumulation of branched fibres. Branched fibres are highly inefficient, being unable to contract properly and, especially in DMD, have a further susceptibility to contractile induced injuries (145).

1.4 Inflammation in DMD

1.4.1 The initiation of inflammation

Although inflammation is integral for muscle repair (146), the actual initiation and subsequent processes of inflammation, especially in DMD, are still widely unknown (147-149). However, regardless if healthy or dystrophic, myofibres that become stressed, damaged or undergo cell death, release various stimuli,
ranging from danger-associated molecular patterns (DAMPs) (150, 151) to pro-inflammatory cytokines such as TNFα, IL-6 and IL-8 (152, 153) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (154), all of which attract neutrophils and monocytes from the circulation. Furthermore, these cytokines may activate additional cells within the surrounding matrices to intensify the chemotactic signals. For instance, TNFα can stimulate tissue resident macrophages to release neutrophil chemoattractant and monocyte chemoattractant protein 1 (MCP-1) (148) thereby contributing to the onset of inflammation.

The initiation of the inflammatory surge is further amplified by pro-inflammatory cytokines, such as TNFα, which upregulate inflammatory cell adhesion molecules, such as E- and P-selectin, upon the endothelial surface of blood vessels (147, 155). These selectins capture inflammatory cells which roll across the endothelial surface, promoting contact with pro-inflammatory cytokines, and stimulate the expression of integrins upon the inflammatory cell surface (156). This allows the inflammatory cells to bind to endothelial receptors, such as intercellular adhesion molecules (ICAMs), and allows for other adhesion molecules to interact, such as platelet-endothelial cell adhesion molecule (PECAM) (157). These receptors enable neutrophils and monocytes to pass through the endothelial blood vessel wall (157).

1.4.2 Inflammatory cell infiltration of damaged muscle

Once out of circulation, neutrophils and monocytes are attracted towards chemoattractant gradients, comprised of the cytokines and proteins secreted by myofibres and resident macrophages, directing their migration to the injury (158). Neutrophils and monocytes first need to traverse the endothelial matrix, a specialised basement membrane comprised of proteins such as laminins and collagen IV, which surrounds blood vessels (159). The migration across this matrix is controversial and the exact processes remain largely unknown (160, 161).
Although this matrix is seen to temporarily impede inflammatory cell migration (160, 162), the cells eventually locate specific regions containing low amounts of matrix proteins, which they then migrate through (159, 163). Additionally, observations that neutrophils may require proteases during this step, whereas monocytes do not and can freely migrate even without neutrophils present (159, 163), further highlights a need to investigate the mechanisms of inflammatory cell migration through endothelial basement membranes (160, 161). Once the neutrophils and monocytes leave the dense venular basement membrane, they traverse the interstitial matrix with ease, being able to squeeze and twist through tight spaces, and use tension to create temporary openings to move through without any damage to the interstitial matrix (161, 164).

### 1.4.3 Overview of inflammatory cells during muscle repair

#### 1.4.3.1 Neutrophils

Neutrophils are the first inflammatory cell to infiltrate the injured muscle, with an increase in numbers seen in as little as 1 to 6 h after muscle damage (165, 166) and are relatively short lived, approximately 2-5 days (165, 167). The main role of the neutrophil is to remove and phagocytose damaged myofibre debris (168), which is facilitated by the secretion of a range of proteases (see section 1.4.4.2). A key component of neutrophil phagocytosis is the respiratory burst, a process in which superoxide and hydrogen peroxide levels are increased to facilitate the degradation of phagocytosed material (169).

Neutrophils have been shown to exacerbate contraction induced injuries in muscle (170), and are shown to be greatly increased in *mdx* mice (171), showing a link between prolonged production and lifespan of neutrophils with the chronic inflammation seen in DMD. Indeed, neutrophils were associated with increased myeloperoxidase (MPO), which generates hypochlorous acid (HOCL), and exacerbates existing muscle damage, further contributing to degeneration in dystrophic *mdx* mice and golden retriever dogs, another animal model of DMD (172, 173). Furthermore, as part of the continuing inflammatory response, pro-inflammatory cytokines such as IL-8, released from damaged muscle fibres and
surrounding tissue, stimulates neutrophils to release their own cytokines, adding to the chemoattractant trail for incoming migratory monocytes (174).

1.4.3.2 Infiltrating and resident monocytes

After approximately 8 h, monocytes begin to infiltrate the site of injury (148). Monocytes were once considered to be a single cell type that differentiated into pro- and anti-inflammatory macrophages (175). This is now known to be more complicated. There are different monocyte subsets; in humans there are inflammatory classical and non-classical monocyte subsets and in mice there are the inflammatory Ly6C\(^{hi}\) (Ly6C\(^{+}\)) alternative/patrolling and Ly6C\(^{lo}\) (Ly6C\(^{-}\)) monocytes (176, 177).

The pro-inflammatory Ly6C\(^{hi}\) monocyte subset infiltrate damaged muscle in approximately 1.5 h, and reaches peak accumulation at around 1-3 days (178, 179). Once at the site of muscle injury, they are able to phagocytose cellular debris and degrade the contents via their own respiratory burst, albeit weaker than that observed in neutrophils (180). The inflammatory Ly6C\(^{hi}\) subset is increased in \textit{mdx} mice (181), which may further contribute to the excessive inflammatory response of dystrophic muscles. Though Ly6C\(^{lo}\) monocytes are observed to patrol the blood vessels, there is some evidence that they may be precursors to resident macrophages in the matrix (182, 183). Furthermore, this subset is suggested to play a role in the inflammatory process, potentially originating from the inflammatory Ly6C\(^{hi}\) subset, as their number steadily increased and peaked at day 7 post injury, and they develop an anti-inflammatory role well suited for muscle repair, while Ly6C\(^{hi}\) numbers dropped (178, 179).

Another added complexity, and one that is not very well studied or understood, is the resulting fate of these monocyte subsets. One theory is that the monocytes are able to differentiate depending on cell type, as the pro-inflammatory Ly6C\(^{hi}\) monocytes are similar to the pro-inflammatory M1 macrophages, while the anti-inflammatory and reparative Ly6C\(^{lo}\) monocytes are relatable to the anti-inflammatory M2 subset (178, 182, 184). Another theory is
that the Ly6C\textsuperscript{hi} subset gives rise to the resident macrophages (177, 185), while others suggest the Ly6C\textsuperscript{low} are the macrophage progenitors (178, 179).

Ultimately, through whichever way the process does occur, it is widely accepted that monocytes differentiate into another inflammatory cell type called a macrophage (175, 186)

**1.4.3.3 Pro-inflammatory M1 macrophages**

After a period of approximately 24-48 h (179), a cytokine influx of IFN-\(\gamma\), TNF\(\alpha\) and GM-CSF stimulates the inflammatory monocytes to differentiate into pro-inflammatory M1 macrophages (87, 187-189). M1 macrophages remove myofibre debris via phagocytosis (190), and are able to generate high amounts of ROS, nitric oxide and pro-inflammatory cytokines (191-193). M1 macrophages can exacerbate existing muscle damage by producing pro-inflammatory cytokines such as IL-1, IL-6 and TNF (194), which intensifies the response of macrophages as well as recruit additional inflammatory cells. Indeed in DMD, M1 macrophages remain persistently elevated from a young age, and persist through the entire lifespan of the patient (195, 196). This may be due to the influx and overlapping of cytokines during the inflammatory response. One cytokine example is IFN-\(\gamma\), which is elevated in DMD and strongly promotes M1 macrophage inflammation, inhibiting muscle repair in \textit{mdx} mice (197). M1 macrophages are actually an important part of the inflammatory process, by phagocytosing cellular debris, they prepare the area for muscle regeneration (198).

**1.4.3.4 Anti-inflammatory M2 macrophages**

At approximately 3 days post injury, and when most neutrophils have been phagocytosed, anti-inflammatory M2 macrophages begin to appear, becoming the predominant inflammatory cell at approximately day 5 (199, 200). There is debate in the literature regarding M2 macrophage origin and propagation. However, current theories suggest that the source of M2 macrophages is through the differentiation of M1 macrophages in response to specific cytokine signals (201).
A major limitation associated with most research is that the focus is on M2 macrophages as a whole, but this is actually very inappropriate. The M2 macrophage population can be further broken down into 3 subsets - M2a, b or c, with each subset having a very different and distinct function.

M2a macrophages, also known as alternately active macrophages, arise through an increase in IL-4 or IL-13. The appearance of M2a macrophages is a sign that the inflammation is about to switch to a regenerative process, and are associated with areas of tissue repair (175). The M2a macrophage population is important to repair, because an increase in IL-4, which stimulates M2a macrophage activation, also contributes to the deactivation of pro-inflammatory M1 macrophages (202). M2a macrophages are also able to suppress the ROS produced by M1 macrophages and reduce further muscle membrane damage, especially relevant in DMD where increased ROS levels are present (201, 202). Furthermore, M2a macrophages can secrete TGFβ and fibronectin to promote tissue repair and wound healing (203, 204). However this process can be dysregulated. M2a macrophages are known to be pro-fibrogenic (205), and have been associated with fibrosis in aged diaphragm muscle from mdx mice (206) and patients with DMD (207, 208).

The immunoregulatory M2b macrophages are activated through increases in MCP-1 (209) or apoptotic cells (210). The M2b macrophages secrete the anti-inflammatory cytokine IL-10, but can also secrete the pro-inflammatory cytokines IL-1β, IL-6 and TNFα (210, 211). In dystrophic mdx mice, IL-10 produced by M2b macrophages suppresses the pro-inflammatory M1 macrophage population and activates the M2c phenotype, thereby dampening inflammatory processes and shifting the micro-environment towards muscle repair (212).

The M2c macrophages are strongly activated by IL-10 cytokine secretion by M2b macrophages, through interactions with TGFβ1 or glucocorticoids (189, 199). They are considered to be anti-inflammatory, and can be further broken down into 3 groups: those activated by IL-10; those activated by both glucocorticoids
and IL-10; and those activated by glucocorticoids only (189, 213). Like the M2b subset, the M2c macrophages are also able to secrete IL-10, further suppressing pro-inflammatory processes and increasing phagocytosis of cellular debris and apoptotic cells (213, 214), and hence can potentially attract more M2c macrophages. This can be beneficial as the M2c macrophages can promote muscle repair in mdx mice by increasing myoblast proliferation (202). Additionally, M2c macrophages are able to influence matrix composition during repair through the synthesis and remodelling of ECM components, such as versican (192, 199). However, they also secrete high levels of TGFβ (215), and when large numbers of M2c macrophages are present, this may stimulate fibroblasts to produce copious amounts of collagen, and potentially result in fibrosis. Indeed, in other diseases, such as obesity, M2c macrophages, along with TGFβ and IL-10, are observed to be located in fibrotic areas (216).

Altogether, when carefully regulated, the inflammatory process is a necessary part of effective muscle repair. However, when in excess, as is seen in dystrophic muscles, inflammation can be detrimental to muscle health and function, and is observed to compromise regeneration and exacerbate damage and fibrosis. The role of inflammation in muscle repair is extremely intricate and complex, and much is still unknown, both in healthy and dystrophic muscles. Hence, an emerging and important area of research is the interactions of the matrix with inflammatory cells.

1.4.4 Inflammatory cell interactions with the ECM

The endomysial and interstitial matrices of skeletal muscle are complex structures (217). In response to muscle damage, the synthesis and remodelling of muscle ECM proteins by inflammatory cells is therefore key for an effective inflammatory and regenerative response.

1.4.4.1 Synthesis of ECM by inflammatory cells

As a part of remodelling the ECM, inflammatory cells synthesise a wide range of proteins depending on context and cellular type. Neutrophils are the first inflammatory cell to arrive after injury (166), with proteomic analysis being
suggestive that they do not appear to produce or contribute to matrix components (218), instead synthesizing and secreting a range of proteases which facilitates ECM degradation and phagocytosis of cellular debris after injury (see section 1.4.4.2). Neutrophils are well known to interact with the ECM and its components (219), and have been observed in areas with high versican expression (220).

Macrophages can also synthesize, secrete and interact with various ECM components. For instance, the alternatively activated M2a macrophage can secrete fibronectin (204), and anti-inflammatory M2c macrophages can produce versican (192). In moderation, these ECM proteins are crucial for a normal healthy matrix and tissue repair (81), however when they are overproduced, they may contribute to fibrosis in inflammatory diseases. Indeed, fibronectin (221) and versican (90) have both been observed, along with tenascin-c, in the same fibrotic areas of a lung with idiopathic pulmonary fibrosis (222). Surprisingly, these three proteins are known to be components of a transitional matrix, upregulated after injury and crucial for muscle repair (73, 74, 77).

These observations hint at an obvious interaction of versican with inflammatory cells. However, these inflammatory cells do not just secrete ECM proteins, they also synthesize a variety of specific proteases and inhibitors that allows for the degradation and removal of cellular debris after injury, as well as keeping protein synthesis in check.

1.4.4.2 Proteases and inhibitors secreted by inflammatory cells

By secreting enzymes and proteases, inflammatory cells such as neutrophils and macrophages remodel the cellular matrix environment following injury (175, 223). Neutrophils release proteases such as neutrophil elastase, and matrix metalloproteases (MMP-8 and MMP-9) which degrade matrix proteins such as collagens (224). Neutrophil elastase is increased within chronically inflamed dystrophic muscle, and has been suggested to potentially impair myogenic regenerative processes (171).
Activated macrophages secrete neutral proteases (eg: MMPs), acid hydrolases (eg: hyaluronidase), and lysozymes (225-227). Relevant to this thesis, macrophages are also able to secrete ADAMTS1, -4 and -5 (228), which are known to degrade versican. In addition to this, macrophages are also able to secrete various protease inhibitors, such as furin, which cleaves the pro-domain off ADAMTS5 in order for it to become proteolytically active (229, 230). Additionally, macrophages also secrete tissue inhibitors of metalloproteinases (TIMPs), such as TIMP-3, which inhibits the proteolytic activities of ADAMTS4 and -5 (175, 231, 232). Therefore, proteases and their inhibitors work in conjunction to keep the balance of ECM synthesis and degradation in check. Thus, matrix proteases, most importantly the MMPs and ADAMTS (233), are capable of remodelling matrix components, such as versican, when required.

The matrix remodelling by these proteases can release previously bound cytokines and growth factors (81). A well characterised example is cleavage of the proteoglycan decorin by MMPs, which leads to TGFβ release and increased availability (44, 234). This suggests a role for inflammation in the progression of fibrosis, particularly in DMD, where levels of TGFβ are high and known to drive fibrosis (235).

Proteases can also create bioactive fragments of matrix components that can elicit various effects upon the surrounding cells and signalling pathways (236). A well characterised example is the degradation of hyaluronan during inflammation. Hyaluronidase, potentially secreted by macrophages (227), and ROS are able to remodel the normal, high molecular weight hyaluronan into smaller fragments of low molecular weight hyaluronan (236-239). These low molecular weight hyaluronan fragments are considered to be pro-inflammatory. In activated macrophages, they stimulate increased expression of pro-inflammatory genes, further potentiating the inflammatory response (240, 241). The fragments also increase the expression of macrophage metalloelastase (MMP12) (242, 243), further promoting ECM remodelling by selectively cleaving basement membrane proteins such as fibronectin and CS proteins (244), as well as dampening the inflammatory process (245). Hence, the role of bioactive ECM
fragments during inflammation and regeneration is a complex, yet important and emerging field of research.

1.5 Dysregulated muscle repair in DMD

1.5.1 ECM remodelling and regenerative myogenesis following muscle injury

The intracellular signalling pathways regulating regenerative myogenesis have been intensely studied (246). Less understood is the regulation and remodelling of ECM during regenerative myogenesis. However, what is clear from DMD research is that effective regenerative myogenesis cannot occur if the ECM is dysregulated. Briefly, regenerative myogenesis depends on the activation, proliferation, migration and fusion of satellite cells with the injured myofibre.

Satellite cells are located between the basal lamina and the sarcolemma of muscle fibres (247) in a quiescent state (248). To maintain satellite cells in this low maintenance form, they are surrounded by a specialised niche, a unique ECM, which is able to interact and therefore regulate cell behaviour (71) (see section 1.2.4.3). Activation of satellite cells has been extensively characterised (249, 250) and as such, will not be discussed here in detail. Briefly, in response to muscle damage, the quiescent satellite cells are activated and while some of the progeny will go back to being quiescent satellite cells and self-renew the population (251), the others will go on to repair damaged muscle myofibres.

Satellite cell proliferation occurs between 24 h and up to a week after the injury has occurred (252, 253). Proliferating satellite cells, now referred to as myoblasts (254), migrate to the site of injury where they either fuse together to form a new multinucleated myofibre, or fuse to damaged fibres in order to repair them (255). As such, myoblast fusion is a series of complex processes involving numerous signalling pathways (246), however appropriate ECM remodelling during these processes is necessary. Versican, for example, can regulate the proliferation of myoblasts (20) yet requires cleavage by ADAMTS5 in order for myoblasts to fuse together during myogenesis (17). Overall, the processes involved in satellite cell activation through to myoblast differentiation allows for
the repair of damaged myofibres, and ultimately results in a fully functional muscle.

However, satellite cells can have other roles within muscle. It is well known that upon activation, satellite cells subsequently begin to remodel the surrounding niche and synthesize proteins (256), such as fibronectin, found to be essential for proliferation (257). Furthermore, satellite cells are able to regulate fibroblast derived production of collagens and fibronectin in satellite cells (258), potentially through the expression of protease inhibitors, as shown in healthy 6 week old adult mice (259). Interestingly, the same study showed that age matched, dystrophic *mdx* mice produced a combination of proteases, such as MMP-2 and ADAMTS1, along with protease inhibitors such as TIMP-2 and -3 (259), which can inactivate ADAMTS1 activity (260). Hence, satellite cells may be linked to fibrosis in DMD through the dysregulation of proteases, leading to increased ECM production.

Furthermore, age also can affect satellite cells. In youth, the muscle repair processes run smoothly, however with increased age come increased problems. For instance, it is well known that increased age correlates with reduced satellite cell numbers (261), as well as an increased expansion of the basement membrane (262). This may inhibit or potentially impair the muscle regeneration process.

As ECM remodelling still remains largely unstudied, it can be concluded from the literature that after damage, muscle repair occurs by first clearing damaged fibres, perhaps before activated satellite cells migrate and fuse to repair the damage. The basement membrane is subsequently reformed and the muscle has completed reparation. However, no studies to date have ever studied this process in detail, in youth or with age, or in inflammatory diseases such as DMD.

1.5.2 ECM remodelling and fibroblast function following muscle injury

As discussed above, ECM synthesis and remodelling are critical for effective inflammatory and regenerative myogenesis responses following muscle injury,
such that repair is successful and muscle function is restored. Fibroblasts play an important role in ECM synthesis and remodelling during muscle repair. Fibroblasts regulate the composition of the matrix, during the synthesis of transitional proteins, such as versican and hyaluronan (74, 263), as well as mature matrix proteins, such as collagens I and III (264). These fibrillar collagens are structural proteins that provide support in the interstitial matrix. Fibroblasts are also able to produce various ECM proteases, including MMPs and ADAMTSs (265, 266), which degrade and remove matrix components, along with their inhibitors, such as TIMPs (267) and furin (268).

Upon muscle injury, fibroblasts are able to infiltrate the site of injury during the initial inflammatory wave, alongside neutrophils and monocytes (264). This occurs at around 1-8 h after injury (165, 264). Through stimulation by chemokines and growth factors, such as IL-1β and TGFβ1, the fibroblasts are able to differentiate into myofibroblasts (195, 269). This myofibroblast transition occurs in an environment that contains versican (265). Myofibroblasts are able to be discerned from fibroblasts by increased expression of protein markers like α-smooth muscle actin (αSMA) (270), and by exhibiting changes in phenotype and function. (271).

TGF-β is able to promote α-smooth muscle actin (αSMA) upregulation, which can associate with stress fibres by increasing their numbers and thickness (271). This is important as the stress fibres provides structure and contractile forces required for wound healing after injury (272-274).

Furthermore, myofibroblasts produce fibrillar collagens I and III found in the interstitial matrix, and collagen IV found within the basement membrane (195). As the inflammatory response dampens and switches to an anti-inflammatory response associated with cellular repair, the cellular signals that stimulate ECM production by myofibroblasts are decreased, eventually forcing them to undergo apoptosis via IL-1β signalling, leaving behind a healed wound and regulated matrix (275, 276).
1.5.3 Dysregulation of myogenesis and fibroblast function in DMD

In DMD, the persistent damage and inflammation causes a dysregulation of ECM remodelling (107), due to fibroblast and myofibroblast dysfunction. Fibroblasts obtained from dystrophic patients revealed abnormal changes in morphology (277-279) and function, skewing the fibroblast population towards a myofibroblast phenotype. TGFβ is upregulated in dystrophic muscles (280-282), where it potentiates myofibroblast formation (271) and inhibits their apoptosis (276). This may contribute to the increased production and accumulation of ECM in DMD. This expansion of the matrix is observed in very young infants with DMD, in the absence of inflammation, suggesting that fibrosis arises as a primary consequence of dystrophy (94). However, as inflammatory pathways are activated in infants (281), the possibility remains that inflammation may add to the dysregulated nature of fibrosis during the progression of DMD pathology.

Fibrosis exacerbates dystrophic pathology by increasing the ECM stiffness which interferes with normal cellular processes and compromises muscle function (93), hence the presence of fibrosis can predict functional decline (94, 283). Eventually the fibrotic tissue, along with infiltrating adipose tissue, results in the severe muscle weakness seen in DMD patients (107).

In addition to a dysregulated and expanded ECM, contributing to the poor capacity of dystrophic muscles is the fact that muscle damage is chronic. This leads to asynchronous regeneration, where multiple bouts of damage, inflammation and regenerative processes, at differing stages, happen within close proximity (284, 285). This involves the possibility of overlapping cell signalling, with the expectation that this environment would include a dysregulated ECM. A section of muscle stained with toluidine blue O showed that rat muscle underwent asynchronous regeneration by showing two distinct stages of differentiation that were occurring within the same area (284). Furthermore, like asynchronous regeneration, there are also multiple areas of inflammation and degeneration that overlap in dystrophic muscles (286). Ultimately, muscle will not be repaired efficiently if the delicate balance between inflammation and repair is disrupted. In DMD, the inadequate repair of
muscle contributes to the overall disease pathology, resulting in muscle loss and fibrosis (107). Together with chronic inflammation and fibrosis, this ultimately means that the repair process for a damaged muscle is not as straightforward as it seems.

The dystrophin protein is also integral to satellite cells (114). The loss of dystrophin in the activated satellite cells within dystrophic muscle was associated with abnormal defects that resulted in the generation of dysfunctional satellite cells, further adding to the impaired muscle repair seen in DMD (114).

Further adding to this, as the dystrophic muscle undergoes continuous repair and degradation, the regenerative processes will eventually be unable to compensate for the damage (287). It is speculated that the supply of satellite cells required for muscle repair is exhausted or somehow loses the capacity to repair the damaged fibre (87), perhaps due to the loss of dystrophin (114). However, a study suggested that the depletion of satellite cells was not the primary cause for failed regeneration, casting doubt over the validity of this theory (287). It was suggested that failed regeneration in the advanced stage of DMD was possibly a result of the inhibition of myoblast differentiation caused by an interaction between TGFβ and myogenic regulatory factors (46, 287), however this is still largely unknown. Therefore, as myogenesis requires remodelling (17), it can therefore be hypothesised that the dysregulation of ECM remodelling contributes to the exacerbation of inflammation and muscle degeneration in DMD.

It can be easy to speculate that if one area of muscle is resolving inflammation, and a neighbouring area is starting to have an invasion of inflammatory cells, any healthy regenerating fibres in between these areas will be affected by the ‘spill over’ of cytokines and chemokines from the surrounding degenerating fibres, inflammatory cells and the ECM. This chaotic environment worsens inflammation, leads to inefficient repair and ultimately results in the permanent deposition of fibrotic tissue. Consequently, the damage obtained throughout the
course of disease progression is not just due to asynchronous regeneration, but also includes the inhibition or impairment of satellite cells, chronic inflammation, abnormal cellular signalling, and fibrosis with its highly expressed protein components, such as versican. As versican is also able to influence inflammation (288), it may prove to be a target, along with its cleaving ADAMTS proteases, in future research involving dystrophic muscle.

1.6 Versican and its remodelling by ADAMTS versicanases

1.6.1 The structure and regulation of versican

Versican is a large chondroitin sulphate proteoglycan (289) and an important transitional extracellular matrix protein (74), which is found in abundance throughout the body. It is located within the endomysial and interstitial matrices and is produced by a wide range of cells including myoblasts, fibroblasts and inflammatory cells (17, 265, 290). As such, versican is a crucial protein that plays a role in many different processes including development, cell maintenance and disease.

1.6.1.1 Versican splice variants

The different forms of versican arise through the alternate splicing of exons 7 and 8, which encode the GAGα and GAGβ regions (289, 291). There are five major splice variants of versican, designated V0 through V4, with the potential of a sixth (V5) variant (48, 289). These variants of versican differ by their structure and components. Versican has four known protein domains consisting of a N-terminal globular (G1) domain, two chondroitin sulphate chain binding regions (GAGα and GAGβ), and a C-terminal globular (G3) domain (292) (Figure 1.3).

1.6.1.2 The G1 and G3 domains of versican

The domains of versican are important in regulating cellular and matrix properties. It is well known that versican can bind to hyaluronan through the G1 domain (293), which in turn is bound to the CD44 receptor upon the cell membrane (50), forming a stable link within the matrix. The G3 domain is equally important, being able to interact with macrophages by binding TLR-2 (294) or
the P-selectin ligand (295). The G3 domain can also bind proteins, such as tenascin-R, and fibulins 1 and 2, providing a link to other proteins with the matrix (288, 292), providing matrix stability.

**1.6.1.3 The G2 domain of versican and its CS GAG chains**

The G2 domain of versican is a region comprised of CS GAG side chains (Figure 1.3). Versican GAG chains are arranged in an array of positions that allows them to influence and mediate various matrix and cellular properties. For instance, versican can bind directly to CD44 via its GAG chains (296). Furthermore, GAG chains have a highly negative charge that attracts water molecules, causing the matrix to swell and become more rigid and stiff. This is important to the interstitial environment as the GAGs help cushion the forces created during contraction (297). As versican accumulates, so would the amount of GAG chains. Therefore it is important to note that any effects may not necessarily be from the core of the versican protein, but may instead be caused predominantly by the GAG chains themselves. The excess accumulation of proteoglycans that cause matrix swelling can negatively affect cells, as increased stiffness of the matrix may disrupt and prevent cellular processes, such as migration, adhesion and differentiation, from occurring (298, 299).

Additionally, during matrix remodelling, such as during cellular injury, the endomysium is able to sequester and regulate the availability of signalling molecules from proteoglycans, including versican. Versican GAG chains are able to bind and potentially act as reservoirs for a variety of cytokines, chemokines and growth factors (51, 84, 300, 301). Furthermore, the CS chains can also bind to cell receptors such as L- and P-selectins, allowing for leukocyte and macrophage adhesion, which may influence inflammatory processes within the endomysium (53, 295).
Figure 1.3 The five known splice variants of versican.

All splice variants of versican contain the G1 and G3 domains. The variants differ in appearance by the presence or absence of GAGα and/or GAGβ within the G2 domains, therefore the amount of CS GAG chains is also different for each variant. Also note the truncated GAGβ domain on the V4 variant.

Figure has been modified from (289), using (302, 303).
1.6.1.4 The V0/V1 versican variants

The V0 and V1 forms of versican are abundant in a range of tissues, particularly within skeletal muscle (17). The versican V0/V1 variants differ not only on GAG domain type, but also on size and number of CS chains (Figure 1.3). The V0 variant contains both GAGα and GAGβ domains and is therefore the largest versican type, whereas the V1 variant contains only the GAGβ domain (291, 302). This difference in GAG domains allows these two forms of versican to be distinguished, as not only do the GAG domains determine the total size of the versican isoform, but they also contain differing number of chondroitin sulphate chains. It has been determined that the GAG-α can bind up to 8 CS chains and GAGβ can bind up to 15 chains (302). Therefore V0 versican GAG domains can contain up to 23 CS chains, while the V1 form contains up to 15 CS chains (Figure 1.3). This difference in CS chain number may have a potential impact on binding properties between these two variants. Furthermore, the V0 and V1 forms also differ through the size and properties of their cleavage products after proteolytic processing by ADAMTS (see section 1.6.2.1).

1.6.1.5 The V2, V3 and V4 versican variants

Although this thesis focuses only upon the V0 and V1 variants of versican, it is important to recognise the other variants that are present in other tissues aside from muscle. The V2 and V3 variants have been observed in the mouse brain, with the V3 variant also in adult human brains (302, 303). The V2 variants is also observed in the central nervous system and neuronal tissues (289, 302, 304). Furthermore, the V3 form has also been observed in other human tissues such as the adult retina and stomach, and also in foetal livers (303). The newly described V4 variant has only been observed in human breast cancer lesions (305).

These variants of versican differ by their GAG domains. The V2 variant contains a GAG-α domain with up to 8 CS chains, while the V4 variant contains a truncated GAG-β domain with up to 5 predicted CS chains (302) (Figure 1.3). In contrast, the V3 variant contains no GAG regions, being comprised purely by the G1 and G3 domains only (303) (Figure 1.3). These differences between GAG domains, or
absence of, and being tissue specific, is therefore suggestive that these variants have very different roles to each other.

**1.6.1.6 The spatio-temporal regulation of versican**

Little is known about the regulation of versican in various tissues or in the stages of disease, in particular the spatio-temporal regulation and transcription of versican in development and pathology. What is currently known is widely based on a general understanding of versican regulation through various signalling and transcriptional pathways. However, as it has been extensively reviewed (306), it will not be described in great detail here. Briefly though, versican can be stimulated through signal transduction pathways such as the receptor tyrosine kinase pathway, the phosphatidylinositol 3-kinase (PI3K) – protein kinase B (PKB/Akt) pathway, the canonical wingless pathway, and the sonic hedgehog pathway (306). The regulation of versican is also dependent on a number of transcription factors, such as P53 or the formation of a complex between β-catenin and T-cell factor/lymphoid-enhancer factor which activates the versican promoter (306). Furthermore, the expression of versican can be suppressed through the downregulation or inhibition of the various signalling pathways, or may be supressed by DNA methylation (306).

The transcription factors regulating versican gene expression during development are not well described, as research has focused on the localisation of versican during development and its role in tissue morphogenesis (307, 308). In developing skeletal muscle, versican was suggested to act as a barrier to these tissues, ensuring that the muscle and nerve patterning occurred in the correct positions, with the expression of versican decreasing as the embryo developed (307), and being almost non-existent in an adult mouse (17). The specific patterning of versican during the embryonic period was determined to be regulated by developmental processes (307). In the study, it was suggested that V0/V1 versican was involved in the developmental skeletal muscle patterning, with V2 versican thought to be staining the developing nerves (307). However, it should be noted that staining was performed by using antibodies specific to GAGα and GAGβ regions (307), and was therefore not specific staining for
individual versican variants. Another study aimed to localise versican during the period of growth from 5 days postnatal to 42 day old adult rats (309). This study found that versican was differentially expressed at different times and regions of the brain (309). However, both of these studies however this study have a limitation in that only total versican was assessed, not the individual versican splice variants.

Little is also known about the transcription and regulation of versican in diseased states, however emerging roles in respiratory, cardiovascular disease (310-312) and cancer (313) are becoming apparent. However, the mediation of versican during developmental and regenerative myogenesis, and fibrotic and inflammatory processes in DMD will not be included here as they are discussed in Section 1.7 of this thesis.

Overall, these studies indicate that the splice variants of versican are located in variant-specific tissues with differential expression patterns highly dependent upon age. This is highly suggestive that each variant has a specific purpose in each tissue. However more detailed characterisations involving all versican variants during developmental, injury and diseased states are required. This would allow for better understanding of the differential expression patterns of versican and how it’s regulated during these states. Another important state to consider however, is the cleavage of versican by proteases.

1.6.2 The ADAMTS proteases and remodelling of versican

There are 19 members in the A Disintigrin And Metalloproteinase with ThromboSpondin motifs (ADAMTS) protease family (314, 315), however only the proteoglycanases ADAMTS1, -4, -5, -9, -15 and -20 can cleave versican (315-319). ADAMTS8 belongs to the same versicanase clade (320), and is therefore presumed to cleave versican, although this has not yet been proven (321). The known ADAMTS versicanases contain multiple domains, however they differ only in the number of thrombospondin motifs located at the C-terminal end of the protein (322). ADAMTS proteases are secreted in an inactive form as a protective mechanism and hence require proteolytic removal of the pro-domain to become
active proteases (323). ADAMTSS for example, can be cleaved by furin on the cell membrane, or by pace4 in the extracellular matrix (230, 324, 325), whereupon becoming active, can cleave proteins such as versican.

1.6.2.1 The V0/V1 versican fragments produced by ADAMTS versicanases

The ADAMTS versicanases can cleave V0 versican at the Glu\textsuperscript{1428}-Ala\textsuperscript{1429} bond, while the V1 form is cleaved at Glu\textsuperscript{441}-Ala\textsuperscript{442}, in order to form bioactive fragments (315, 316) (Figure 1.4). The resulting fragments span the G1 domain until just before the first CS chain on the GAG\textbeta domains (289). The V0 variant, known only as G1-DPEAAE\textsuperscript{1428}, still contains CS chains within the GAG\textalpha domain (Figure 1.4), and has been observed to differ during injury and repair (321), however this versican fragment still remains largely unstudied.

However, the V1 form, G1-DPEAAE\textsuperscript{441}, now known as versikine, contains no CS chains (315) (Figure 1.4). Versikine is the best characterised fragment in regards to function. However, the fate of the remaining C-terminus fragments containing the rest of GAG\textbeta and the G3 domain is still largely unknown. They may also have important signalling roles (326), or they may be endocytosed and lysed internally like aggrecan (327), however, any such mechanism remains to be determined.

It is important however, to note that ADAMTS versicanases produce versikine, they are not the only ECM protease that can cleave versican. Other proteases, such as the MMPs, specifically MMP-1, -2, -3, -7 and -9, are also able to cleave versican (328-330). However, the main versican protease remains the ADAMTS versicanases, particularly ADAMTSS. The ADAMTS proteases are complex, being shown to exhibit differing expression patterns in different biological contexts such as myogenesis (17) and fibrosis (321). Regardless, the main role of an ADAMTS versicanase is to process versican, forming the much smaller bioactive fragment, versikine.
Figure 1.4. The cleavage of versican by ADAMTS versicanases.
A) Versican V0 and V1 can be cleaved by ADAMTS versicanases just before the first CS chain on the GAGβ. The cleavage site is depicted as a dotted black line, and ADAMTS as a pair of scissors. B) The cleavage of versican by ADAMTS versicanases produces two fragments - the as yet unnamed V0 G1-DPEAAE$^{1428}$, and the V1 G1-DPEAAE$^{441}$, which is more commonly known as versikine. Figure 4 has been modified from (289), using (302, 321).
1.7 Versican in skeletal muscle development, repair and DMD pathology

1.7.1 Versican and its remodelling during development

During development, many meticulously timed processes occur that are integral to the developing muscle. Such processes include proliferation, cell adhesion, migration, differentiation and apoptosis of cells, along with extensive matrix remodelling during these processes (Figure 1.5). As such, many extracellular proteins, such as versican, play a large role in developmental processes. Indeed, a complete absence of versican is fatal in the heart defect mouse, with embryos dying in-utero due to malformed hearts (331). Therefore, versican and its proteolytic cleavage are especially important during development. This has been recently reviewed (289, 311) and will not be described here in great detail. Briefly, versican is important for the heart (331), as well as being present elsewhere within the mouse embryo (307), particularly in the embryonic hindlimb skeletal muscles (17).

Versican has been linked to myogenic processes, such as satellite cell proliferation, and is upregulated during proliferation of myoblast cultures obtained from turkey skeletal muscle (20). Furthermore, the cleavage of versican is required for the differentiation of C2C12 myoblasts in culture (17), and is thought to be involved in the even spacing of myotubes during development (20). Additionally, in another biological context, versican has also been found to direct migration, acting as a barrier to ensure the correct migration route of neural crest cells (18). Therefore, these processes may all help facilitate the expansion of skeletal muscle growth during the developmental phase.

Furthermore, the remodelling of V1 versican, through the proteolytic activity of ADAMTS versicanases, to produce versikine, is also instrumental during developmental processes. The remodelling processes occurring during development are widely exhibited around the body, and can include processes such as synovial joint formation (332), the formation of the cardiac outflow tract and endocardial cushions (308, 333), and provides a link between apoptosis and
digit formation (334). Furthermore, versican cleavage also aids myogenesis (17). Another study linked rounded, densely packed cells within the embryonic endocardium with co-localised versikine staining (333). A rounded cell is indicative of the mitotic phase of proliferation, the rounded shape providing strength and structure for the mitotic spindle (335), for which versikine has been shown to be a crucial component (336), proving that versikine is itself an important protein.

1.7.2 The role of versican during regenerative myogenesis

Versican is highly upregulated during skeletal muscle development, whilst being very scarce in the ECM of healthy adult muscles (17, 337). However, versican can be studied in cell cultures where stress can be implemented through mechanical strain or wounding, with results being suggestive that versican increases after injury (338, 339). Indeed, after injury a special transitional matrix, very similar to that seen in development, is synthesized (73) (see Figure 1.5 and section 1.2.5). This transitional matrix supports the reconstruction of a new mature matrix after injury. As versican is increased after injury, it may be a component of the transitional matrix in response to the normal regenerative processes (77, 338), where it may influence many cellular processes, such as those seen during development (see section 1.7.1) to facilitate the reparation of damaged muscle tissue.

1.7.3 Versican as a novel mediator of fibrosis and inflammation in DMD pathology

Versican is upregulated in inflammatory diseases, such as asthma (340) and chronic obstructive pulmonary disease (COPD) (341), where inflammation is chronic and fibroblast matrix remodelling is abnormal. The uncontrolled expansion of the extracellular matrix is also seen within DMD pathology. Indeed, in dystrophy, fibrotic endomysial expansion and inflammation is observed shortly after birth (94, 281), with versican and CS GAGs found overexpressed within endomysial areas of dystrophic skeletal muscle (90, 116), Figure 1.5.
Figure 1.5. Known and potential roles of versican in development, muscle regeneration and disease.

It is well known that versican directs the migration of cells during development, and is involved in the proliferation of satellite cells. Furthermore, the remodelling of versican by ADAMTS versicanases produces the fragment called versikine, which has previously been linked to apoptosis. This remodelling event is required for the fusion of myoblasts to form multinucleated myotubes, an essential step in muscle regeneration. Another aspect of regeneration is the increase of versican, as part of the transitional matrix, in response to injury. This specialised matrix both nurtures and directs cells important to the repair process, such as myoblasts and inflammatory cells. Versican is known to influence inflammatory cells, and is thought to be involved in the transition of monocytes to macrophages, and even in the transition of fibroblasts to myofibroblasts. Versikine is also associated with inflammation, being able to act as a DAMP and could potentially could instigate the inflammatory process. Versican may also be one of the factors causing the exacerbation of inflammation in DMD. Indeed, DMD is characterised by a vicious cycle of inflammation, degeneration and regeneration, ultimately resulting in the inefficient repair of muscles and the formation of fibrosis, which can also impair regeneration. As myofibroblasts are thought to be persistently activated in DMD, depositing copious amount of ECM, they could increase the amount of fibrosis in the endomysium. This is particularly interesting given that dystrophic muscles contain an upregulation of versican in the endomysium, and may therefore be implicated in fibrosis. Hence, versican has many known and potential roles in muscle processes and DMD pathology. Red arrows and text indicate the dysregulation in DMD. Figure has been created using (17, 18, 20, 90, 107, 116, 265, 285, 288, 290, 334, 338, 339, 342-344)
1.7.3.1 Potential interaction between TGFβ and versican in skeletal muscle fibrosis

In muscular dystrophy, fibrosis is driven through the increased production of TGFβ (345), of which TGFβ1 is the predominant form (119). TGFβ stimulates fibroblasts to synthesise and deposit matrix components. Based on in-vitro data from fibroblasts, this increase in matrix protein synthesis includes versican (346). Versican is upregulated in dystrophic muscles at the gene (347) and protein level (90). Given that the core protein of versican can bind TGFβ (52), it may therefore have an important role in fibrosis and dysregulated muscle repair.

Indeed, an excess of versican appears to stimulate the transition of fibroblasts into myofibroblasts (348). Myofibroblasts have been described as being persistently activated in DMD (107), expanding areas of fibrosis by secreting proteins such as collagens I, III and IV (271). As the myofibroblast deposition of collagen increases, the amount of versican is decreased, suggesting that the versican is being degraded and replaced by a collagen rich mature matrix (41). It currently remains unknown what effect versikine has on the myofibroblast, and hence should be investigated in future studies.

1.7.3.2 The potential for versican and versikine to mediate inflammatory processes in DMD

There is emerging support throughout the literature that versican can influence and mediate inflammatory responses (288). For instance, versican can bind specific chemokines that attract leukocytes (51), or may directly interact with adhesion molecules on leukocytes themselves (49), thereby promoting inflammatory responses. Versican can also bind macrophages, either though the G3 domain, or via the CS GAG chains found on the G2 domain (see sections 1.6.1.2 and 1.6.1.3). Furthermore, versican is also known to modulate T cell activation (343). Hence, versican can be linked to many inflammatory processes, and can be thought as being an important mediator for many inflammatory cells.

Furthermore, the cleavage of V1 versican to form versikine (see section 1.6.2.1) is also becoming an important factor for inflammation. Indeed, the signalling pathways regulated by versikine are attracting increasing research interest,
though little is known about versikine in skeletal muscle or within DMD. Versikine has recently been revealed as a damage-associated molecular pattern (DAMP) (342, 343), which are produced upon tissue injury and help instigate the inflammatory process. It can also act as a pro-inflammatory protein, being able to induce expression of IL-1β and IL-6 in myeloma-associated macrophages, while both versican and versikine differentially mediated T cell activation (342, 343). Perhaps versican and versikine might also exhibit similar effects on other inflammatory cells, such as neutrophils, monocytes, and the pro-inflammatory M1 and anti-inflammatory M2 macrophage subsets, however this remains to be seen, as do any studies involving dystrophy in this cellular model.

Hence the interactions of versican and versikine, although extremely complex in regards to dystrophic pathology, are both important to consider and understand, as they exert very different effects. As of yet, the relevance of versican within dystrophy is largely unstudied, and as increased gene expression of versican is observed in human dystrophic muscle (90), it only further highlights versican as being an important transitional and mediating protein. An overview of versican and its possible interactions and roles is summarised in Figure 1.5.

1.8 The function of ADAMTS versicanases in healthy and dystrophic muscle

1.8.1 Relevance of ADAMTS versicanases to muscle

As ADAMTS versicanases are part of an emerging field, little is known about their specific roles within skeletal muscle. However ADAMTS1, -4, -5, -9, -15 and 20 mRNA transcripts are expressed in developing wild type mouse hindlimb muscles at E12.5 – E15.5 (17, 334). Furthermore, ADAMTS5 is the most highly expressed versicanase in developing embryonic muscle, and has also been associated with dystrophic pathology, being highly expressed in serum from both DMD patients and dystrophic mdx mice (349). However a well characterised expression timeline is still needed for the ADAMTS versicanases, not only during development and growth to adulthood, but also in diseased states, such as DMD, to better understand the role these proteases have in growth and disease.
pathology. What is well known though, is the proteolytic interaction between ADAMTS versicanases and V1 versican results in the formation of the bioactive fragment versikine (see section 1.6.2.1). Both versican and versikine have been shown as being integral proteins within cellular processes and regulation of the matrix, as documented throughout this review.

It is important to remember that versican and versikine are also important developmental proteins. In some instances, the degradation of versican is required for proper development to occur (see section 1.7.1). For instance, ADAMTS cleavage of versican, forming versikine and providing a link to apoptosis, is integral for web regression and the formation of digits in the embryo (334). Furthermore, similar roles involving the cleavage of versican are the development and maturation of embryonic synovial joints (332).

In other contexts, ADAMTS5 and -15 play a prominent role in skeletal muscle myogenesis, being observed in culture to proteolytically clear a versican-rich matrix during myoblast fusion, enabling the differentiation of the cells into multinucleated myotubes (17). This is a crucial process, not only for normal muscle growth, but also for muscle repair after injury.

1.8.2 Relevance of ADAMTS to DMD pathology

The expression and function of ADAMTS versicanases have not yet been well characterised in dystrophic muscle. However, ADAMTS5 is upregulated in serum from both the dystrophic mdx mouse and DMD patients (349), hinting at an upregulation in DMD. However the expression of ADAMTS seems to be context dependent, as when 8 week mdx mice were treated with batimastat, an inhibitor of MMPs, the mRNA expression of Adamts1, -5 and -8 was observed to be decreased compared to wild type mice (350). It may be that at this age, because no fibrosis has yet occurred in the mdx mice, the dysregulation of ADAMTS versicanases has yet to begin.

Hints about ADAMTS versicanases can be obtained through other disease types. For instance, the inflammatory cytokines, IL-1β, TNFα, and IL-6 appear to play a role in the upregulation of ADAMTS versicanases (351). As these same cytokines
are also upregulated in dystrophy (344), perhaps these may also upregulate ADAMTS in this context. However in differing cell types that are not dystrophic, the expression of ADAMTS versicanases appears to be quite conflicting. For example, even though Adamts5 mRNA expression was found to be increased by IL-1\(\alpha\) treatments in cultured mouse chondrocytes, with no change observed in ADAMTS4 expression (352, 353), the opposite occurred in cultured mouse synovial fibroblasts treated with IL-1\(\alpha\), where no changes were observed in Adamts5 expression (354). Furthermore, in human synovial fibroblasts, no change in mRNA expression was seen with treatments of IL-1\(\beta\), TNF\(\alpha\), or TGF\(\beta\), whereas Adamts4 mRNA was seen to increase upon addition of TGF\(\beta\) (355). These studies appear to suggest that in different cells, tissue types, and perhaps contexts, the ADAMTS versicanases are stimulated differently, potentially due to their different structures and roles, by inflammatory mediators. This is further supported by studies in myogenesis, hepatic fibrosis and macrophages, which show that the versicanases are differentially upregulated (17, 321, 356).

It may be that the ADAMTS versicanases have these differences in regulation as a natural safeguard. If all were to have similar expression and activity, then the chance of a dysregulated matrix would be high. The ADAMTS are known to be compensatory, such as ADAMTS15 being able to cleave versican in the absence of ADAMTS5 (17). It is most likely however, that the differences in expression observed are due to the requirement of processing before they become catalytically active (see section 1.6.2), or through the regulation of ADAMTS inhibitors, such as the TIMPs.

After injury, TIMP mRNA levels rapidly rise, but decrease quickly 48 h after injury, whereas TIMP protein, initially present before injury, was decreased from 3-24 h, before expression increased from 48 h onwards (357). This suggests that not only are ADAMTS proteases carefully regulated, but that also TIMP itself could be regulated as another measure of careful control of matrix remodelling. Furthermore, in mdx mice, regardless of utrophin expression, all dystrophic hearts were observed to have dysregulated TIMP (358). This suggests that the regulation of ADAMTS versicanases may be dysregulated in DMD, which would
have significant impact on their substrates. Indeed, versican is seen to be greatly increased in DMD, particular in endomysial areas (90) indicating a potential dysregulation of proteases and their inhibitors in dystrophic fibrosis.

Other potential roles for ADAMTS in DMD are also coming to light. Fibrosis for instance, gains a major contribution of ECM components from fibroblast and myofibroblast dysregulation. Fibroblasts are known to express ADAMTS-5 (265) and after differentiating into myofibroblasts, they appear to continue expressing ADAMTS5, as versican expression appears to be degraded and replaced by collagen (41). Furthermore, cultured human macrophages stimulated with IFN-γ, TNF-α or IL-1β were observed to regulate Adamts1, -4, -5, -8 and -9 mRNA in differing ways (228, 356), suggesting that the ADAMTS versicanases may still be differentially expressed. These indicate that ADAMTS regulation is also important during inflammatory processes, and may implicate ADAMTS protease dysregulation in the inflammatory and degenerative processes in DMD.

1.9 Current strategies and future directions for DMD treatments: The importance of targeting the ECM

1.9.1 Current therapies used for DMD treatments

The loss of dystrophin in DMD leads to a multitude of persistent muscle degenerative, inflammatory and fibrotic processes (90). There is no cure for DMD and the most obvious, but also most complex treatment would be to correct the genetic defect. Current methods, extensively reviewed, include exon skipping, nonsense stop codon read-through, and viral mediated gene therapies (359, 360). However, the DMD gene is the largest known human gene (361). This restricts the use of some gene therapies, as the DMD gene is too large to insert in viral cassettes (362). Furthermore, there are currently 7,149 different types of DMD gene mutations that can cause DMD (359). As there are so many mutations, major drug companies would presumably only focus on the most common mutations. Therefore, any treatments mentioned above would be targeted towards specific mutations and would only benefit patients with the corresponding mutation. This is the case with a drug called eteplirsen (Exondys
51), recently approved for the treatment of DMD (363). This drug allows the skipping of exon 51 on the DMD gene, restricting it for use in approximately 15% of all DMD patients (363, 364).

Other therapies are compensatory, aiming to help relieve the pathology of dystrophy rather than attempting to fix the mutations. These include the upregulation of utrophin to counter the loss of dystrophin, increasing muscle mass by delivery of IGF-1, or inhibiting myostatin (360).

Despite much research effort, there is no cure for DMD. At the present, despite significant side-effects, glucocorticoids remain the only effective treatment in clinical use for all DMD patients. Glucocorticoids work by reducing inflammation and facilitating muscle repair (365). To date, the cell signalling pathways regulated by glucocorticoids such as the downstream consequences on inflammation, fibrosis and muscle function in DMD are not fully understood. A better understanding of these pathways will allow for a better understanding of the process involved in muscle repair, and therefore to identify potential new targets. For gene and cell therapies to work and to get the best out of compensatory strategies, the abnormal matrix therefore must be targeted.

1.9.2 Glucocorticoids in the treatment of DMD

Glucocorticoids influence many physiological processes within the body and have the ability to regulate glucose, protein and fat metabolism (307). They are anti-inflammatory and are able to suppress inflammatory processes (366). For this reason, synthetic glucocorticoids such as prednisone and prednisolone are commonly used for the treatment and alleviation of inflammatory disorders such as asthma and allergies (285), and also autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and type 1 diabetes (367). Glucocorticoids are predominantly used to treat inflammation. Because of this, they can be used to alleviate symptoms in other diseases that are not predominately mediated by the immune system, but are associated with significant inflammation due to tissue damage, such as DMD.
There are many review articles that compile the extensive clinical trials using glucocorticoids in DMD patients (368-370), and hence will not be reviewed in great detail here. Briefly, in patients with muscular dystrophy the benefits of glucocorticoids outweigh the side effects, such as weight gain and osteoporosis. Glucocorticoids improve respiratory function and muscle strength, reduce scoliosis, prolong ambulation (368-370) and potentially reduce fibrosis, all which indicate a delay in the progression of the disease.

Although clinical trials utilize different corticosteroids and a range in dosages, the recommended treatments are 0.75 mg/kg of prednisone or 0.90 mg/kg of deflazacort (368, 371). The treatments vary from consistent daily dosages to intermittent dosages such as alternate days or 10 days on/10 days off (368). To date there is no optimal standardised glucocorticoid treatment for patients with DMD. Indeed, a study in 2013 found clinicians had high variations between their methods of treating DMD patients, and proposed that a high level of uncertainty was present towards the benefits and side effects of glucocorticoids treatments (372). As such, there is still an urgent need for an optimal treatment regime. However this may soon be rectified as a clinical trial, Finding the Optimum Regimen for Duchenne Muscular Dystrophy (ClinicalTrials.gov Identifier: NCT01603407), is currently underway. This trial aims to identify which is the best treatment (daily or intermittent (10 days on/off) 0.75mg/kg of prednisone, or daily 0.90 mg/kg of deflazacort) for increasing muscle strength with the least side effects.

However, these human clinical trials are predominantly aimed at finding the best treatment regime, and so the mechanism as to how glucocorticoids work or affect the extracellular matrix are still relatively unclear. However, studies into the effects of glucocorticoids on arthritis found that they inhibit the destruction of the matrix surrounding the cartilage, by inhibiting protease (ADAMTS4 and ADAMTS5) expression and activity in the inflamed area (373). Furthermore, in a study investigating cartilage proteoglycan degradation, short term treatment with a glucocorticoid (dexamethasone) was found to inhibit matrix degradation by inhibiting the GAG cleavage of aggrecan (374). These studies show that
glucocorticoids are beneficial in other pro-inflammatory states other than DMD. In addition to this, glucocorticoid treatment during allergen exposure in asthma resulted in an increase of versican (375). Therefore the biological context is important, especially in inflammatory diseases.

Glucocorticoids are currently used to treat inflammation within DMD, but it currently remains unknown as to how glucocorticoids affect the ADAMTS-versican axis within muscle. Although glucocorticoids are beneficial for alleviating inflammation and can help prolong the lifespan of boys with DMD, their use comes at a cost. Long term glucocorticoid use has numerous side effects ranging from osteoporosis, skin atrophy, gastrointestinal ulcers, cataract formation, increased adipose tissue, excessive hair growth, psychosis, insomnia and increased appetite (365, 376). Therefore clinicians typically tend to prescribe low dose treatments, or treatments that are staggered, with dosage being dependent upon the condition being treated (365). The future directions for glucocorticoids however, lie in the creation of new synthetic glucocorticoids, such as VBP15 (377), that have all the benefits of reducing inflammation, but with minimal side effects.

### 1.9.3 Alternate therapeutic strategies to target fibrosis

Currently, other anti-fibrotic therapies are available. Dystrophic hearts can be treated with ACE inhibitors and angiotensin receptor blockers, currently used for treating cardiomyopathies (378). An ACE inhibitor called perindopril was able to maintain cardiac output by delaying left ventricle dysfunction in DMD patients (379). Therefore, these treatments may improve heart complications observed in DMD and help prolong the longevity of the patient.

A complication in DMD is the beginning of pathology and the accumulation of fibrosis, mediated in part by MMPs. MMP expression is dysregulated in DMD and may lead to the progression of fibrosis, therefore MMP modulators may be a useful treatment in this regard (36). Additionally, other anti-fibrotic treatments are available that inhibit signalling pathways before disease pathologies become complicated. An example is the NFκB pathway. NFκB has been elucidated as
being expressed by activated macrophages to stimulate inflammation and necrosis in muscle (380). In addition, NFκB can also impair muscle regeneration by inhibiting satellite cell activation.

Another target for inhibition is TGFβ, being upregulated in dystrophic muscles (280-282). TGFβ upregulates fibrosis by stimulating myofibroblasts (271) and is linked to inflammatory cells, such as M2c macrophages which also secrete TGFβ, and which further influences dystrophic pathology (215, 216). Suramin is a TGFβ inhibitor, and when mdx mice were treated with the drug, it was found to have a protective role where it lessened fibrosis and necrosis in muscle, and improved muscle repair (381).

From the treatment strategies reviewed in this section, it is clear that there is an urgent need for therapeutic strategies which target fibrosis, and are safe for long term use and have minimal side effects; in particular cardiac side effects. It would be best if any strategy to target fibrosis could be administered when satellite cells still have a good regenerative potential. This would be so the ECM can be regulated from the beginning and hence the fibrosis could be controlled - it should not expand, nor should it worsen any pathological outcomes. Regardless of the treatment, it is integral that it keeps ECM synthesis and remodelling balanced to minimise degeneration and excessive inflammation.

1.10 Overview and rationale of thesis

The ECM is a diverse, complex and dynamic scaffold that surrounds all cells and tissues (231). It is comprised of a variety of proteins including collagens, glycoproteins including laminin and fibronectin, and proteoglycans such as versican (382). Key to the structure and function of the ECM are the integrins and syndecans, transmembrane proteins that mediate matrix and cell adhesion. All matrix proteins can be remodelled by ECM proteinases, such as MMPs and ADAMTS proteoglycanases.

Beyond structural support, the ECM can regulate cell fate and behaviour, through the synthesis and remodelling of specific ECM proteins, integrin and
syndecan mediated signalling (14), and through the regulation of growth factor, cytokine and chemokine bioavailability (231, 383, 384). For optimal cell health and function, the integrity of the ECM must be maintained and its composition and remodelling need to be tightly regulated. If the ECM is damaged, or if synthesis and remodelling is dysregulated, then the health and function of the cell will be compromised.

The ECM is an important component of skeletal muscle, and aberrant ECM synthesis and remodelling is associated with muscle pathology. This can lead to degeneration, weakness and muscle loss, as is seen in DMD (141). DMD is also characterised by aberrant ECM synthesis and remodelling, resulting in fibrosis, which is both a cause and consequence of the disease (87, 94).

Anti-fibrotic treatments, such as those reviewed in section 1.9.3, are generally aimed at reducing fibrosis. Indeed, anti-fibrotic treatments, drugs such as Losartan and Suramin, or injections of decorin, administered to rats or mice with muscular injuries or diseases, were found to have reduced fibrosis and overall improved muscle regeneration and function (385, 386). There is evidence in the literature that perhaps a reduction in fibrosis improves regeneration (381, 386) and also lessons degeneration. For instance, mdx mice were treated with a TGFβ inhibitor called Suramin, which was found to reduce fibrosis and necrosis in dystrophic muscles (381). Therefore, it can be theorised that anti-fibrotic therapeutics not only lessen fibrosis, but may also be beneficial in reducing inflammation and degradation in healthy and diseased muscles alike.

Skeletal muscle comprises approximately 40 % of the human body and is essential for locomotion, respiration, posture and metabolism (387). Muscle is a highly adaptive tissue and when damaged has the capacity to undergo repair. The carefully regulated remodelling and synthesis of muscle ECM is important for successful repair following injury, because of its effects on inflammation, and satellite cell function (86).

Whilst there are numerous ECM proteins involved in effective muscle regeneration, it can be hypothesised that the carefully regulated synthesis and
remodelling of the ECM proteoglycan versican is essential. Versican is a chondroitin sulphate proteoglycan and is synthesised as part of a transitional matrix (49, 74, 388). In skeletal muscle, versican expression is upregulated during early myogenensis and its subsequent cleavage by ADAMTS versicanases facilitate the fusion of myoblasts into muscle fibres (17), a process critical for successful repair. A versican-rich matrix also has an emerging role in regulating inflammation (48). Versican is able to bind and store inflammatory cytokines and chemokines, thus attracting monocytes (389). These bind to versican, become activated and transition into macrophages. Both monocytes and macrophages can synthesize and secrete versican, and add to the already versican-rich area (290, 390, 391).

In healthy skeletal muscle versican expression is low. Whereas, in DMD, high levels of versican expression are observed, particularly in areas of damage, inflammation and fibrosis (90). To date, glucocorticoids are the only treatment with clinical efficacy in DMD, however the exact mechanisms as to how they work remain unclear. Furthermore, it remains unknown as to how aberrant versican synthesis and remodelling may affect the pathology and function of dystrophic muscle fibre types during growth.
Therefore, the aims of this thesis were to investigate the hypotheses that:

(1) Whether the expression of versican and versikine in dystrophic \textit{mdx} mice was similar to that observed in wild type mice.

(2) Whether dexamethasone decreases versican expression in cultured muscle cells and thus enhances myogenesis, potentially leading to the identification of a potential mechanism of action for glucocorticoids.

(3) Whether the genetic reduction of versican in dystrophic \textit{mdx} mice could improve the pathology and muscle function of the diaphragm, the muscle most representative of the human form, through an attenuation in inflammation and fibrosis, as well as improve regenerative myogenesis.

(4) Whether the genetic reduction of versican in dystrophic 6 and 26 week old \textit{mdx} mice could improve the pathology and muscle function of fast and slow twitch muscles of the hindlimb, through an attenuation in inflammation and fibrosis, as well as improved regenerative myogenesis.
Chapter 2: Methods

2.1 Cell maintenance and experiments

2.1.1 Overview of C2C12 myoblasts

C2C12 myoblasts are a well characterised model of mouse satellite cells capable of differentiating into multinucleated myotubes. C2C12 myoblasts were originally extracted and isolated by Helen Blau in 1981 (392). These cells produce an extracellular matrix which includes proteins such as collagen (393), fibronectin, hyaluronan (394) and versican (17). In DMD, an increase in collagen, fibronectin and versican is seen and is associated with increased fibrosis (119). Furthermore, versican is thought to affect myoblast migration, proliferation and differentiation (17, 395, 396). Therefore, C2C12 cells may be a useful tool for investigating ECM remodelling during myogenesis in-vitro.

2.1.2 C2C12 myoblast maintenance

In order to effectively culture C2C12 myoblasts, they need to be maintained, with optimum parameters, to ensure good growth or differentiation. Below describes the general requirements for growing or differentiating C2C12 myoblasts into multinucleated muscle fibres. C2C12 myoblasts were grown in a HERAcell I50 CO₂ incubator (ThermoFisher Scientific), at a temperature of 37 °C with 5 % CO₂ levels. C2C12 myoblasts were proliferated up to 70 % confluency in 75 cm² flasks with Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) with 10 % FBS (Invitrogen) (hereby referred to as growth media). When passaging, cells were loosened by the addition of 1 ml of trypsin (Gibco), then resuspended in growth media. Cells were passaged at a 1:30 dilution to maintain the cell line. To differentiate C2C12 myoblasts, the growth media was replaced with differentiation media (DMEM supplemented with 2 % horse serum). Differentiation media was refreshed daily for up to 4 d.

2.1.3 Cell Counting

For all in-vitro experiments, cells were trypsinised and resuspended in media. A 1 mL volume was transferred to an eppendorf tube, and were stained with trypan
blue (Amresco) before being manually counted using a haemocytometer and a handheld counter. This was done to get the number of cells per mL, which was needed to calculate the appropriate volume of resuspended cells required for seeding into a 6, 12 or 96 well plate for experiments.

2.1.4 C2C12 culture with dexamethasone treatments

Differentiating C2C12 myoblasts were treated with 25 and 100 nM dexamethasone, which is considered to be a low dose treatment and has previously been shown to increase myoblast fusion (397).

To assess the effect of dexamethasone on myoblast proliferation, C2C12 myoblasts were seeded in a 96 well plate (Corning) at a density of 10,000 cells/cm² per well, and were left to proliferate for 24 h or 48 h in growth media supplemented with 0 nM, 25 nM or 100 nM dexamethasone. Media and treatments were refreshed daily. After 24 h or 48 h, the WST-1 assay was performed (see section 2.1.6).

To assess the effect of dexamethasone on myoblast differentiation, C2C12 myoblasts were seeded in a 12 well plate (Corning, USA) at a density of 25,000 cells/cm² per well, and were left to proliferate for 48 h. Myoblasts were then treated with 0 nM, 25 nM or 100 nM dexamethasone in differentiation media for 72 h. The media and treatments were replaced daily. After 3 or 4 days, the cells were either fixed in 4 % PFA for the determination of the fusion index (see section 2.1.9) or were stored at -80 ºC for RNA extraction and subsequent gene expression analysis (see sections 2.2.1, 2.2.3 and 2.2.5).

2.1.5 Conditioned Media Preparation

2.1.5.1 Preparation of constructs

To create conditioned media which was to treat differentiating C2C12 myoblasts with excess versican or versikine, it is first necessary to insert the relevant constructs of required proteins into Escherichia coli (E.coli). As E.coli replicates quickly over a few hours duration, it becomes an easy and economical way to produce a large quantity of plasmid DNA, specific for each protein construct.
needed. Constructs listed in Table 2.1 were diluted using nuclease free water (Ambion) before being added to DH5α E.coli (Invitrogen) and placed upon ice for a period of 20 min. Afterwards, the E.coli and DNA constructs were heat-shocked for 50 s using a heat block at 42 °C and placed onto ice for 2 min. Using aseptic technique, Luria Broth (LB) (Amresco) was added to each tube, which was then placed into an orbital shaker set to a speed of 250 rpm, at 37 °C for 1 h. Aliquots of each sample and ampicillin were spread upon LB agar plates (Amresco) allowing for the easy selection of E.coli colonies. Any bacterial colonies that grow will have ampicillin resistance, gained through the successful incorporation of the DNA construct. The plates were allowed to dry slightly, then were inverted and placed within an incubator for 16 h at 37 °C. Afterwards, single colonies were collected, dropped into a 14 mL Falcon tube of LB broth. Tubes were then placed into an orbital shaker at a speed of 250 rpm, at 37 °C for 16 h to allow E. coli to replicate.

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Purpose of conditioned media</th>
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<tbody>
<tr>
<td>Empty vector (EV)</td>
<td>The empty vector is used as a control for every cell culture experiment utilising conditioned media.</td>
</tr>
<tr>
<td>V1 versican</td>
<td>Most published research does not distinguish between versican and versikine. C2C12 cell culture experiments are a useful tool to begin to dissect these differences. Therefore, C2C12 myoblasts were treated with conditioned medias and observed for changes in proliferation, migration and fusion of myoblasts.</td>
</tr>
<tr>
<td>G1-DPEAAE (Versikine)</td>
<td></td>
</tr>
</tbody>
</table>
2.1.5.2 Purification of the plasmid DNA using mini prep extractions

To purify the plasmid DNA for transfection into mammalian cells, overnight *E. coli* cultures (see section 2.1.5.1) were centrifuged to obtain bacterial cell pellets. The PureLink Quick Plasmid Miniprep Kit (Invitrogen) was then used to isolate plasmid DNA as per the manufacturer’s instructions, and allowed the removal of bacteria and waste. The purified plasmid DNA was eluted into 1.5 mL microtubes with TE buffer, and the concentration (ng/μl) was quantitated using the NanoVue spectrophotometer (GE Healthcare), with A260/280 rations of >8 being acceptable for use. Plasmid DNA was stored at -20 °C for future use.

2.1.5.3 Lipofectamine transfection of Hek293T cells for conditioned media synthesis

Lipofectamine disrupts the cell membrane allowed the purified plasmid DNA to be incorporated into Hek293T cells. Following transfection, Hek293T cells were cultured in serum free media to ensure that the resulting secreted protein would not be drowned out by other serum proteins, such as albumin. Conditioned media containing versican, active or inactive ADAMTS5 and ADAMTS15 has been successfully used as treatment for C2C12 myoblasts in a similar study investigating matrix clearance in myogenic regeneration (17). Hek293T cells were used to make the conditioned media, because they are readily transfected and secrete a high yield of versican, versikine, and both the active and inactive forms of ADAMTS5 and -15 with good efficiency (17).

Hek293T cells were grown in a 75 cm² flask (Corning) using DMEM (Gibco) plus 10 % FBS in a HERAcell i50 CO₂ incubator (ThermoFisher Scientific) at 37 °C with 5 % CO₂. Once 70 % confluent, they were transfected with 8 μg of plasmid DNA, Lipofectamine-2000 reagent (Invitrogen, USA) and serum free DMEM (Gibco). Cells were placed back in the incubator to grow for 5 h, upon which the flasks were aspirated and serum free DMEM was added. Cells were left to grow for an additional 48 h, after which all media was collected and centrifuged to remove cell debris. The conditioned media was immediately placed upon ice and stored at -80 °C.
2.1.6 WST-1 assay

The WST-1 assay was used to assess C2C12 myoblast numbers after being treated with dexamethasone or conditioned media containing empty vector (EV), versican or versikine. The WST-1 reagent contains tetrazolium salts that are cleaved on the cell surface through the trans-plasma membrane electron transport chain by electron carrier (1-methoxyPMS) that is driven by a reduction in NAD(P)H from mitochondria (398, 399). The cleavage of the tetrazolium salts forms a coloured formazan which is an indication of viable cells. This colour change can be measured by a plate reader, with a stronger colour having more viable cells. This means that the WST-1 assay is a relatively easy and simple way to generate an approximate cell number. However, a limitation to this is that if mitochondrial function is impaired or increased within individual cells, then the assay may not accurately reflect cell number.

Figure 2.1 Example of a WST-1 standard curve.

A known amount of cells are plated and the absorbance is read to enable the creation of a standard curve. A trendline with a high $R^2$ value, such as 0.983, is considered to be very good, and allows for the determination of the slope equation, which is used to calculate the cellular concentration of unknown experimental samples.
2.1.6.1 WST-1 Standard Curve

A standard curve of known cellular concentrations is produced for the WST-1 assay so that it can be used to calculate the amount of cells in the samples with unknown concentrations. An example of a WST-1 standard curve can be viewed in Figure 2.1. In order to produce a standard curve, C2C12 myoblasts were serially diluted using densities ranging from 0 to 100,000 cells/cm². Myoblasts were seeded into a 96 well plate (Corning) and left to adhere for 7 h in growth media. Following the supplier’s protocol, WST-1 reagent (Roche) was added to each well and incubated at 37 °C for a further 2 h. An Alpha Fusion plate reader was used to read the plate at 450 nm, the results enabling the creation of a standard curve with known concentrations.

2.1.6.2 WST-1 experiment

Following 24 h or 48 h of proliferation, WST-1 reagent (Roche) was added to each well and the plate was placed back into the incubator for an additional 2 h. An Alpha Fusion plate reader was used to read the plate at 450 nm. The results were used to calculate cell number based on the standard curve produced above (see section 2.1.6.1). Three biological experiments were performed, with 8 experimental replicates per treatment.

2.1.7 Wound migration assay

In-vivo, myoblasts are required to migrate to the site of injury to repair it. A wound migration assay is a simple method to assess the effects of the treatments on cell migration in-vitro. For experimental consistency, care was taken to make scratches of a consistent width.

2.1.7.1 Cell maintenance for the wound migration

C2C12 myoblasts were seeded at a density of 8,500 cells/cm² in 6 well plates (Corning), and left to proliferate for 3 days. The growth media (Gibco, USA) was refreshed once after 48 h. After 3 days, the wound migration assay was performed.
2.1.7.2 Wound migration experiment

After 72 h, a wound was made through the confluent monolayer, down the length of each well. PBS was added so wounds could be viewed under a microscope, allowing for a digital image of the 0 h time point to be captured. Each well was marked in 3 places along the wound, to be used as a guide when taking digital images at 6 h and 10.5 - 11 h post wounding. Once the initial image was captured, the PBS was aspirated and the relevant treatment was added to each well (N = 2-3 wells per treatment). For the three control wells, only growth media was added. For the treatment wells, the relevant conditioned media (EV, versican or versikine) was diluted 1:4 with growth media and added to the appropriate wells. The plates were returned to the incubator for 6 h. Photos were again captured at the 6 hour timepoint, with the final sets of photos taken at the 10.5 - 11 h time point. An example of the progression of cellular migration can be viewed in Figure 2.2. Digital images were captured using a IX71 Olympus inverted fluorescence microscope and a XM10 camera. Using the digital images, the distance between the edges of the scratches over the three time points was measured using the program ImageJ 1.47v, a program freely available on the internet (https://imagej.nih.gov/ij/).

Figure 2.2 Example of the progression of a migration assay.
A) Named time point 0 h, this is an image taken straight after the scratch was performed. B) An image taken 6 h, and C) 10.5-11 h after the start of experiment to view the rate of wound closure by measuring distance of cellular migration over a set time period.
2.1.8 Dexamethasone and conditioned media treatments

To assess the effects of dexamethasone on myotube formation in the presence of excess versican or versikine, C2C12 myoblasts were seeded in a 6 well plate at a density of 20,000 cells/cm² per well and were left to proliferate for 24 h or 48 h in growth media. After 24 h or 48 h, differentiation media was added and, depending upon the treatment required, was supplemented with either 0 nM, 25 nM or 100 nM dexamethasone, and/or EV, versican or versikine conditioned media (diluted 1:4). Differentiation media and all treatments were refreshed and applied daily. After 3 or 4 days, cells were fixed in 4 % PFA for determination of the fusion index (section 2.1.9). Additional experiments using the same parameters, but in a 6 well plate, were used for the creatine kinase and BCA assays (see section 2.1.10), with cell lysates used for western blots (see section 2.2.6).

2.1.9 Fusion index

The fusion index, a marker of myoblast differentiation efficacy, is the proportion of fused compared to total myonuclei (17). Myotube fusion has two distinct stages. Individual myoblasts must fuse together to form a nascent myotube, which contains less than 3-4 nuclei. Subsequent myotube growth and secondary fusion require individual myoblasts to further fuse with the nascent myotube forming mature myotubes (17, 400-402). Myotube maturity can be assessed by counting how many fused nuclei each myotube contains.

Following the fixation of C2C12 myotubes in 4 % in PBS, cells were stained with AlexaFluor 568 phalloidin (Invitrogen, diluted 1:50), which stains cytoskeletal actin. The plate was kept in the dark, on a rocker for 20 min at room temperature. Nuclei were counterstained using DAPI blue fluorescent nuclear stain (Invitrogen; diluted 1:20), the plate was again kept in the dark, placed on the rocker for 20 min at room temperature. Images were captured on an Olympus 1X71 Inverted Fluorescence Microscope with an XM10 camera. A view with well differentiated myotubes was located and an image was captured, followed by two random images within 1 field of view of this initial image (17).
This process was repeated for a total of three times, yielding a total of 9 images per well for each experimental condition, performed in duplicate for each of the 3 biological replicates. To determine the fusion index, individual nuclei were counted and designated as either fused or unfused, with myotubes also being counted and classified using the program ImageJ 1.47v, as either a nascent (3-4 fused nuclei), mature (5 - 10 fused nuclei) or super mature (10 + fused nuclei) myotubes.

The fusion index was used to assess the myotube maturation. For differentiating myoblasts treated with 25 nM or 100 nM dexamethasone, 3 biological replicates in duplicate were performed, with 8897 ± 222 nuclei and 270 ± 23 myotubes counted per experimental condition for each biological replicate. For differentiating myoblasts treated with dexamethasone, and/or conditioned media, a total of 5 biological replicates in duplicate were performed, with 3156 ± 96 nuclei and 110 ± 3 myotubes counted per experimental condition for each biological replicate.

2.1.10 Creatine kinase and BCA assays – C2C12 lysates

The creatine kinase (CK) assay was used as a measure of C2C12 myoblast differentiation. C2C12 cells (section 2.1.8) were lysed, one aliquot was used in a CK activity assay (Abcam) to company specifications, and another aliquot of lysate was used in a BCA assay (ThermoFisher Scientific) to measure protein levels within the samples, also to company specifications. The BCA assay was used to normalise the CK assay results to protein levels.

2.2 Molecular biology

2.2.1 Total RNA isolation from cell culture

TRIzol breaks down cells within moments of adding to the samples and therefore is used to ensure high quality yields of RNA are extracted. TRIzol (Ambion) was added to each sample and cells were lysed by pipetting up and down before being collected into an eppendorf tube and stored at -80 °C. The RNA was extracted by following the manufacturer’s protocol. Briefly, chloroform was added to each sample and were centrifuged at 12,000 g for 15 min at 4 °C. After
centrifugation, the samples separated into three distinct layers. The top aqueous phase contains RNA, which was carefully transferred into a sterile eppendorf tube to avoid contaminating with the white organic phase which contains DNA. Then isopropanol was added to each sample and spun down 12,000 g for 15 min at 4 °C to form a pellet at the bottom of the tube. The isopropanol was removed and samples were washed with 75 % ethanol before being centrifuged again. As much ethanol as possible was removed before the RNA pellets were dried by incubating on a heat block at 65 °C, then resuspended in nuclease free water (Ambion) by gentle pipetting. The samples were further incubated on the 65 °C heat block for 5 min to ensure that RNA was thoroughly dissolved and resuspended. The RNA concentration and purity was determined using the NanoVue spectrophotometer (GE Healthcare) with an A260/A280 nm ratio of ≥ 1.8 deemed acceptable for downstream analysis. The extracted RNA samples were then stored at -80 °C.

2.2.2 Total RNA isolation from skeletal muscle tissue
EDL, soleus and diaphragm muscles (see Section 2.3.7) were homogenised in 1 mL of TRIzol using a handheld homogeniser. Chloroform was added to each sample and centrifuged at 12,000 g for 15 min at 4 °C to allow the formation of 3 distinct layers. The top aqueous phase containing RNA was carefully transferred into an eppendorf tube with the addition of isopropanol. The samples were then transferred into RNeasy spin columns (Qiagen) and the manufacturer’s protocol was followed. Samples were eluted in 30 µL or nuclease free water. RNA concentration and purity was determined using the NanoVue spectrophotometer (GE Healthcare) with an A260/A280 nm ratio of ≥ 1.8 deemed acceptable for downstream analysis. The extracted RNA samples were then stored at -80 °C.

2.2.3 Reverse transcription
Reverse transcription was used to synthesize cDNA from extracted RNA. The defrosted RNA (up to 1µg) was reverse transcribed to cDNA using the manufacturer’s protocol from the iScript CDNA Synthesis Kit (BioRad). For one
sample, the following master mix was made; 5x iScript reaction mix (4 µl), iScript reverse transcriptase (1 µl), 1 µg of RNA template, with nuclease free water (Ambion) being used to make up a total of 20 µl. For multiple samples, each volume was multiplied according to the number of samples to be used. The reactions were incubated on the Bio-Rad T100 ThermoCycler at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min before being held at 4 °C. The cDNA samples were stored at -20 °C until required.

2.2.4 cDNA quantitation via OliGreen assay

All gene expression data were normalised to cDNA concentration. The cDNA concentration was quantified using the Quant-iT OliGreen ssDNA reagent kit (Invitrogen) as per the manufacturer’s protocol. Briefly, a black well, clear bottom 96 well plate (Thermofisher) was set up so that the oligonucleotide standards and cDNA samples could be added in triplicate. Tris-EDTA (TE) buffer (95 µl) was added per well, followed by 100 µl Quant-iT Oligreen ssDNA reagent. The plate was wrapped in aluminium foil and incubated for 5 min in the dark at room temperature. The fluorescence was measured using a microplate reader with the following fluorescent wavelengths: excitation 485 nm and emission 535 nm. The cDNA concentration was determined by firstly constructing a standard curve from the absorbance values of the oligonucleotide standards, and then using the slope equation on the standard curve to determine the cDNA concentrations of the unknown samples. A CV value of 5 % or less was used to screen out any outliers.

2.2.5 Real time PCR

RT-PCR is a semi-quantitative way to assess gene expression in cells, however the results need to be normalised to OliGreen for cDNA content. The primers used (Table 2.2) have all been previously tested and found to work efficiently at 55 °C. Additionally, any peaks other than the dissociation curve graph (or background noise near baseline) produced at the end of the program run will indicate the presence of any contamination or primer-dimers. As a further control measure, non-template controls (NTC’s) that contain everything but
cDNA, are run alongside the samples to act as a safeguard against contamination.

Samples were added in duplicate to clear 96 well plates (4titude). Following manufacturer protocol, iTaq Universal SYBR Green Supermix (BioRad), forward and reverse primers of interest, nuclease free water (Ambion) and the relevant cDNA template was added to each well. The plate was sealed using 8-strip flat caps (4titude) and loaded into a Stratagene Mx3000P QPCR System (Agilent Technologies). The following conditions were used: 10 min at 95 °C, followed by 40x (30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C), followed by 1x (1 min at 95 °C, 30 s at 60 °C, 30 s at 95 °C). Relative changes in mRNA levels were calculated using the 2ΔCt method normalised for cDNA concentration using an OliGreen assay (section 2.2.4).
<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Primer name</th>
<th>5’ Forward</th>
<th>3’ Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_007392.3</td>
<td>Acta2</td>
<td>CAGGCATGGATGCGATCAATCAC</td>
<td>ACTCTAGCTGTGAAAGTCAGTGTCG</td>
</tr>
<tr>
<td>NM_009621.5</td>
<td>ADAMTS-1</td>
<td>CCTGTGAAGCCCAAAGGCATTG</td>
<td>TGCAACAGACAGAGGTAGAGT</td>
</tr>
<tr>
<td>NM_011782.2</td>
<td>ADAMTS-5</td>
<td>GCTACTGCACAGGGAAGAGG</td>
<td>GCCAGGACACCTGCATATTT</td>
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<tr>
<td>NM_001329420.1</td>
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<td>GTCATCTGCGGACGCAAT</td>
<td>CAGCCAGCCTTGATGCACTT</td>
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<tr>
<td>NM_007542.5</td>
<td>Biglycan</td>
<td>GAGATTCCTCCAAACCTGCC</td>
<td>GGATTCCCGCCCATCTCAAT</td>
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<td>NM_007710.2</td>
<td>Creatine Kinase muscle (CKm)</td>
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<tr>
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<tr>
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<tr>
<td>NM_009931.2</td>
<td>Collagen 4a1 (Col4a1)</td>
<td>TGTTGATGGCCTATTCCTCTC</td>
<td>ATGGGGGCCTTCTAACTCT</td>
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<td>NM_008907.2</td>
<td>Cyclophilin</td>
<td>CCCACCGTGTTCTTCGCA</td>
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<td>Decorin</td>
<td>TCAAGAAGATGGGGAGGAC</td>
<td>AGTAGGGCACATGGGAG</td>
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<tr>
<td>NM_010043.2</td>
<td>Desmin</td>
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<td>CTGTGTAGCCTCGGCTG</td>
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<tr>
<td>NM_008216.3</td>
<td>Hyaluronan synthase 2 (Has2)</td>
<td>GGGACCTGGTGAGACAGAAG</td>
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<tr>
<td>XM_006511645.3</td>
<td>Hyaluronidase 2 (Hyal2)</td>
<td>AGCCGCAACCTTGTGACAGTT</td>
<td>GAGTCTCCTGGGTTAGTGG</td>
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<tr>
<td>NM_001355722.1</td>
<td>F4/80</td>
<td>AAGCATCCGAGACACACACA</td>
<td>GGCAGACATACACAGGGAG</td>
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<tr>
<td>XM_011250822.2</td>
<td>Furin (Pcsk3)</td>
<td>CAGCGAGACCTGAATGTGA</td>
<td>CAGGGTCATATTGCTGGCT</td>
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<td>Gene Accession</td>
<td>Gene Name</td>
<td>Enriched Motif</td>
<td>Enriched Sequence</td>
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<td>----------------------------</td>
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<td>NM_011333.3</td>
<td>Macrophage Chemoattractant Protein 1 (MCP1)</td>
<td>CCAATGAGTGAGCTGGAGA</td>
<td>TCTGGACCCATTCCTTCTT</td>
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<tr>
<td>XM_017314318.1</td>
<td>Myosin Heavy Chain (MyHC)</td>
<td>GCTCAAGCCTGTTACC</td>
<td>CATAGACGGCTTTGGCTAGG</td>
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<td>NM_010866.2</td>
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<td>NM_031189.2</td>
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<td>Pax7</td>
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<td>GTCACAAGCTGGGTTAGATG</td>
</tr>
<tr>
<td>NM_001291184.1</td>
<td>PACE4 (Pcsk6)</td>
<td>ATTTCCCCACCTCGTCTCT</td>
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<td>NM_011577.2</td>
<td>TGFβ1</td>
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<td>CTTCCAGGTCAGCAAATCA</td>
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<tr>
<td>NM_019389.2 (V1)</td>
<td>Versican (V0/V1)</td>
<td>ACCAAGGAGAAGTTCGAGCA</td>
<td>CTTCCAGGTCAGCAAATCA</td>
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</tbody>
</table>
2.2.6 Western blotting

Western blotting was used to confirm that the transfected Hek293T cells had successfully incorporated the plasmid DNA of interest and were secreting the relevant protein. Versican conditioned media and cell lysates first underwent a 1 μl addition of chondroitinase ABC (Seikagaku, Tokyo, Japan) and a 2 h incubation at 37 °C to remove GAG chains as described (319). All samples were then combined with 1x loading dye (BioRad).

Conditioned media samples were loaded onto 1.5 mm 7.5 % acrylamide gels and subjected to reducing sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) (30 mamps per gel, for 1 hour) before being transferred (150 mamps per gel, for 1 hour) onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5 % skim milk powder / Tris Buffered Saline containing 0.1 % Tween-20, then incubated with primary and secondary antibodies (Table 2.3).

The dexamethasone treated C2C12 cell lysates underwent SDS-PAGE using pre-cast Mini-PROTEAN TGX Stain-Free Protein Gels (BioRad) to allow imaging of protein in lanes using stain-free technology on benchtop Chemidoc XRS+ (BioRad), followed by electrophoresis using pre-cut Trans-Blot Turbo Mini PVDF Transfer Packs (BioRad) utilizing a Trans-Blot Turbo Transfer System (BioRad).

Visualisation of conditioned media was facilitated by an anti-rabbit or anti-mouse horseradish peroxidase (HRP)-labelled secondary antibody (Table 2.3). Bands were exposed manually by covering PVDF membranes with 750 ul of ECL prime western blotting detection reagent (GE Healthcare) for 1 min, then drained and added to a cassette for manual exposure, or placed on the quartz surface within the chemidoc system. The film was exposed in the dark for 30 s to 10 min, with developed films being scanned to obtain a digital image. The exposure time used with the Chemidoc was calculated automatically by the software.
<table>
<thead>
<tr>
<th>Antibodies used for Western blotting</th>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1-DPEAAE (Versikine)</td>
<td>Versican V0, V1 Neo Antibody (ThermoFisher Scientific; PA1-1748A)</td>
<td>1:1000</td>
<td>Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Empty vector and Versican</td>
<td>Anti-Versican Antibody, a.a. 1360-1439 of mouse versican (Merck; AB1033)</td>
<td>1:200</td>
<td>Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)</td>
<td>1:5,000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Anti-Glyceraldehyde-3-Phosphate Dehydrogenase Antibody, clone 6C5 (Merck; MAB374)</td>
<td>1:10,000</td>
<td>Anti-mouse IR680, (Sigma Aldrich)</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>
For cell lysate visualisation, an anti-rabbit horseradish peroxidase (HRP)-labelled secondary antibody (Table 2.3) was used. An equal volume (750 μl) of part A and B chemoluminescent (BioRad) was used to cover the membrane, before being drained and placed into the Chemidoc, with the computerized system automatically calculating exposure times. Downstream analysis of the cell lysate bands was undertaken through the use of the accompanying ImageLab software.

2.3 Animal studies

2.3.1 Mouse rationale and husbandry

All animals and experiments used in Chapters 4 and 5 were approved by the Deakin University Animal Ethics Committee. (Applications A79-2011 and G06/2015). Female C57BL/6 and C57BL/10 wild type mice and C57BL/10ScSn-Dmd<sup>mdx</sup>/Arc mice, hereafter referred to as mdx mice, were obtained from the Animal Resource Centre (Canning Vale, Western Australia). Male versican<sup>heART</sup> <sup>defect/+</sup> (Vcan<sup>hdf/+</sup>) were obtained from Hoffman-La Roche Pharmaceuticals. All mice were maintained on a 12 hour light-dark cycle, with standard mouse chow and water provided ad libitum.

The Vcan<sup>hdf/-</sup> strain, homozygous for the deletion of versican, is embryonically lethal at E10.5. Therefore, as the heterozygous Vcan<sup>hdf/+</sup> mouse has one functional versican allele, they are a viable strain (331) and were therefore used in this thesis as a source of genetically reduced versican. The Vcan<sup>hdf/+</sup> mice, hereafter known as hdf mice, were maintained on a C57BL/6 background. Female C57BL/6 mice were bred with male hdf mice in order to produce more hdf males. The hdf males on a C57BL/6 background were bred with female mdx mice on a C57BL/10 background to generate male pups that were mdx (dystrophic with normal versican) or mdx-hdf (dystrophic with reduced versican) and on a mixed C57BL/6 and C57BL/10 background. Mice were bred as required, with pups being weaned at 3-4 weeks of age. At 5 weeks of age, female pups were humanely, culled, while males were housed until reaching 6 or 21-26 weeks of age for experiments.
The mouse ages were chosen specifically, as at 6 weeks of age, *mdx* mice are past their period of spontaneous degeneration and are still in a period of postnatal growth (403), which is thought to be an additional stress on dystrophic muscles (404). The 6 week mice also approximately equate to 14-18 human years of age (129), a period where patients with DMD lose ambulation. Adult *mdx* mice between 21-26 weeks of age were also chosen as an age point, as although mice have stopped growing, at this point the dystrophic pathology steadily progresses, especially in the diaphragm, and muscles fibres have undergone numerous cycles of degeneration and regeneration (403). These adult mice equate to approximately 35 years in humans (129), an age that has severe complications from pathology and is towards the end of lifespan in DMD patients.

### 2.3.2 Mouse genotyping

As there were no obvious phenotypical differences between the mice, the male pups therefore required genotyping to be able to know which mice were *mdx* and which were *mdx*-hdf (133, 405, 406). From 5 weeks of age, male pups had an ear punch biopsy taken for genotyping. A 3-4 mm sized ear punch biopsy was placed into an eppendorf tube containing 75 µl of ice-cold extraction buffer (25 mM NaOH, 0.25 mM EDTA, distilled water), and immediately placed on ice. Samples were placed on a heat block at 100 °C for 30 min, after which they were placed on ice to cool down before the addition of 75 µl of neutralisation buffer (40 mM Tris-HCl, pH 5) to each tube. Samples can be stored at -20 °C if required for further use. Samples were run alongside a 1000 kDa EZ load molecular ruler (BioRad), on a 1 % agarose gel at 100 amps for 45 min, and visualised bands of interest were imaged on the Chemidoc XRS+. If mice are *mdx*-hdf, there will be a band at 400 kDa due to the LacZ promoter insertion, which causes the partial knockdown of versican (Figure 2.3). If the male is *mdx*, then there will be no band present at this location.
2.3.3 Assessment of body composition - EchoMRI

Directly prior to function testing, all mice were weighed and placed into an EchoMRI for rodents (Body Composition Analyser ESF-005, EchoMRI). The EchoMRI tests for body composition of each individual mouse at the time of testing, relevant to its own bodyweight. Specifically it measures the following: total body fat content measures fat within the body; lean content measures the tissues (muscle) containing water within the body excluding fat and bones; total water content measures all water within the body; and free water content measures the water in the bladder.

2.3.4 Ambulation and respiratory measures using calorimetric cages

The results obtained from calorimetric cages allowed for investigations into respiratory and metabolic functions, and also detailed analysis for spontaneous physical activity during the duration the experiment. The calorimetric cages are kept in a room that has the exact same parameters as the mice re normally exposed to, they were kept at the same temperatures and light/dark periods as normal. Mice were acclimatised to the metabolic cages (Accuscan Fusion v3.6;
Columbus Instruments International, OH, USA) for a 3 h period, 48 h prior to contractile function testing. The day before function testing, mice were placed into metabolic cages (Columbus instruments, USA) for 24 h in order to measure the volumes of oxygen (VO₂) consumed, carbon dioxide (VCO₂) released and respiratory exchange rate (RER) per mouse. As the respiratory data is recorded every minute during the 24 h experiment, it was therefore possible to breakdown the data into the 12 h light and dark periods that the mice undergo normally. Additionally, infrared sensors lining the cages allowed for the measurement of spontaneous activity by recording the total (crossing 2 or more infrared beams, ie: walking) and ambulatory (crossing only 1 infrared beam, ie: twitching) movement of each mouse. As the counter connected to the infrared sensors was only basic and could only tally the movements, only total data for the 24 h period could be obtained. Afterwards, mice immediately underwent contractile function testing (section 2.3.6).

2.3.5 Echocardiography

The results obtained through echocardiography allowed for more detailed investigations into heart function. Both DMD patients and mdx mice have a dilated cardiac myopathy and myocardial fibrosis as a consequence of the disease. More importantly, however, is that versican is very important for heart development, a complete absence being embryonically lethal at E10.5 due to a malformed heart, whereas heterozygous hdf mice are viable (331). Therefore it was necessary to investigate the interaction between reduced versican and heart function. A veterinarian (Dr Richard Wooley) performed echocardiography on 25 week old mice using a HD15 purewave ultrasound system (Phillips), as previously described (407). By placing the mouse nose into a nosecone, mice were lightly anaesthetised by inhalation of a maintained gas flow of 1.5 % isoflurane in oxygen. The parameters acquired were measured in M-mode and included interventricular septal dimension (IVSd, IVSs), left ventricular internal diameter (LVIDd, LVIDs), left ventricular posterior wall dimensions (LVPWd, LVPWs); where d = diastole and s = systole. Other important parameters acquired included the stroke volume, ejection fraction and fractional shortening for each heart.
2.3.6 Muscle function testing

After removal from metabolic cages (section 2.3.4), mice were anaesthetised using medetomidine (0.5mg/kg), midazolam (5mg/kg) and fentanyl (0.05mg/kg), administered via an IP injection in ~1 ml sterile saline. Once mice were unresponsive to tactile stimuli, the hind limb was shaved to remove fur and an incision was made between the knee and ankle. The extensor digitorum longus (EDL) was carefully excised by firstly locating the proximal tendon, which was then tied with a surgical silk suture. The surrounding membrane, nerve and blood vessels were very carefully removed from the muscle surface so as not to damage the muscle during this process. Another suture was tied around the distal tendon, and the EDL muscle was then dissected free. The EDL muscle was then tied to a dual mode force transducer/servomotor for muscle function testing and assessment.

Then, the 6 and 26 week mouse hind limbs were rotated to allow for the dissection of the soleus by firstly tying the proximal aponeuroses, a type of connective tissue that connects the soleus to the tibia and fibula bones near the knee, and the distal tendon (Achilles tendon) at the back of the foot. Once both ends of the soleus were tied, it was carefully excised starting at the proximal tendon and tied to a dual mode force transducer/servomotor for muscle function testing and assessment.

The 21 week old mice were dissected to obtain access to the diaphragm. Once dissected free, the diaphragm and surrounding ribcage was placed into a glass dissecting dish full of ringers and bubbled carbogen whilst finer dissections were undertaken. The diaphragm was pinned down and the ribs removed. Then the diaphragm was cut in half again pinned carefully. From this, a small ~1 cm strip was carefully cut, leaving the rib and central tendon on the muscle strip which were then tied with sutures. The small strip of diaphragm was then tied to a dual mode force transducer/servomotor for muscle function testing and assessment.
Figure 2.4 Examples of muscle tracings at different frequencies. 
A) The tracing of a single twitch at 1 Hz. B) A tracing of 4 twitches after a stimulation of 10 Hz, and C) after 30Hz, a series of twitches can be seen, which leads to D) complete tetanus is was achieved after stimulation at 100 Hz.

To assess muscle function, the muscles were transferred to the muscle function bath, which was filled with Kreb’s ringer buffer (NaCl 137 mM, NaHCO₃ 24 mM, D-glucose 11 mM, KCl 5 mM, NaH₂PO₄·H₂O 1mM, MgSO₄ 1 mM, CaCl₂ 2 mM, d-tubocurarine chloride 0.025 mM), continuously bubbled with Carbogen (medical grade 95 % CO₂ and 5 % O₂; BOC gases) and thermostatically maintained at a temperature of 25 °C. Each muscle was attached to the muscle function machine by firstly tying the distal tendon end to an immovable pin, while the proximal tendon was tied onto the lever arm of the transducer, as recommended in the standard operating procedure (SOP), entitled: Measuring isometric force of isolated mouse muscles in vitro (SOP ID number: DMD_M.1.2.002). This is SOP is freely available on the TREAT-NMD website (http://www.treat-nmd.eu/research/preclinical/dmd-sops). All muscles were stimulated using a voltage of 40 mV, and supramaximal square wave pulses of 350 ms in duration used for the EDL and diaphragm, with 1200 ms duration used for the soleus. All
stimulation parameters and contractile responses were controlled, measured and recorded on a desktop computer, using Dynamic Muscle Control Software (DMCv5.415) along with transducer control/feedback hardware (Aurora Scientific). The optimal muscle length was obtained from a series of twitches until a force plateau was reached. Then muscle length was measured using a set of digital callipers. The maximal isotonic force ($P_o$) was determined from a force-frequency curve; a series of stimulations comprising of 1, 10, 30, 50, 60, 80, 100, and 120 Hz for diaphragm and EDL, with 1, 10, 20, 30, 50, 60, 80, 100 and 120 Hz for the soleus. Some examples of the muscle tracings can be found in Figure 2.4.

To assess muscle fatigue, each muscle was stimulated once every 5 s for a total of 4 min at the optimal length and a submaximal stimulation frequency of 60 Hz (408-411). The recovery of $P_o$ was determined by stimulating the muscles at 60 Hz at 2, 5 and 10 min after the fatigue protocol was run.

As $P_o$ is highly dependent on the size of each muscle, $P_o$ values were subsequently normalized to muscle cross-sectional area and were expressed as specific force ($sP_o$; kN.m$^{-2}$). Muscle cross-sectional area was calculated by dividing the muscle weight by the optimum fibre length ($L_f$) and 1.06 mg/mm$^3$, which is the density of mammalian muscle. The $L_f$ is calculated by multiplying $L_o$ with previously determined $L_o$:$L_f$ ratios of 0.44 for the EDL, and 0.71 for the soleus (127, 412).

### 2.3.7 Sample collection and storage

Following muscle function testing the EDL, soleus and diaphragm muscles were dissected free of any tendons and connective tissue, then weighed on an analytical balance. The muscles were then snap frozen in liquid nitrogen and stored in the -80°C freezer for analysis of gene expression.

Muscles from the contralateral hindlimb and diaphragm, not used for function testing, were used for histological and immunohistochemical analysis. They were stretched out to $L_o$ and covered with a layer of OCT medium (VWR International), and frozen in thawing isopentane.
Blood was obtained by cardiac puncture and stored at 4 °C for 24 h to allow clot formation. Blood was centrifuged at 13,400 rpm for 2 min to separate the red blood cells from the serum, in the form of a pellet. Serum was carefully extracted and stored at -80 °C.

Hearts were weighed and subsequently stored in 4 % PFA for 24 h, before being stored in PBS in a fridge, until they were embedded in paraffin. Embedded hearts were stored at -20 °C.

### 2.3.8 Creatine kinase assay - mouse serum

The creatine kinase assay was used as an unspecific, whole body measure of muscular damage in the *mdx* mice. Serum obtained from mice (section 2.3.7) was used in a creatine kinase (CK) activity assay (Abcam), to company specifications. Note that the assay measures brain, heart and muscle isoforms of creatine kinase within the serum, and is not specific for the muscle isoform.

### 2.3.9 Histology

#### 2.3.9.1 Haematoxylin and Eosin staining

In order to view the muscle morphology, each muscle was sectioned and stained with haematoxylin and eosin (H&E). Eosin stains muscle fibres and cytoplasm bright pink and haematoxylin stains nuclei purple, so the muscular structures become very easy to differentiate. Frozen muscle sections of 8 µM thickness were fixed in 70 % ethanol for 3 min and rinsed in running tap water, then washed with distilled water. Sections were stained in Mayer’s haematoxylin (Sigma) for 1 min, rinsed under running tap water for 30 s and placed in 0.05M Tris Buffered Saline (TBS) for 1 min to allow the colour to develop. Sections were rinsed underrunning tap water for 30 s, following by distilled water. The slides were stained with aqueous Eosin (Sigma) for 15 s and immediately rinsed in tap water. Slides were dehydrated by dipping 10 times in 70 % ethanol, 10 times in 95 % ethanol (repeated twice), 10 time in 100 % ethanol (repeated twice) and 10 dips in xylene (repeated twice). Muscle sections were mounted using DPX (Sigma) and glass coverslips.
2.3.9.2 Muscle morphology analysis

As H&E staining allows for the easy viewing of muscle structures, it is possible to then analyse the muscle fibres and areas of damage. An example of the dystrophic pathology found in *mdx* mice can be found in Figure 2.5. Two images of muscle H&E stained muscles were captured at 100x magnification on a camera mounted Leica microscope. The program Image-Pro Plus (version 7) was used to assess the mean cross surface area (CSA) and centrally nucleated myofibres were counted to assess recently damaged and repaired myotubes. Cells located in the ECM surrounding the muscle fibres were assumed to be inflammatory cells and fibroblasts, while areas of thick connective tissue deposits (clear or very pale pink in colour) were assumed to be fibrosis. Muscle damage was analysed by identifying and circling areas of mononuclear infiltration and degeneration (413, 414), which includes inflammatory cells, adipose and connective tissues, and expressing the circled area as percentage of the total muscle area. Furthermore, additional images were also taken on the same microscope at 200x magnification in order to produce a representative figure panel.
Figure 2.5 Example of *mdx* muscle stained with H&E.

This *mdx* muscle section gives a good overview of dystrophic pathology. Given the varying size of muscle fibres in the muscle, both small and large fibres are highlighted. Undamaged muscles and recently regenerated fibres, those with central nuclei, are also present. Areas of inflammation and necrosis can also be seen, as well as the infiltration of fibrosis and adipose tissue.
2.3.10 Immunohistochemistry

2.3.10.1 Versican and versikine

Immunohistochemistry was undertaken in order to investigate the cellular localisation of versican and versikine within muscle sections, and also to assess their expression level by analysing the proportion of the muscle section immunoreactive for versican and versikine. Frozen muscle sections were left to defrost at room temperature for 15 min, after which they were fixed in 4 % PFA for 10 min at 4 °C. Sections were rinsed in PBS twice. Muscles were permeabilized using 1 % Triton X-100 in PBS for 10 min. Sections were placed in a small container with wet tissues at the bottom for humidity, and were blocked at room temperature with 200 µl of protein block (Dako) for 30 min. This was followed by the addition of 100 µl of primary antibody for versican (Millipore, AB1033), or versikine (Pierce, PA1-1748A) per slide, added at a dilution of 1:200 in antibody diluent (Dako), kept in the dark, at room temperature for a period of 1 hour. The negative control slide was not treated with a primary antibody, and was instead incubated with antibody diluent. Sections were washed 4x 10 min using 1 % NGS in PBS. The fluorescent Alexafluor goat-α-rabbit 594 secondary antibody (Life Technologies) was added at a dilution of 1:750 using antibody diluent (Dako), and the humid box containing the muscle section was carefully placed at 4 °C overnight to incubate. Sections were washed 6x 10 min in PBS and were mounted using fluoroshield mounting media with DAPI (Abcam), covered with a glass coverslip (ProSciTech) and sealed with nail polish. Slides were kept in a foil covered box (away from light) at 4 °C. Two non-overlapping images for each slide were imaged at 60x magnification on a confocal microscope (Olympus Fluoview FV10i, All in One Confocal Laser Scanning Microscope). Staining was quantified by using Image-Pro Plus (version 7), expressing the area of staining as a percentage of the total muscle area in the field of view.

2.3.10.2 Myosin heavy chain (MyHC) IIa

This experiment and analysis was performed by Alex Addinsall.

To explain the increase in endurance observed in the mdx-hdf diaphragm, it was hypothesised that perhaps they have an increased amount of fast twitch type IIa
fibres. Analysis of the proportion of MyHC I is also planned. Diaphragm sections from *mdx* and *mdx*-hdf mice were stained with anti-MyHClIa (Developmental Studies Hybridoma Bank; SC-71; diluted 1:50), and incubated for 2 h. Sections were then reacted with Alexa Fluor 488 goat anti-mouse IgG1 (Thermo Fischer Scientific; A21121; diluted 1:500) fluorescent secondary antibody at room temperature, with nuclei being counter stained with DAPI containing mounting medium (Vectashield). Two non-overlapping images for each slide were imaged on a Zoe Fluorescent Imager at 200x (Bio-Rad). To control for non-specific staining, negative controls incubated with the secondary antibody were included with all analyses.

2.3.10.3 Wheat germ agglutinin

As wheat germ agglutinin is a quick and easy stain that has been shown to be an effective marker of fibrosis (415), it was used to stain the diaphragm sections. As WGA is a fluorescent stain, this allowed for the easy viewing of fibrotic tissue and its subsequent morphometric image analysis. The limitations of WGC staining are that the sarcolemma of muscle fibres also stains positive for WGA, and this limits the sensitivity of the technique. Frozen diaphragm sections from *mdx* and *mdx*-hdf mice were defrosted at room temperature for 15 min, after which they were fixed in 4 % PFA for 10 min at 4 °C. Sections were rinsed 3 times in distilled water for 30 s. Muscles were permeabilized using 0.5 % Triton X-100 in PBS for 10 min, then stained with a fluorescent Wheat Germ Agglutinin Conjugate (Invitrogen; 1:50). Sections were rinsed twice in PBS and mounted using fluoroshield mounting media with DAPI (Abcam), covered with a glass coverslip (ProSciTech) and sealed with nail polish. Two non-overlapping images for each slide were imaged on an Olympus 1X71 Inverted Fluorescence Microscope with an XM10 camera. Staining was quantified by using Image-Pro Plus (version 7), expressing the area of staining as a percentage of the total muscle area in the field of view.
2.4 Statistical analysis

Statistical analyses were performed using either 1- or 2-way General Linear Model (GLM) ANOVA and Tukey’s post hoc analysis, or an independent sample T-test, where appropriate. The statistical test used has been indicated within each of the relevant figure legends. Minitab 16 statistical software (Version 16.2.1) was used to calculate the GLM ANOVA, with Microsoft Excel used for independent t-tests. All data were expressed as the mean ± SE, with p < 0.05 considered statistically significant.
Chapter 3: Dexamethasone improves myogenic differentiation *in-vitro* by suppressing the synthesis of versican, a transitional matrix protein overexpressed in dystrophic skeletal muscle

The majority of results in this Chapter was incorporated into a manuscript which was published in December 2017. Details of the publication are as follows:


N. McRae performed all experiments, analysed the majority of data, and prepared all figures. The contributions of the co-authors are stated within the methods of this Chapter where appropriate.

### 3.1 Introduction

Duchenne muscular dystrophy, is an x-linked muscle degenerative disease, characterised by persistent inflammation and repeated bouts of ECM remodelling due to muscle degeneration and regeneration occurring in different locations within the same muscle (284, 344). This ultimately results in a permanent expansion of ECM components, such as collagens, fibronectin and versican, and is collectively known as fibrosis. Fibrosis in turn causes further damage during dystrophic pathology, being both a cause and consequence, as fibrotic tissue creates solitary microenvironments and increases ECM stiffness, thereby interfering with normal cellular processes and compromising muscle integrity and function (93).

The accumulation of fibrotic tissue becomes very severe in DMD, eventually affecting striated skeletal, respiratory and cardiac muscle in a detrimental
manner. Within their limited lifespan, dystrophic patients loose ambulation in the limbs, and eventually succumb to respiratory and cardiac failure by approximately 30 years of age (109, 111, 416). Fibrosis is usually thought of as an endpoint to dystrophic pathology, but it is important to note that the accumulation of endomysial components precedes the degeneration of muscle (417) and may actively contribute to the degeneration of dystrophic muscles (119, 207, 418).

The mature ECM of normal healthy skeletal muscle is composed of proteins such as collagens and proteoglycans containing HS and CS/DS GAG side chains (116). Endomysial fibrosis in DMD is associated with the increased expression of not only these mature ECM proteins (107, 419), like decorin and biglycan (40), but also transitional ECM proteins such as versican (90), hyaluronan (420) and tenascin C (421). These transitional matrix proteins, through their synthesis and remodelling, are able to regulate cell behaviour during normal development and regeneration, functioning as a scaffold for the deposition of mature ECM components (73, 289).

The role of the matrix is not well recognised during development and within tissue repair processes, and as such, has not been fully elucidated in the context of fibrosis. Therefore strategies to understand the formation of fibrosis and its inhibition are of great therapeutic interest within DMD. Given the importance of a transitional matrix during tissue repair, proteins such as versican should be of particular interest as potential therapeutic targets (74).

However much still remains unknown about versican and its implications upon fibrosis. Versican has previously been established as an important protein associated with various cellular processes. Versican is associated with the proliferation of myoblasts (20), promotes migration in valvular interstitial cells (338), and upon cleavage, is able to help facilitate myoblast fusion (17).

Furthermore, versican also has a role in inflammatory processes (288), being able to bind both chemokines and inflammatory cells via its CS GAG chains (51, 53), as well as being associated with the differentiation of fibroblasts to
myofibroblasts (265). Myofibroblasts secrete prolonged and increased levels of ECM proteins, including collagens (422), and are a major contributor to fibrotic areas, especially in DMD (107) where versican is also known to be highly upregulated (90). Additionally, ADAMTS5, a protease capable of cleaving versican, is upregulated in serum from both \textit{mdx} mice and DMD patients (349), and increased \textit{Adams5} mRNA transcripts were observed in \textit{mdx} hindlimb muscles compared to wild type mice (423). These are suggestive that ADAMTS5 may also be implicated in fibrotic regions of dystrophic tissues.

There is currently no cure for DMD, or for the debilitating fibrosis. However steroids known as glucocorticoids are currently the only treatment of clinical beneficence, and offer some comfort for the dystrophic patients. Glucocorticoids are known to reduce inflammation and are already used for treating an assortment of inflammatory disorders, such as asthma and allergies (285), autoimmune diseases such as rheumatoid arthritis (424), multiple sclerosis and type 1 diabetes mellitus (367). Because of the wide range of potential beneficial effects, they are used to treat DMD patients to help alleviate disease symptoms. Glucocorticoids can delay the progression of DMD by improving muscle strength and respiratory function, increasing ambulation, and by reducing scoliosis (366, 425). These beneficial effects may be due to membrane stabilisation and a reduction in inflammation, along with decreases in muscle necrosis and fibrosis, and improved regeneration of muscle (285, 426).

Although patients are treated with glucocorticoids, it still remains largely unknown how the treatment affects the genes able to regulate transitional matrix synthesis and remodelling, the cellular processes important for regenerative myogenesis. It is generally thought that the administration of glucocorticoids can dampen the inflammatory response, effectively resetting the process, and thereby allowing muscle repair processes to occur normally (365). Indeed, glucocorticoids have been previously shown to modulate ECM proteins in other pro-inflammatory diseases. In cultured rat mesangial cells, glucocorticoids were shown to decrease \textit{versican} and \textit{decorin} gene expression (427). Another glucocorticoid, budesonide, was also observed to decrease
versican, along with perlecan and decorin protein levels in human lung fibroblasts stimulated with serum, but whereas versican gene expression was decreased, no effect was observed for perlecan or decorin (428). Studies into the effects of glucocorticoids on arthritis found that they inhibit the destruction of the matrix surrounding the cartilage, by inhibiting protease (ADAMTS4 and ADAMTS5) expression and activity in the inflamed area (373). These studies suggest that the observed effects of glucocorticoids are quite complex, being tissue and context dependent.

Glucocorticoids can also improve muscle repair, as shown in both in-vivo and in-vitro studies. In dystrophic mdx mice, high dose treatment with the glucocorticoid deflazacort suggested an increase in satellite cells during myofibre formation following crush injury in the TA (429). Additionally an increase in muscle fibre diameters in the uninjured TA and diaphragm, suggested that the treatment may have an effect on muscle growth (429). Another study observed that when mdx mice were treated with prednisone or VBP15, a dissociative glucocorticoid, they suppressed TGFβ and fibrotic networks, which included a reduction in collagen 1A1, 3A1 and 6A1 gene expression, leading to improved muscle repair (285). Glucocorticoids have also been shown to enhance myofibre formation in primary wild type and dystrophic mdx myoblasts, as well as in C2C12 cells in-vitro (397, 430).

However, the downstream effects of glucocorticoids on important processes, such as the regulation of genes and ECM components, cellular migration and muscle regeneration, remain largely unstudied. However, being steroids, they also come with a wide range of undesirable side effects, and therefore are only administered for a brief period or at low dosages (365, 376). Thus, understanding how glucocorticoids work can lead to the development of better therapeutic strategies, especially those targeting aberrant matrix synthesis and remodelling.
3.2 Methods

3.2.1 Versican and versikine expression in mdx and WT mouse skeletal muscles

Transverse frozen sections from 12 week old C57/BL10 and mdx mice were already prepared and kindly provided by Chris Van der Poel (LaTrobe University), and Nicole Stupka. Slides were mounted with 8 µm thick sections from the mid-belly of the TA, or strips from the diaphragm, stored at −80 °C until analysis. The muscles were stained with versican or versikine. Four representative digital images were captured on a confocal microscope of each muscle section at 600× magnification and analysed for area of immunoreactivity using Image-Pro Plus software (version 7). While N.McRae quantified the area of staining in the majority of images, both L. Forgan and B. McNeill quantified some images and thereby contributed to the analysis in this experiment. Resultant data were expressed as % immunoreactivity. A more detailed version of this protocol is found in section 2.3.10.1.

3.2.2 C2C12 cell culture experiments

3.2.2.1 C2C12 cell culture conditions

C2C12 myoblasts were maintained in growth media (DMEM plus 10 % FBS) in atmospheric O2 and 5 % CO2 at 37 °C. A different passage number was used for each replicate to ensure individuality and to avoid duplicating results. When differentiation of myoblasts was required, growth media was replaced with differentiation media (DMEM plus 2 % HS).

3.2.2.2 The effects of dexamethasone treatments on C2C12 myoblast differentiation

To determine the effects of dexamethasone on myoblast differentiation, myoblasts were seeded at 25,000 cells/cm²; in duplicate wells for fusion index determination, and in triplicate wells for gene and protein expression and creatine kinase activity analyses. Following 48 h of proliferation (and at 90 % confluence), myoblasts were treated with 0 nM, 25 nM or 100 nM dexamethasone in differentiation medium (DMEM plus 2 % HS) for 72 h,
refreshed every 24 h. Following this, the cells were harvested for biochemical analyses (as below), or fixed with 4 % paraformaldehyde and stained with phalloidin and DAPI for 20 m.

The fusion index was used to assess myoblast differentiation. Therefore to quantify the differentiated myotubes collected from culture, the small nascent myofibres were those with 3-4 myonuclei, and mature myofibres containing 5-9 nuclei or those with 10+ myonuclei, were quantified as previously described (17, 431). For the fusion index, 3 biological replicates in duplicate were performed, with 8897 ± 222 nuclei counted per experimental condition for each biological replicate. To assess myofibre maturation, 270 ± 23 myofibres were classified and counted per experimental condition for each biological replicate. While N.McRae counted myotubes in two experimental replicates, A. Addinsall contributed to this experiment by counting myotubes in one experimental replicate. In order to support the fusion index data, muscle creatine kinase (Ckm) and myosin heavy chain (MyHC), both gene markers of myoblast differentiation, were performed along with a creatine kinase enzyme activity assay kit, as per manufacturer’s instructions.

3.2.2.3 RNA extraction, cDNA analysis and RT-PCR of C2C12 cells treated with dexamethasone

To observe the effect of dexamethasone on the synthesis and processing of a versican and hyaluronan rich pericellular matrix, C2C12 myoblasts were harvested in triplicate wells, collected in TRIzol and stored at −80 °C. Upon thawing, RNA was extracted and 1 μg of total RNA was reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and oligonucleotide primers for the murine genes of interest (Table 2.2). Relative changes in mRNA levels to untreated myofibres were calculated using the 2ΔCt method. Real time data was normalised to cDNA content, as determined using a Quant-iT Oligreen ssDNA reagent kit.
**3.2.2.4 HEK293 cell culture conditions, and preparation of constructs and conditioned media**

HEK293T cells were grown in DMEM (25 mM glucose) containing 10 % FBS in atmospheric O₂ and 5 % CO₂ at 37 °C. Cells were transfected using Lipofectamine 2000 with constructs encoding 1) V1 versican construct (kindly provided by Professor Dieter Zimmermann), 2) the G1-DPEAAE versikine fragment, produced by the insertion of a stop codon in the V1 versican construct after the Glu⁴⁴₁-Ala⁴₄² peptide bond cleavage site (334), and 3) an empty vector control (pcDNA3.1MycHisA+). Serum-free conditioned media was collected from the HEK293 cells for use in the myoblast differentiation experiments, as previously described (17). The conditioned media underwent western blotting for confirmation of V1 versican and versikine protein expression.

**3.2.2.3 Dexamethasone and versican or versikine on differentiating C2C12 myoblasts**

To assess the effects of dexamethasone treatments on myofibre formation in the presence of excess versican or versikine, C2C12 myoblasts were seeded at 20,000 cells/cm² in duplicate wells for 24 h or 48 h. Afterwards, differentiation media (DMEM plus 2 % horse serum) was added for 3 or 4 days, respectively. Depending on the experimental conditions, the differentiation medium was supplemented with 0 nM or 100 nM dexamethasone, and serum-free versican, versikine or empty vector conditioned media (diluted 1:4; refreshed daily). Fusion index and myofibre number were then determined as described above. Five biological replicates in duplicate were performed, with a total of 3156 ± 96 nuclei and 110 ± 3 myofibres counted per experimental condition for each biological replicate.

**3.2.2.3 Western blotting and protein quantification**

The versican and versikine conditioned media, and the dexamethasone treated C2C12 cell lysates from the creatine kinase enzyme activity assay, were used for analysis and quantification of versican and versikine protein expression. The GAG chains on versican were removed by adding of 1 µl of chondroitinase ABC (Seikagaku) to versican conditioned media and to the cell lysates from the
creatine kinase enzyme assay for 2 h at 37 °C. The serum free conditioned media containing versikine was subjected to the western blotting as previously described (319). The versican conditioned media and C2C12 cell lysates underwent SDS-PAGE using pre-cast Mini-PROTEAN TGX Stain-Free Protein Gels (BioRad), which were transferred onto PVDF membranes. Primary antibodies used were versican, versikine and GAPDH, and secondary antibodies used were peroxidase AffiniPure goat anti-rabbit IgG and anti-mouse IR680 (Table 2.3). Proteins were visualised either manually by film, or by chemiluminescence on a Chemidoc XRS+ and analysed using ImageLab software. Versican and versikine protein levels were normalised to total optical density of all protein bands on the TGX Stain-Free Protein Gel. A more detailed version of this protocol is found in section 2.2.6.

3.2.2.4 The effect of dexamethasone and versican or versikine upon C2C12 myoblast migration and proliferation

C2C12 myoblasts were seeded at 8,500 cells/cm² in triplicate wells and 72 h later (when 100 % confluent) a scratch wound assay was performed (432). Growth media was supplemented with serum-free versican, versikine or empty vector conditioned media (diluted 1:4) for up to 11 h. To determine migration rate, digital images (three per well with duplicate or triplicate wells) were captured at 0 h, 6 h and 11 h post-wounding with a IX71 Olympus inverted fluorescence microscope and a XM10 camera. The distance between the edges of the scratches was measured using ImageJ. Migration rate calculated in pixels/m.

To assess the effects of versican and versikine on myoblast proliferation, myoblasts were seeded at 10,000 cells/cm² in growth media was supplemented with serum-free versican, versikine or empty vector conditioned media (diluted 1:4; refreshed daily) for 48 h. Myoblast number was assessed using the WST-1 cell proliferation reagent, as per the manufacturer’s directions.

3.2.3 Statistics

For the quantitation of immunoreactivity against versican and versikine in TA and diaphragm muscles, independent T-tests were used to assess differences in
immunoreactivity for each muscle type. For the cell culture, biochemical and fusion index analyses, independent T-tests, or 1-way or 2-way general linear model ANOVA were performed as indicated and followed by Tukey's post-hoc analysis where appropriate. All data are presented as mean ± S.E. and were considered statistically significant when p < 0.05.

3.3 Results

3.3.1 Versican, but not versikine, protein levels were increased in dystrophic muscles and correlate with the severity of pathology

The pathology of TA muscles from adult mdx mice is moderate compared to that of the human disease. Specifically, in hindlimb muscles from mdx mice there is minimal fibrosis, lower levels of inflammation and effective regeneration (129), although muscle strength is compromised (127). The pathology of mdx diaphragm muscles is more representative of DMD, with high levels of endomysial fibrosis and greatly impaired contractile function (433, 434).

Here, versican protein levels were upregulated 6-fold in mdx TA muscles and 21-fold in mdx diaphragm muscles compared to those of the wild type muscles, as quantified in Figure 3.1 I. In particular, the endomysial versican staining was more extensive in the mdx diaphragm than the TA muscles. In the mdx diaphragm and TA muscles, versican staining was also associated with regions of mononuclear infiltrate. This is consistent with the synthesis and secretion of versican by various cells such as inflammatory cells (48), satellite cells (20), and fibroblasts (348).

Remodelling of versican by ADAMTS proteoglycanases yields the bioactive versikine fragment (289). Versikine protein levels were similar in TA and diaphragm muscles from mdx and wild type mice, as quantified in Figure 3.1 J. Areas of mononuclear infiltrate within the mdx TA and diaphragm sections were also observed to contain versikine staining. This is interesting given the association of versikine with inflammation (342).
Figure 3.1 Versican and versikine staining in TA and diaphragm muscle cross-sections from 12 week old wild type and mdx mice.

Versican staining is red; versikine staining is red; and nuclei are stained blue with DAPI. A-H) Representative images from TA and diaphragm muscles from wild type and mdx mice. I) Quantification of versican immunoreactivity revealed an upregulation in TA (*p = 0.001; T-test) and diaphragm muscles (#p = 0.0001; T-test) from mdx mice when compared to wild type mice. J) Versikine immunoreactivity was similar in mdx and wild type TA or diaphragm muscles. White asterisks denote areas of mononuclear infiltration. Immunoreactivity analysis was determined from n = 5 wild type mice and n = 5 mdx mice. Scale bar = 100 µm. Error bars = S.E.
3.3.2 Dexamethasone enhances myoblast fusion and myotube formation

To characterise dexamethasone mediated effects upon transitional matrix synthesis and remodelling, during myogenic differentiation, C2C12 skeletal muscle myoblasts were used. Like skeletal muscle development in-vivo, myogenic differentiation of C2C12 myoblasts is associated with an upregulation of transitional matrix genes, including Vcan (versican), Adamts1, Adamts5, Adamts15, Pcsk6 (17), Has2 and Hyal2 (73, 435). Differentiating C2C12 myoblasts were treated with 0 nM, 25 nM or 100 nM dexamethasone, see Figure 3.2 for representative images. These are low and clinically relevant concentrations, which have previously been shown to increase the efficiency of myoblast fusion (397).

Following 72 h of treatment, a two-fold increase in muscle creatine kinase (CKm) gene expression (Figure 3.3 A), was observed along with dose dependent increases in total CK activity (Figure 3.3 B), suggesting that dexamethasone may improve differentiation in C2C12 cells. Indeed, CKm is a known myogenic differentiation marker and has been shown to be increased alongside myosin heavy chain (MyHC) during myogenic processes (436, 437). However, MyHC mRNA transcripts, although tending to increase, were not significant in this study (Figure 3.3 C).

Therefore, the fusion index was used to further assess myoblast differentiation. To analyse the differentiated myotubes collected from culture, total myotube numbers and nuclei were counted. The fusion index involves counting small nascent myofibres containing 3 or 4 myonuclei, and growing myofibres undergoing secondary fusion containing 5-9 myonuclei or 10+ myonuclei, quantified as previously described (17, 431). The myotubes that formed following 25 nM and 100 nM dexamethasone treatments were larger and contained a greater number of myonuclei compared to untreated cells (Figure 3.3 D-F). Specifically, the fusion index, which is the proportion of nuclei fused into multinucleated myotubes compared to total nuclei, was increased in a dose dependent manner following treatment with 25 nM and 100 nM dexamethasone.
(Figure 3.3 D). This was associated with a dose dependent increase in myotube number following dexamethasone treatment (Figure 3.3 E). Myotube formation is a two-step process with differentiating myoblasts initially fusing to form nascent myotubes, with further growth achieved through secondary fusion of additional myoblasts, which ensures the maturation of the myotube (438, 439). The dexamethasone treatments also appeared to enhance secondary fusion. There were more myotubes with 5-9 nuclei and 10+ nuclei in cells treated with 25 nM and 100 nM dexamethasone versus untreated cells. Specifically, the increase in the number of mature myotubes with 5-9 nuclei was dose dependent, with more myotubes with 5-9 nuclei observed following 100 nM compared to 25 nM dexamethasone treatment (Figure 3.3 F). Interestingly, the 100 nM dexamethasone treatment also increased the formation of nascent (3-4 nuclei) myotubes (Figure 3.3 F). This is a significant observation as distinct signalling pathways mediate the formation of nascent and mature myotubes (400, 440, 441), and the increased formation of nascent myotubes by dexamethasone suggests a possible upregulation of these early myogenic pathways.
Figure 3.2 Representative images for differentiating C2C12 cells treated with dexamethasone.
Representative images of differentiating C2C12 myofibres treated with A) and D) 0 nM dexamethasone, B) and E) 25 nM dexamethasone, or C) and F) 100 nM dexamethasone. Cells were stained with phalloidin for F-actin (red) and DAPI for nuclei (blue). Scale bar = 200 µm.
Figure 3.3 Low dose dexamethasone treatment for 72 h increased myogenic differentiation efficacy in C2C12 myoblasts.

A) The gene expression of myogenic differentiation marker creatine kinase (Ckm) was increased 2-fold in cells treated with 25 nM and 100 nM dexamethasone treatment (*p < 0.01; T-test). B) CK enzyme activity in cell lysates was also increased following treatment with 25 nM (*p = 0.02; T-test) and 100 nM (#p = 0.01; T-test) dexamethasone. C) Dexamethasone (Dex) treatment did not significantly increase myosin heavy chain (MyHC) mRNA transcripts. D-E) Fusion index (FI; *p < 0.001; 1-way GLM ANOVA) and myofibre number (*p < 0.01; 1-way GLM ANOVA) were greater in C2C12 cells treated with 25 nM and 100 nM dexamethasone compared to untreated control cells, and this increase was dose dependent (+p < 0.05; 1-way GLM ANOVA). F) When compared to control cells, treatment with 100 nM dexamethasone increased the formation of nascent myofibres containing 3-4 myonuclei (*p < 0.001; 1-way GLM ANOVA). The number of myofibres with 5-9 nuclei was greater in cultures treated with 25 and 100 nM versus 0 nM dexamethasone (+p < 0.02 and †p = 0.03, respectively; 1-way GLM ANOVA), indicated that this increase was dose dependent. The number of myofibres with 10+ myonuclei was also increased following dexamethasone treatment (‡p < 0.001; 1-way GLM ANOVA) when compared to control cells. CK enzyme activity was calculated from n = 3 biological replicates performed in quadruplicate. The fusion index and myofibre number were calculated from n = 3 biological replicates performed in duplicate. Gene expression was determined from n = 3 biological replicates in triplicate. Error bars = S.E.
3.3.3 Dexamethasone regulates the expression of genes associated with transitional matrix synthesis and processing during myogenic differentiation

Glucocorticoids have previously been described as being able to enhance myogenic differentiation by regulating the expression of genes associated with transitional matrix synthesis and processing. Specifically, 72 h of treatment with 25 nM and 100 nM of dexamethasone reduced \( Tgfb1 \) mRNA transcripts by 50% and 54%, respectively (Figure 3.4 A). This is consistent with data from cultured hepatic stellate cells, where low doses of dexamethasone also reduced \( Tgfb1 \) gene expression and protein secretion (442). TGF\( \beta \) is a potent inhibitor of myogenesis which is reversible upon its decreased expression or removal (443), so a decrease in \( Tgfb1 \) gene expression suggests increased myogenesis, a result indeed observed. Furthermore, TGF\( \beta \) is known to drive networks that increase the synthesis of ECM components such as collagens (285), and cultured human bronchial smooth muscle cells stimulated with Tgfb1 were also observed to have increased levels of collagen 1a1, fibronectin and versican (444).

In accordance with increased myogenesis, the decreased \( Tgfb1 \) transcripts (Figure 3.4 A) were associated with a reduction in \( V0/V1 \) Vcan gene expression by up to 56% (Figure 3.4 B) and Has2 (Figure 3.4 C) gene expression by up to 58% following glucocorticoid treatment. Has2 is the primary Has gene involved in hyaluronan synthesis in skeletal muscle (445) and hyaluronan is an important versican binding partner (18), so a decrease in hyaluronan is potentially suggestive for a decrease in versican.

Furthermore, versican is cleaved by ADAMTS versicanases (316), and consistent with the decreased versican, \( Adamts1 \) mRNA transcripts were increased up to 3-fold in dexamethasone treated C2C12 cells (Figure 3.4 D). This effect was isoform specific, as \( Adamts5 \) and \( Adamts15 \) mRNA levels were not significantly increased following dexamethasone treatment (Figure 3.4 E-F). This was unexpected as \( Adamts5 \) is the most highly upregulated ADAMTS transcript during myogenesis (17).
Figure 3.4 The expression of genes associated with the synthesis and processing of a versican-hyaluronan rich pericellular matrix is modulated by dexamethasone in differentiating C2C12 myoblasts.

A-C) Compared to untreated control cells, 25 and 100 nM dexamethasone treatment decreased Tgfb1 (*p < 0.0001 and *p = 0.0001, respectively; T-test), Vcan (V0/V1 variants) (*p = 0.005 and *p = 0.0006, respectively; T-test) and Has2 mRNA transcripts by approximately 2-fold (*p < 0.0001 and *p = 0.0001, respectively; T-test). D) Adamts1 gene expression was increased up to 3-fold in response to 25 and 100 nM dexamethasone treatment (*p = 0.03 and *p < 0.001, respectively; T-test). E-F) Adamts5 and Adamts15 mRNA transcripts were not significantly increased. G-H) Pcsk3, but not Pcsk6, mRNA transcripts were decreased approximately 2-fold following treatment with 25 and 100 nM dexamethasone (*p < 0.0001; T-test). I) Hyal2 mRNA levels were not altered by dexamethasone treatment. Gene expression was determined from n = 3 biological replicates in triplicate. Error bars = S.E.
Adamts1 is synthesised as a zymogen (pro-ADAMTS1) which undergoes proteolytic processing to form the active enzyme. Both Pace4 and Furin can cleave the zymogen to produce the mature form of Adamts1, but Furin is the most efficient (230). Treatments of 25 and 100 nM dexamethasone decreased Pcsk6 (Pace4) gene expression by 65 % and 68 %, respectively (Figure 3.4 G), whilst Pcks3 (Furin) mRNA levels were not significantly altered (Figure 3.4 H).

Dexamethasone also had no effect on hyaluronidase-2 (Hyal2) mRNA transcripts (Figure 3.4 I), the enzyme necessary for HA degradation (446). Therefore the effects of dexamethasone on transitional matrix synthesis and cleavage, including any consequences regarding the catalytic activation of ADAMTS versicanases, particularly Adamts1, during myogenic differentiation require further investigation.

Of all the gene expression changes, the effect of dexamethasone on the expression of versican was the one of most interest as it was overexpressed in the dystrophic mdx muscles, especially in the more severely affected diaphragm (See Figure 3.1 I). Indeed, western blotting further confirmed the gene expression data and that the dexamethasone treatments reduced versican protein levels (Figure 3.5 A-B) in a dose dependent manner. Dexamethasone treatment did not appear to affect versican remodelling, as indicated by versikine, the cleaved form of versican, remaining unchanged across all treatments (Figure 3.5 C).
Figure 3.5 Versican and versikine protein expression in differentiating C2C12 myoblasts following dexamethasone treatment.

A) Representative western blots stained for versican and versikine, with the respective stain free protein gel image to demonstrate even protein loading. B) A dose dependent decrease in versican protein expression was observed following 25nM (*p = 0.00002; T-test) and 100nM (*p = 0.0000001; T-test) dexamethasone treatment, and between 25nm and 100nm dexamethasone (#p = 0.026; T-test). C) Versikine levels were not altered by dexamethasone treatment. Versican and versikine protein expression analysis was calculated from n = 3 biological replicates performed in quadruplicate. Error bars = S.E.
3.3.4 Dexamethasone rescues myofibre formation in differentiating myoblasts treated with exogenous versican and versikine

Versican expression is increased in dystrophic muscles from patients with DMD (90) and in mdx mice (Figure 3.1 I). Excess versican may impair regenerative myogenesis, as versican processing is implicated in myoblast fusion (17). Reduced versican synthesis and favourable processing may contribute to the clinical efficacy of glucocorticoids in DMD and the improvement in regeneration observed in-vitro and in-vivo. To test this, differentiating C2C12 myoblasts were treated with V1 versican, versikine or empty vector conditioned media supplemented with 0 nM or 100 nM dexamethasone, see Figure 3.6 for representative images. These are challenging experimental conditions and it is worth noting that the increase in fusion index in response to dexamethasone treatment (see Figure 3.3), was blunted by the addition of versican and versikine conditioned media, ultimately decreasing myoblast fusion. This decrease in fusion was ameliorated upon treatment with 100 nm dexamethasone (Figure 3.7 C). In agreement with the fusion index data, treatment with versican and versikine conditioned media also decreased myofibre number (Figure 3.7 D). Again, this effect was improved by glucocorticoid treatment.

Alignment of myoblasts is essential for fusion, and this depends on carefully regulated migration (431, 432, 447). Versican is known to modulate cell migration and depending on the biological context, the effects can be stimulatory (448, 449) or inhibitory (450). Myoblast migration is known to be necessary for myoblast fusion and myotube formation (432, 447), but to date, the effects of versikine on myoblast migration have not been well characterised. Therefore, to investigate effects on myoblast migration C2C12 myoblasts were treated with versican or versikine conditioned media for up to 11 h, which resulted in myoblast migration rate being reduced by 12 % and 13 %, respectively (Figure 3.7 E). Thus, excess versican and versikine may impair regenerative myogenesis through a reduction in myoblast migration.

Myoblast viability and number can be a confounding factor in determining the efficacy of myogenic differentiation. It is worth noting that an increase in cell
number is an indication of cell proliferation and in cell viability. Versican is associated with proliferation in various biological contexts (49, 336), including within primary myoblasts from turkey muscles (20), whilst versikine has been shown to be associated with apoptosis during interdigital web regression in embryonic mice (334). However no changes were observed in cell number within actively proliferating C2C12 myoblast cultures following 24 and 48 h of treatment with conditioned media (Figure 3.7 F), meaning cells remained viable upon both treatments, however, they also did not proliferate. These in-vitro observations in C2C12 myoblasts, including those mentioned above highlight the complexity and the importance of cellular context with regards to versican biology.
Figure 3.6 Representative images for differentiating C2C12 cells treated with dexamethasone and conditioned media.

Representative images of differentiating C2C12 myofibres treated with A-B) EV conditioned media and C-D) EV conditioned media and 100 nM dexamethasone; or E-F) Versican conditioned media and G-H) Versican conditioned media and 100 nM dexamethasone; or I-J) Versikine conditioned media, and G-H) Versikine conditioned media and 100 nM dexamethasone. Cells were stained with phalloidin for F-actin (red) and DAPI for nuclei (blue). Scale bar = 200 µm.
Figure 3.7 Dexamethasone ameliorates the impairment in myogenic differentiation associated with excess versican.

A-B) Representative western blots of conditioned media containing versican and versikine, along with representative images of stain free protein gels and GAPDH as a loading controls. C) The addition of versican and versikine conditioned media compromised myoblast differentiation, as assessed by fusion index (\(^*p < 0.001\) and \(^*p < 0.02\), respectively; main effect conditioned media; 2-way GLM ANOVA with factors being conditioned media and dexamethasone). Whilst, dexamethasone enhanced myoblast fusion in cells treated with empty vector (EV), as well as versican and versikine, conditioned media (\(^#p < 0.001\); main effect dexamethasone; 2-way GLM ANOVA with factors being conditioned media and dexamethasone). D) Myofibre number was also reduced in versican compared to EV conditioned media treated cells (\(^*p < 0.001\); main effect conditioned media; 2-way GLM ANOVA with factors being conditioned media and dexamethasone). Again, dexamethasone increased myofibre number in cells treated with EV, as well as versican and versikine, conditioned media (\(^#p < 0.01\); main effect dexamethasone; 2-way GLM ANOVA with factors being conditioned media and dexamethasone). E) Reduced cell migration may contribute to the impairment in myogenic differentiation efficacy associated with excess versican, as versican or versikine conditioned media reduced the migration rate of C2C12 myoblasts compared to EV conditioned media (\(^*p = 0.01\) and \(^*p = 0.04\), respectively; T-test). F) Myoblast cell number was not different following 24 h and 48 h of treatment with versican, versikine or EV conditioned media. Fusion index and myofibre number were calculated from \(n = 5\) biological replicates performed in duplicate. Migration rate was measured from \(n = 5\) biological replicates performed in duplicate or triplicate. Myoblast proliferation was assessed from \(n = 3\) biological replicates performed in 8 wells. Error bars = S.E.
3.4 Discussion

The co-ordinated synthesis and remodelling of ECM proteins is essential for efficient skeletal muscle regeneration and myotube formation. The ECM regulates the bioavailability of growth factors, such as TGFβ, whilst signals from specific matrix proteins control muscle stem cell activation, proliferation, migration and fusion (451). Excess synthesis and inappropriate processing of ECM proteins leads to fibrosis and degeneration, and compromises muscle function (85, 107).

Similarities between ECM expansion in patients with DMD and mdx mice most likely contribute to the impaired regenerative capacity and function of these muscles (285). For example, increased expression of collagen 6α1 has been observed in human DMD muscle biopsies (452), as well as in mdx mouse diaphragms (453). Furthermore, the proteoglycan versican is highly upregulated in muscle biopsies from patients with DMD compared to healthy controls (90), as is the deposition of chondroitin sulphate GAG side chains (116), of which V0 and V1 versican forms both contain (48).

The significance of versican in the generation and activity of a transitional matrix during skeletal muscle development and regeneration is continuing to gain recognition (74). Indeed, the carefully regulated synthesis and processing of a versican rich transitional matrix is an important factor in differentiating between successful regenerative myogenesis, or degeneration and fibrosis. A better understanding of versican function in DMD is needed if progress is to be made in targeting the dysregulated ECM, a hallmark of DMD pathology. Versican is increased in the diaphragm and TA muscles of mdx mice compared to wild type diaphragm muscles (Figure 3.1). Despite greater levels of versican protein, low levels versikine were observed in mdx diaphragms. It has previously been shown that versican processing by ADAMTS5 helps facilitate myoblast fusion (17). The diaphragm muscle best resembles the human form of DMD in terms of muscle function and fibrosis (454, 455), with fibrosis being progressive and becoming more prominent in aged mdx mice (221). The fact that significantly increased
versican was observed in the 12 week old mdx diaphragms is therefore interesting, particularly as it matches a suggestion that a provisional matrix is persistently secreted in DMD during the early stages of fibrosis (107).

*In-vivo*, centrally nucleated fibres are indicative of recent damage and repair, with myoblast fusion being essential for effective regeneration. In diaphragm muscles from *mdx* mice, the proportion of centrally nucleated fibres is much lower, up to 2-3 fold when compared to dystrophic TA muscles (433, 456). It was therefore hypothesised that excess versican accumulation and inadequate clearance, as indicated by the lack of increase in versikine levels, contributes to the impaired regeneration in the *mdx* diaphragm muscles.

It is well known that versican is cleaved by ADAMTS versicanases, forming the fragment called versikine (316, 319). An increase of *Adams5* mRNA was reported in injured hindlimb muscles from *mdx* mice compared to wild type mice (423), which may enhance versican processing and promote more effective muscle repair. In support of this, myoblast fusion and the formation of multinucleated myotubes is associated with a reduction in chondroitin sulphate chains, which may be attributed to versican processing (457). Indeed, the data contained within this Chapter show a concentration dependent increase of CK gene expression, along with increased myoblast fusion following low dose, 25 nM and 100 nM, dexamethasone treatment. This is in accordance with a number of studies reporting positive effects of glucocorticoids on myogenesis *in-vitro* (397, 430) and muscle regeneration *in-vivo* (285, 429).

However, glucocorticoids need to be used with caution as side effects, including muscle atrophy, compromised muscle function and impaired myogenesis can occur (26,74,75). Nevertheless, the benefits are usually greater. Therefore disease context and glucocorticoid dosage need to be considered for optimal results for the patient. For instance, a high concentration of dexamethasone (10 µM) was found to inhibit myogenic differentiation of C2C12 and primary mouse myoblasts through the activation of GSK-3β, a negative modulator of myogenic differentiation and adult muscle growth (458). This concentration of
dexamethasone was 100-400 fold higher than the low dose treatment used within Chapter 3. Furthermore, with lower concentrations of 10 nM and 100 nM dexamethasone, no significant decrease in myogenin or myosin heavy chain protein expression was reported (458), matching the observation that MyHC gene expression did not change (Figure 3.3 C).

The increase in myotube formation following glucocorticoid treatment was associated with an approximate 50 % decrease in Versican, Has2 and Tgfb1 gene expression and a 3-fold increase in Adamts1 mRNA transcripts (Figure 3.4). These data suggest that in differentiating C2C12 myoblasts, dexamethasone may attenuate transitional matrix synthesis and enhance remodelling, thus facilitating membrane contact during fusion. The effects of glucocorticoids on reduced ECM synthesis and remodelling have previously been described in various biological contexts. Glucocorticoid induced skin atrophy is associated with reduced proteoglycan (459) and hyaluronan synthesis (460, 461). Relevant to the findings here in Chapter 3, glucocorticoids have also been reported to decrease versican expression in cultured rat mesangial cells and airway fibroblasts (428, 458).

Myoblast fusion and myofibre number were reduced when differentiating C2C12 myoblasts were treated with versican or versikine conditioned media, further supporting the hypothesis that excess versican impairs regenerative myogenesis. Indeed, inefficient cleavage by ADAMTS5 leading to an excess of versican has previously been suggested to impair myoblast fusion (17). Additionally, the observation that excess versikine impaired myoblast fusion is also not unexpected, given that hyaluronan may bind to versikine via the G1 N terminus link module (462), thus potentially contributing to the expansion of the pericellular matrix. However this reduction in fusion was ameliorated upon the addition of 100 nM dexamethasone (Figure 3.7 D), further highlighting the significance of V0/V1 versican reduction by glucocorticoids.

To further investigate how versican can compromise myoblast fusion, the effects upon myoblast migration was assessed. Myoblast migration is critical for
myoblast fusion as cells need to migrate and align before fusion can occur (432, 447). The observations from Figure 3.7 E showed that excess versican or versikine impairs the migration of myoblasts. Versican can signal through the CD44 receptor, either by binding directly to the receptor or through its binding to hyaluronan, to regulate myoblast migration during myogenic differentiation (296, 447, 463).

These *in-vitro* observations support the hypothesis that excess accumulation of versican in dystrophic muscles contributes to their poor regenerative capacity. The accumulation of versican in dystrophic muscles may be attributed to elevated TGFβ levels. It has previously been confirmed that TGFβ centred signalling networks, induced by asynchronous regeneration in response to chronic contraction-induced injury, are key drivers for fibrosis and failed regeneration in dystrophic skeletal muscles (285). These TGFβ networks were suppressed when dystrophic *mdx* mice were treated with glucocorticoids (285). Indeed, consistent with this finding, *Tgfβ1* gene expression was observed to decrease (see figure 3.3A). Therefore, the observation that dexamethasone regulates the expression of genes involved in transitional matrix synthesis and remodelling, in particular V0/V1 Vcan, Has2 and *Adamts1*, are highly relevant with regards to the potential of improving regenerative myogenesis in dystrophic muscles.

The *in-vitro* studies upon versican in this Chapter are promising and ultimately highlight the need for regulated versican synthesis. This potentially may be beneficial within the context of dystrophic pathology, where a regulated transitional matrix would be crucial for efficient muscle repair. It may be that the conclusions drawn from this Chapter may also translate to other pro-inflammatory diseases as well, further emphasising the importance of a regulated matrix. Therefore, future studies should look into reducing the level of versican *in-vivo* within *mdx* mice and investigate cellular processes crucial to inflammation, muscular damage and myogenesis, to ascertain that the *in-vitro* effects suggested within this Chapter are the same as those observed within a dystrophic murine model.
Chapter 4: Genetic reduction of versican improves fatigability and strength of diaphragm muscles from dystrophic mdx mice

4.1 Introduction

DMD is a paediatric disease arising from a mutation on the X chromosome leading to the loss of dystrophin protein and the dystrophin associated protein complex (108). This renders muscle fibres prone to persistent contraction induced damage. Muscle repair is inadequate, leading to the loss of muscle fibres, expansion of fibrosis, and adipocyte infiltration (107). Skeletal, respiratory and cardiac muscles are affected in DMD; leading to a loss of ambulation, and becomes life threatening when cardiac and respiratory function becomes compromised. The life expectancy of patients with DMD is approximately 30 years of age, whereupon they succumb to cardiorespiratory failure (109, 111, 416).

Like patients with DMD, the mdx mouse also has a loss of dystrophin protein expression (464), and undergoes persistent cycles of muscle damage, degeneration and repair (126). The diaphragm muscles of mdx mice are most severely affected and mimic the pathology of DMD well (125, 454). As mdx mice age, there is a progressive increase in diaphragm fibrosis, which is associated with impaired contractile function (455).

Fibrosis occurs when chronic and persistent muscle damage disrupts the balance between ECM synthesis and remodelling leading to the expansion of endomysial and interstitial matrices (87). Endomysial fibrosis is not just a consequence of dystrophy, it precedes overt degeneration within the muscle and expands during the course of the disease (94, 107). This fibrosis is comprised of various mature and transitional ECM proteins, including collagens (89) and proteoglycans. Versican, a proteoglycan decorated with CS GAG chains, is overexpressed in DMD (90, 347) and from Chapter 3, is highly upregulated in diaphragm muscles from mdx compared to wild type mice (465), highlighting an association of
versican with dystrophic pathology. Versican is decreased by dexamethasone in C2C12 muscle cells (465) and in various other biological contexts where heightened inflammation is a problem (427, 428). It is well recognised that the ECM is important for cell signalling and function. An abnormal matrix, however, disrupts cell signalling and function and ultimately results in pathology. Given that versican is such an important transitional matrix protein in development and disease (74), it can be assumed that its persistent overexpression contributes to DMD pathology, as has been observed in pulmonary and cardiovascular disease (341, 466).

Versican is highly expressed in developing skeletal muscles, where together with hyaluronan, is a key component of the transitional matrix (74). To date, versican has been associated with various cellular processes relevant to the pathogenesis of muscular dystrophy. These processes include satellite cell proliferation (20), myoblast fusion and myofibre formation (17), and inflammation (51, 288). Indeed, versican can be linked to inflammation in a variety of mechanisms, though binding MCP-1 which attracts infiltrating monocytes (51), being involved in the monocyte to macrophage transition (290), and may bind leukocytes through interactions with its CS GAG chains (53). Versican is also strongly associated with TGFβ, which is highly overexpressed in DMD and thought to be a major driver of fibrosis (235, 280). TGFβ1 upregulates versican synthesis in smooth muscle airway cells (444). Similarly, in fibroblasts TGFβ1 can induce secretion of various ECM proteins, including versican (346). Overexpression of versican stimulates the differentiation of fibroblasts into myofibroblasts (348), and these expand fibrotic areas by secreting ECM proteins such as collagens I, III and IV (195).

Given that versican is part of a transitional matrix (74), its remodelling by ECM proteases, including ADAMTS5, is very important for its biological function. During development, versican remodelling regulates interdigital web regression (334), formation of the cardiac outflow tract and endocardial cushions (308, 333) and facilitates myoblast fusion (17). Interestingly, ADAMTS5 levels are increased in serum from mdx mice and patients with DMD (349). Indeed, increased
Adamts5 gene expression has been observed in the hindlimb, specifically the gastrocnemius muscle, from male 3 month mdx mice when compared to those of 1.5 months (423). Similar to versican, ADAMTS5 has been associated with myogenesis (17). It has also been implicated in inflammation, as ADAMTS5 expression can be mediated by pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, (351, 355, 467), and its mediated cleavage of versican is required for inflammatory cell migration (468). Additionally, versikine, the cleavage product formed through ADAMTS cleavage of V1 versican, is also bioactive. Versikine has been associated with apoptosis (334), and more recently has been shown to induce the upregulation of IL-1β and IL-6 in myeloma tumour cells (342). This is particularly interesting and relevant as these same cytokines are increased in dystrophic muscles (344).

Given versican is upregulated in dystrophic muscles (90, 347), particularly in areas of degeneration, inflammation and fibrosis (465), it was hypothesised that it may modulate inflammation, fibrosis and disrupt regenerative myogenesis, thus exacerbating pathology of dystrophic muscles. Therefore, the aim of this chapter was to build upon the findings from Chapter 3, and to test the hypothesis that versican reduction is a relevant therapeutic target for DMD. Consequently, female mdx mice were bred with male heart defect (hdf) mice, which are haploinsufficient for the versican allele, in order to generate F1 male pups – specifically, mdx control littermates with two functional versican alleles and the mdx-hdf mice with a single functional allele and therefore a genetic reduction in versican. These mice have never before been produced and are therefore a novel strain. These mice were then used to assess the phenotypic effects of versican reduction on whole body metabolism, spontaneous physical activity, cardiac function and diaphragm muscle contractile properties, including ex-vivo strength and fatiguability. Histology, immunohistochemistry and qRT-PCR were then used to assess diaphragm muscle structure, versican expression and gene markers of myogenesis, inflammation and ECM synthesis and remodelling.
4.2 Methods

4.2.1 Ethics statement and animal husbandry

All mouse experiments were approved by Deakin University’s Animal Ethics Committee, under project numbers A79/2011 and G06/2015. Female \textit{mdx} mice, obtained from the Animal Resource Centre (Canning Vale, Western Australia), were bred with male \textit{hdf} (heart defect) mice, obtained from Hoffman-La Roche Pharmaceuticals. The \textit{hdf} mice are haploinsufficient for the versican allele, being heterozygous for versican. The resulting F1 generation \textit{mdx} and \textit{mdx}-hdf male pups were confirmed through genotyping. All mice were maintained on an alternating 12 h light and 12 h dark cycle, with standard mouse chow and water provided \textit{ad libitum}. All experimental procedures were completed on mice between 20 to 26 weeks of age.

4.2.2 Echocardiography

Using a nosecone, mice were lightly anaesthetised by inhalation of a maintained gas flow of 1.5 % isoflurane in oxygen. Echocardiography was performed by a specialist veterinarian (Dr Richard Woolley; http://cprvictoria.com.au), using a HD15 Purewave Ultrasound System (Phillips). Mice were replaced back into cages after completion of the procedure. Further details can be found within section 2.3.5.

4.2.3 Metabolic cages and body composition

Mice were acclimatised to the metabolic cages (Accuscan Fusion v3.6; Columbus Instruments International, OH, USA) for a 3 h period, 48 h prior to contractile function testing. Mice were then placed individually into the metabolic cages for 24 h, whereupon volumes of oxygen consumed (\(\text{VO}_2\); mL/min), carbon dioxide exhaled (\(\text{VCO}_2\); mL/min) and respiratory exchange rate (\(\text{RER}\); \(\text{VCO}_2/\text{VO}_2\)) were recorded per mouse. The resting energy expenditure (REE) was then calculated from the obtained \(\text{VO}_2\) and \(\text{VCO}_2\) data by using the abbreviated weir equation (469, 470). Furthermore, infrared sensors (Animal Activity Meter: Opto-Varimex-Mini; Columbus instruments) lining the metabolic cage allowed for the measurement of total spontaneous physical activity for each mouse, as
determined by crossing ≥2 infrared beams. Immediately prior to contractile function testing, mice were weighed and placed into an EchoMRI (Body Composition Analyser ESF-005, EchoMRI; Houston, USA) to measure whole body composition.

4.2.4 Contractile function
Mice were anaesthetised with medetomidine (0.5mg/kg), midazolam (5mg/kg) and fentanyl (0.05mg/kg), administered via an IP injection in approximately 1 ml sterile saline, until unresponsive to tactile stimuli. The diaphragm was removed and a diaphragm muscle strip extending from the rib to the central tendon was prepared for contractile function testing. Using small increments in length and 1 Hz twitches, the optimal length (L₀) was determined. This allowed for the assessment of twitch force normalised to muscle size (sPt), time to peak tension (TPT), ½ relaxation time (½ RT), and maximal rate of force production (max dx/dt). Following which, the maximal force producing capacity (sP₀) for the diaphragm muscle was determined from a force frequency curve ranging from 1 to 120 Hz, with 2 m rest in between each stimulation. To assess fatigability and endurance, the diaphragm was stimulated every 5 s for a total duration of 4 minutes at a submaximal stimulation frequency of 60 Hz. Muscle recovery was then assessed by stimulating the diaphragm at 60 Hz at 2, 5 and 10 m post fatigue. Further details can be found within section 2.3.6.

After contractile function testing had concluded, the diaphragm strip was trimmed of rib and tendon, weighed and snap frozen in liquid nitrogen. An additional 10 mm wide diaphragm section was frozen in thawing isopentane for histology and immunohistochemistry, with the remainder of the diaphragm muscle being snap frozen for gene expression analysis. Heart weight was also determined to complement the echocardiography data.

4.2.5 Histology and immunohistochemistry
Diaphragm strips were sectioned on a cryostat at -20 °C at a width of 8 μm.
Diaphragm cross-sections from mdx and mdx-hdf were allocated as follows: 1) Stained with H&E in order to observe muscle structure, and analyse fibre area
(minimal feret diameter), central nucleation and areas of mononuclear infiltrate. 2) Stained with wheat germ agglutinate in order to observe areas of fibrosis. 3) Were reacted with primary antibodies for versican or versikine. Or 4) Were reacted with a primary antibody for myosin heavy chain IIA (MyHC IIA).

4.2.5.1 H+E staining
For analysis of muscle morphology, two non-overlapping digital images were captured from H&E stained sections. Image analysis was completed using ImageJ 1.47v (https://imagej.nih.gov/ij/) or Image-Pro Plus (version 7). To assess muscle fibre size, all fibres within the field of view were manually traced to obtain the fibre area (minimal feret diameter). Minimum ferets diameter is a method of measuring the cross sectional area minus any experimental discrepancies, such as the orientation of the muscle section (471, 472). The number of fibres analysed per image was 487 ± 18. In addition, the proportion of centrally nucleated fibres, a hallmark of recent damage and repair, was also determined. As a general indicator of degeneration and inflammation, regions of mononuclear infiltrate and degeneration were traced and expressed as a percentage of the total muscle area in the field of view.

4.2.5.2 Wheat germ agglutinin staining
Two non-overlapping images were captured of slides stained with wheat germ agglutinin (WGA), which can be used as a marker for fibrosis (415). Using the program Image-Pro Plus (version 7), the area stained with WGA was quantified by measuring the area of staining and expressing as a percentage of the total area of muscle in the field of view. For a more detailed description, please refer to section 2.3.10.3.

4.2.5.3 Immunohistochemistry for versican and versikine
Four non-overlapping images were captured of muscle cross-sections were immunohistochemically stained with versican and versikine primary antibodies. Using the program Image-Pro Plus (version 7), the areas stained were quantified by measuring the area of staining and expressing as a percentage of the total
area of muscle in the field of view. For a more detailed description, please refer to section 2.3.10.1.

4.2.5.4 Immunohistochemistry for Myosin Heavy Chain IIa (MyHC Ila)
Note that this experimental procedure and analysis was performed entirely by A. Addinsall. Sections from diaphragm muscle strips were immunohistochemically stained with a MyHC Ila primary antibody. The number of fibres expressing MyHC Ila staining, along with the number of MyHC Ila with central nuclei were counted using ImageJ 1.47v (https://imagej.nih.gov/ij/). For a more detailed description, please refer to section 2.3.10.2.

4.2.6 Gene expression analysis
Gene expression was performed using cDNA created from function tested diaphragms that were snap frozen (section 2.3.7). All samples were tested in duplicate. All gene expression data were calculated using the 2ΔCT method and were normalised to Oligreen. For a more detailed description, please refer to sections 2.2.4 and 2.2.5. For primer sequences, please refer to Table 2.2.

4.2.7 Statistics
An independent sample t-test was undertaken for all histology, immunohistochemistry and gene expression analyses. For metabolic cage analyses, a 1- or 2-way General Linear Model (GLM) ANOVA were performed, followed by Tukey’s post hoc analysis where appropriate. For contractile function activity analyses, 2-way general linear model (GLM) ANOVA were performed, followed by Tukey’s post hoc analysis where appropriate. All data are presented as MEAN ± S.E. and were considered statistically significant when p < 0.05.
4.3 Results

4.3.1 Full length versican expression is decreased in \textit{mdx}-hdf diaphragm muscles

As previously observed in 12 week \textit{mdx} diaphragms (465), the 21 week diaphragm muscle cross-sections from the \textit{mdx} mice versican immunoreactivity was also localised to areas of endomysial fibrosis (Figure 4.1 A-B). Versican protein levels were reduced by approximately 50 \% in diaphragm muscles from \textit{mdx}-hdf mice (\( p = 0.0004 \); Figure 4.1 C). Versikine was also localised to endomysial fibrosis and regions of mononuclear infiltrate, with some nuclear co-localisation also observed (Figure 4.1 D-E). Versikine protein expression did not differ between diaphragm muscles from \textit{mdx} and \textit{mdx}-hdf mice (Figure 4.1 F). It is worth noting that versikine is not the end product of versican remodelling and is likely further degraded. For example, versikine immunoreactivity is greater in developing embryonic E13.5 hind limb muscles than in hindlimb muscles from 3 week old wild type mice (17). This could account for the lack of difference in versikine immunoreactivity in \textit{mdx} and \textit{mdx}-hdf diaphragm muscles.

The decrease in V0/V1 versican immunoreactivity was supported with qRT-PCR analysis of diaphragm muscles. Indeed, the mRNA transcript abundance of V0/V1 versican (\textit{V0/V1 Vcan}) was lower in diaphragm muscles from \textit{mdx}-hdf mice compared to \textit{mdx} littermates (\( p = 0.005 \); Figure 4.1 G). Altogether, these results confirm the successful genetic reduction of versican in dystrophic diaphragm muscles.
Figure 4.1 V0/V1 versican and versikine expression in diaphragm muscles from 21 week old mdx and mdx-hdf mice.

Versican and versikine immunoreactivity in red with nuclei stained blue (DAPI). A-B) Representative versican staining in mdx and mdx-hdf mice. C) Versican immunoreactivity was reduced in diaphragm muscles from mdx-hdf mice compared to mdx littermates (*p = 0.0004; T-test). D-E) Representative versikine staining in mdx and mdx-hdf mice. Arrows indicate areas of mononuclear infiltrate and degeneration. F) Versikine immunoreactivity did not differ between mdx and mdx-hdf diaphragm muscles (p = 0.192; T-test). G) V0/V1 Vcan mRNA transcripts were reduced in diaphragm muscles from mdx-hdf mice (*p = 0.005; T-test). N = 11-13 mdx and n = 11-12 mdx-hdf mice were used to assess versican and versikine immunoreactivity, while n = 12 mdx and n = 11 mdx-hdf mice were used to assess V0/V1 Vcan gene expression. Scale bar = 100 µm.
4.3.2. Body weight and composition of 26 week old *mdx* and *mdx*-hdf mice

The genetic reduction of versican did not affect body weight or composition, as lean mass and fat mass values were similar in *mdx* and *mdx*-hdf mice (Table 4.1). Accordingly, the percentage of body fat and lean mass did not differ between mice (Table 4.1).

<table>
<thead>
<tr>
<th></th>
<th><em>mdx</em></th>
<th><em>mdx</em>-hdf</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>32.24 ± 0.50</td>
<td>32.46 ± 0.70</td>
<td>0.80</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>1.44 ± 0.20</td>
<td>1.78 ± 0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>4.41 ± 0.57</td>
<td>5.49 ± 0.44</td>
<td>0.14</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>29.77 ± 0.41</td>
<td>30.22 ± 0.66</td>
<td>0.58</td>
</tr>
<tr>
<td>Lean mass (%)</td>
<td>92.40 ± 0.52</td>
<td>93.10 ± 0.68</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Data are means ± S.E. 
N = 14 *mdx* and n = 16 *mdx*-hdf

4.3.3. The genetic reduction of versican is associated with increased spontaneous physical activity

During 24 h in the metabolic cages, the *mdx*-hdf mice were observed to be more active than their *mdx* littermates (p = 0.024; Figure 4.2 A). Characteristic of dystrophic pathology in *mdx* mice is a reduction in spontaneous physical activity and exercise capacity, along with an increase in lean body weight due to ongoing cycles of degeneration and regeneration, resulting in muscle hypertrophy (470). Given the relevance of these variables to the dystrophic pathology of *mdx* mice, the total movement data were interrogated further by correlating it with body weight. Interestingly, as body weight increased, *mdx* mice moved less (p = 0.0282; Figure 4.2 B). However, this negative correlation was not observed in the *mdx*-hdf mice, where increased body weight was not associated with a reduction in spontaneous physical activity within the metabolic cages (Figure 4.2 C). These data indicate positive effects of versican reduction on the whole body phenotype of dystrophic *mdx* mice.
Figure 4.2 The genetic reduction of versican is associated with an increase in spontaneous physical activity.

The *mdx* and *mdx*-hdf mice were housed in metabolic cages for 24 h and total movement was measured with infrared activity sensors. A) The *mdx*-hdf mice were more active than their *mdx* littermates as indicated by an increase in total movement (*p = 0.024; T-test). B-C) In *mdx* (*p = 0.0282), but not *mdx*-hdf mice (p = 0.4147), a negative correlation between increasing body weight and reduced physical activity was observed. N = 12 *mdx* and n = 15 *mdx*-hdf mice.
4.3.4 The genetic reduction of versican shifts whole body metabolism to a more oxidative phenotype without affecting REE

The dystrophic pathology of *mdx* mice is characterised by increased energy expenditure due to the high metabolic cost of inflammation and muscle regeneration that is associated with chronic contraction induced muscle damage (470). Patients with DMD exhibit both metabolic and mitochondrial dysfunction (90), while oxidative phosphorylation and ATP synthesis is compromised in *mdx* mice (473). As expected, lower RER values were observed during the 12 h light period (6am – 6pm), when mice are sleeping and typically less active, when compared to the more active 12 h dark period (6pm – 6am) (474). The *mdx*-hdf mice had lower RER values during the day and at night when compared to *mdx* littermates (*p* = 0.015 and *p* = 0.040, respectively; main effect strain; 2-way GLM-ANOVA; Figure 4.3 A-B). This is indicative of a shift towards more oxidative metabolism and greater reliance on lipid as a substrate (475) with the genetic reduction of versican. Light or dark resting energy expenditure (REE) did not differ between *mdx* and *mdx*-hdf mice (Figure 4.3 C and D). Increased spontaneous physical activity in the *mdx*-hdf mice (Figure 4.2 A) may have contributed to this lack of difference in REE.
Figure 4.3 Effects of versican reduction on whole body metabolism and energy expenditure.
Mice were singly housed in individual metabolic cages for 24 h. A-B) RER values for the light and dark periods were lower in mdx-hdf mice compared to mdx littermates (*p = 0.015 and *p = 0.040, respectively; main effect strain; 2-way GLM ANOVA with factors being strain and time). C-D) REE during the light or dark periods was not different between mdx and mdx-hdf mice (p= 0.945 and p = 0.574, respectively; 1-way GLM ANOVA). Light Period = 6am – 6pm; dark period = 6pm – 6am; RER = respiratory exchange ratio; REE = resting energy expenditure. N = 9 mdx and n = 12 mdx-hdf mice.
4.3.5 Cardiac function is maintained in *mdx*-hdf mice despite increased heart mass and left ventricular dilatation

Versican is essential for embryonic development of the heart (331). The hdf mice are referred to as the heart defect mice, as homozygous deletion of versican is embryonically lethal at E10.5 due to malformation of the heart, whereas heterozygous hdf mice are viable (331). Due to the loss of dystrophin, patients with DMD, and *mdx* mice, present with a dilated cardiac myopathy and increased myocardial fibrosis (133, 405, 406). It is likely that this increase in myocardial fibrosis is associated with an upregulation of versican (476). Thus, given the association of versican with fibrosis and the importance of versican for heart development, it was important to consider the effects of versican reduction on cardiac function in dystrophic *mdx* mice. This is especially important as it could potentially have implications for spontaneous physical activity.

At 26 weeks of age, hearts from *mdx*-hdf mice were heavier than those from *mdx* littermates (*p = 0.032; Figure 4.4 A), even when normalised to body weight (*p = 0.020; Figure 4.4 B).

![Graph showing heart weight and heart weight normalized to body weight](image)

**Figure 4.4 Genetically reduced versican increases heart weight.**

**A)** The *mdx*-hdf mice had heavier hearts (*p = 0.032; T-test) than their *mdx* littermates at 26 weeks of age; **B)** even when normalised to body weight (*p = 0.020; T-test). *n* = 13 *mdx* and *n* = 16 *mdx*-hdf mice.
Echocardiography was performed to assess how the genetically reduced versican, and the associated increase in heart mass, affected cardiac function. When compared to their mdx littermates, mdx-hdf mice had a greater left ventricular internal diameter (LVIDs; p = 0.03; Table 4.2), a decreased ejection fraction (EF; p = 0.03; Table 4.2), and reduced fractional shortening (FS; p = 0.02; Table 4.2) indicating the mdx-hdf mice had a more dilated left ventricle compared to the mdx mice. However, contractile function did not appear to be severely compromised and these impairments were compensated for, as stroke volume (SV) was maintained in the mdx-hdf mice and did not differ from mdx littermates.
### Table 4.2 Heart echocardiography data from *mdx* and *mdx*-hdf mice hearts at 25 weeks of age

<table>
<thead>
<tr>
<th>Units</th>
<th><em>mdx</em></th>
<th><em>mdx</em>-hdf</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDs (MM)</td>
<td>cm</td>
<td>0.122 ± 0.011</td>
<td>0.173 ± 0.015</td>
</tr>
<tr>
<td>EF (MM-cubed)</td>
<td>%</td>
<td>90.5 ± 2.5</td>
<td>80.7 ± 3.0</td>
</tr>
<tr>
<td>FS (MM-cubed)</td>
<td>%</td>
<td>56.8 ± 4.0</td>
<td>44.1 ± 3.2</td>
</tr>
<tr>
<td>LV Mass (cubed)</td>
<td>g</td>
<td>0.678 ± 0.004</td>
<td>0.697 ± 0.011</td>
</tr>
<tr>
<td>SV (MM-cubed)</td>
<td>ml</td>
<td>0.021 ± 0.002</td>
<td>0.027 ± 0.005</td>
</tr>
</tbody>
</table>

Data are means ± SEM.

LVIDs = left ventricle internal diameter (systole); EF = ejection fraction; FS = fractional shortening; LV = left ventricle; SV = stroke volume.

* Statistical significance at p < 0.05 for all measurements, analysed by 2-tailed T-test.

n = 8 *mdx* and n = 12 *mdx*-hdf.
The genetic reduction of versican improved the diaphragm muscle strength and resistance to fatigue ex-vivo

The genetic reduction of versican had minimal effects on the twitch contractile properties of the dystrophic diaphragm muscles. The optimum muscle length ($L_o$), body mass to muscle mass ratio (BM:MM) and muscle cross sectional area (CSA), were similar between mouse strains (Table 4.3). In response to a 1 Hz stimulation, modelling a single action potential, twitch force ($P_t$), specific twitch force ($sP_t$), rate of force development ($\frac{dx}{dt}$) and $\frac{1}{2}$ relaxation time ($\frac{1}{2}$ RT) did not differ between diaphragm muscle strip from $mdx$ and $mdx$-hdf mice (Table 4.3). Although, the time to peak tension (TPT) was increased in $mdx$-hdf mice (Table 4.3). Factors contributing to this increase in TPT may include a shift to a different fibre type MyHC isoform composition or differences in regeneration efficacy.
**Table 4.3** *Ex-vivo* diaphragm twitch contractile properties from *mdx* and *mdx*-hdf mice at 21 weeks of age

<table>
<thead>
<tr>
<th>Units</th>
<th><em>mdx</em></th>
<th><em>mdx</em>-hdf</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L_o$</td>
<td>6.86 ± 0.16</td>
<td>7.22 ± 0.23</td>
<td>0.26</td>
</tr>
<tr>
<td>BM:MM</td>
<td>0.26 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.43</td>
</tr>
<tr>
<td>CSA</td>
<td>1.12 ± 0.07</td>
<td>1.05 ± 0.08</td>
<td>0.51</td>
</tr>
<tr>
<td>$P_t$</td>
<td>15.83 ± 2.04</td>
<td>18.35 ± 1.74</td>
<td>0.36</td>
</tr>
<tr>
<td>$sP_t$</td>
<td>14.45 ± 1.80</td>
<td>17.97 ± 1.64</td>
<td>0.17</td>
</tr>
<tr>
<td>TPT</td>
<td>0.2301 ± 0.0005</td>
<td>0.2314 ± 0.0004</td>
<td>0.03 *</td>
</tr>
<tr>
<td>$\frac{1}{2}$ RT</td>
<td>0.031 ± 0.002</td>
<td>0.035 ± 0.002</td>
<td>0.11</td>
</tr>
<tr>
<td>Max dx/dt</td>
<td>749.48 ± 99.70</td>
<td>848.76 ± 83.74</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Data are means ± standard error of mean.

* Statistical significance at $p < 0.05$ for all measurements, analysed by 2-tailed T-test.

$L_o$ = optimum muscle length; BM:MM = body mass to muscle mass ratio; CSA = cross sectional area; $P_t$ = peak twitch; $sP_t$ = specific peak twitch; TPT = time to peak tension; $\frac{1}{2}$ RT = half relaxation time; Max dx/dt = maximal rate of force development.

$n = 11$ *mdx* and $n = 15$ *mdx*-hdf.
The ex-vivo maximal force producing capacity (sPo) of the diaphragm muscle strips was determined from a force frequency curve (1 to 120 Hz). Diaphragm muscle strength was increased in *mdx*-hdf mice when compared to *mdx* littermates, as indicated by an upward shift of the force frequency curve (*p < 0.001; Figure 4.5).

Figure 4.5 The genetic reduction of versican increased diaphragm muscle strength in *mdx*-hdf mice.
A force frequency curve was used to assess the strength of diaphragm muscle strips from *mdx* and *mdx*-hdf mice. The genetic reduction for versican increased the force producing capacity of diaphragm muscles, as indicated by the upward shift in the force frequency curve (*p < 0.001; main effect strain; 2-way GLM-ANOVA with factors being frequency and strain). n = 11 *mdx* and n = 15 *mdx*-hdf mice.
Following completion of the force frequency protocol, the diaphragm muscle strips were subjected to a 4 m period of intermittent, submaximal 60Hz stimulations followed by a single 60 Hz stimulation at 2, 5 and 10 minutes post fatigue to assess fatigability and force recovery (408-411). During the 4 m of intermittent submaximal stimulation, diaphragm muscles from \textit{mdx-hdf} mice fatigued less compared to their \textit{mdx} littermates (\(p < 0.001\); Figure 4.6), and force recovery at 2, 5 and 10 m post fatigue was also improved (\(p < 0.001\); Figure 4.6). Altogether, the genetic reduction of versican improved diaphragm muscle function in \textit{mdx-hdf} mice. Therefore, the increase in movement displayed by the \textit{mdx-hdf} mice (Figure 4.2 A) may be mediated by this increase in diaphragm muscle strength and endurance. To gain better insight into how the genetic reduction of versican may improve diaphragm contractile function, muscle morphology, along with gene markers of inflammation, regenerative myogenesis and matrix remodelling were further investigated.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure46}
\caption{Reduced fatigability and improved force recovery in diaphragm muscle strips from \textit{mdx-hdf} mice. During 4 m of intermittent, submaximal stimulation, diaphragm muscle strips from \textit{mdx-hdf} mice fatigued less (\(* p < 0.001\); main effect strain; 2-way GLM ANOVA) and produced more force during recovery (\(* p < 0.001\); main effect strain; 2-way GLM ANOVA with factors being time and strain) when compared to diaphragm muscle strips from \textit{mdx} mice. \(N = 11 \text{ \textit{mdx}}\) and \(n = 13 \text{ \textit{mdx-hdf}}\).}
\end{figure}
4.3.7 The genetic reduction of versican had modest effects on diaphragm muscle fibre size

Morphological analysis of H&E stained diaphragm muscle cross-section was undertaken for mdx and mdx-hdf diaphragms (Figure 4.7 A-B). Typical of dystrophic diaphragm muscles from mdx mice, extensive fibrosis and mononuclear infiltrate, comprising of inflammatory cells, fibroblasts and muscle progenitor cells, were observed. Myofibres were centrally nucleated, indicating recent damage and repair, and fibre size was variable due to ongoing degeneration-regeneration and fibre splitting (477). The number of myofibres per mm² did not differ between diaphragm muscles from mdx and mdx-hdf mice (Figure 4.7 C). Although, the genetic reduction of versican tended to increase the proportion of centrally nucleated fibres in mdx-hdf mice (p = 0.086; Figure 4.7 D).

Given the variability in fibre morphology, minimal ferets diameter was used to assess muscle fibre cross-sectional area, which was binned to better observe differences in fibre size. Diaphragm muscles of mdx-hdf mice had significantly fewer very small myofibres in size (< 9.99 µm; p = 0.031), and all together the data suggests a slight shift towards increased cross-sectional areas when compared to mdx mice (Figure 4.7 E). This could contribute the increase in force production and the upward shift of the force frequency curve in mdx-hdf compared to mdx mice (Figure 4.5), as larger fibres produce more force.

The percentage of muscle cross-section comprised of mononuclear infiltrate did not differ between mdx and mdx-hdf mice (Figure 4.7 F). Given that this mononuclear infiltrate includes inflammatory cells and muscle precursory cells, the effects of versican reduction on gene markers of inflammation and myogenic differentiation were characterised.
Figure 4.7 A reduction in versican had no effect on diaphragm muscle properties.

A-B) Representative H&E stained sections of *mdx* and *mdx-hdf* diaphragms at x20 magnification. C) No differences were observed in the number myofibres (p = 0.450; T-test). D) The percentage of centrally nucleated fibres tended to be increased in diaphragm muscles from *mdx-hdf* mice (p = 0.086; T-test) compared to *mdx* littermates. E) Additionally, analysis of minimal ferets diameter revealed the diaphragm muscles from *mdx-hdf* had fewer very small fibres, less than 9.99 µm in diameter (*p = 0.031; T-test). F) Mononuclear infiltrate and degeneration did not differ between diaphragm muscles from *mdx* and *mdx-hdf* mice (p = 0.791; T-test). Scale bar = 100 µm. N = 11 *mdx* and n = 11 *mdx-hdf* mice.
4.3.8 Genetic reduction of versican and gene markers of inflammation

To gain preliminary insight into the effects of versican reduction on inflammation in dystrophic diaphragm muscles, the mRNA transcript abundance of Has2, Mcp-1, Tgfβ1 and F4/80 were quantified using qRT-PCR. Has2 is the predominant form of hyaluronan synthase in skeletal muscle, involved in the synthesis of hyaluronan (446). Hyaluronan forms a complex with versican, together modulating inflammatory processes by binding inflammatory cells (48, 240). Furthermore, versican also binds MCP-1, an attractant protein for monocytes and macrophages (478). Has2 mRNA transcripts had a trending decrease in mdx-hdf mice (p = 0.057; Figure 4.8 A), whereas Mcp-1, Tgfβ1 and F4/80 mRNA transcript abundance did not differ between mdx and mdx-hdf mice (Figure 4.8 C, E and G). F4/80 is a rather non-specific inflammatory cell marker, being expressed by monocytes, macrophages and other inflammatory cells (479, 480).

The pathology of mdx mice is highly variable, as shown through indicators of damage such as Evans blue dye staining and creatine kinase activity (481). The damage to mdx muscles can therefore significantly impact gene expression data. Further analysis was therefore undertaken to investigate a possible correlation between versican mRNA transcript abundance and gene markers of inflammation, as versican has been associated with a pro-inflammatory state (47, 48). Has2, Mcp-1 or Tgfβ1 mRNA transcript abundance was not correlated with VO/V1 Vcan gene expression (Figures 4.8 B, D and F). However, F4/80 mRNA transcript abundance was positively correlated with VO/V1 Vcan gene expression in mdx (*p = 0.0044; Figure 4.8 H) but not mdx-hdf diaphragms. This is interesting as both monocytes and macrophages can produce versican (290, 390, 391). Therefore, these observations should be followed up in future studies with an assessment of monocyte and macrophage infiltration in mdx and mdx-hdf diaphragm muscles using immunohistochemistry or flow cytometry, along with additional markers specific for neutrophils, monocytes, and pro-inflammatory M1 and anti-inflammatory M2 macrophage subsets.
Figure 4.8 Gene markers of inflammation in diaphragm muscles from *mdx* and *mdx-hdf* mice.

A) *Has2* mRNA transcripts had a trending decrease in diaphragm muscles from *mdx-hdf* versus *mdx* mice (*p* = 0.057); C, E and G) whilst *Mcp-1, Tgfβ1 and F4/80* mRNA transcripts did not differ between diaphragm muscles from *mdx* and *mdx-hdf* mice (*p* = 0.118, *p* = 0.288 and *p* = 0.307, respectively; T-test). B, D and F) The mRNA transcript abundance of *Has2, Mcp-1 or Tgfβ1* did not correlate with *V0/V1* Vcan gene expression in diaphragm muscles from *mdx* and *mdx-hdf* mice; H) whilst *F4/80* mRNA transcript abundance was correlated with *V0/V1* Vcan gene expression in diaphragm muscles from *mdx* mice (*p* = 0.0044) but not *mdx-hdf* mice (*p* = 0.8045). N = 9 *mdx*; n = 11-12 *mdx-hdf* mice.
4.3.9 Genetic reduction of versican and gene markers of regenerative myogenesis

As muscle regeneration has a large role in muscular dystrophy, the characterisation of genes relevant to myogenesis were of particular interest. The mRNA transcript abundance of *Pax7, Desmin, MyoD* and *Myogenin* did not differ between diaphragm muscles from *mdx* and *mdx*-hdf mice (Figure 4.9 A, C, E and G).

Again, given the association of versican with myogenesis (17, 20), a possible correlation between versican mRNA transcript abundance and gene markers of myogenesis was investigated. No correlation between *V0/V1 Vcan* gene expression and *Pax7* or *Desmin* mRNA transcript abundance was observed in diaphragm muscles from *mdx* or *mdx*-hdf mice (Figure 4.9 B and D). *Pax7* is a transcription factor important for regulating satellite cell proliferation (482) and desmin is highly expressed by proliferating satellite cells, as well as newly regenerated myotubes (483, 484). *MyoD* mRNA transcript abundance and *V0/V1 Vcan* gene expression were positively correlated in diaphragm muscles from *mdx* mice (p = 0.0469; Figure 4.9 F). *Myogenin* mRNA transcript abundance also tended to be positively correlated (p = 0.0673; Figure 4.9 H), whereas, in diaphragm muscles from *mdx*-hdf mice, no correlation was observed for *MyoD* or *Myogenin* (Figure 4.9 F and H). The association of versican with a gene marker of satellite cell differentiation and myoblast fusion in *mdx* diaphragm muscles is interesting. Especially given that highest transient upregulation of versican mRNA transcripts is observed in developing embryonic muscles between E14.5 and E15.5 (17), which correlates with myoblast differentiation and fusion (485). No gene correlations tested here were of any significance in the *mdx*-hdf diaphragms. Altogether, these data suggest an association between versican and regenerative myogenesis, although the specific cellular pathways regulated by versican and the consequences of versican reduction on the regenerative capacity of dystrophic muscles requires further investigation.
Figure 4.9. Gene markers of regenerative myogenesis in diaphragm muscles from \textit{mdx} and \textit{mdx-hdf} mice.

\textbf{A, C, E and G)} The mRNA transcript abundance of \textit{Pax7}, \textit{Desmin}, \textit{MyoD} and \textit{Myogenin} were similar in diaphragms from \textit{mdx} and \textit{mdx-hdf} mice (p = 0.286, p = 0.369, p= 0.378 and p = 0.374, respectively; T-test). \textbf{B and D)} No correlation between the mRNA transcript abundance of \textit{Pax7} or \textit{Desmin} and \textit{V0/V1 Vcan} gene expression was observed in diaphragms from \textit{mdx} and \textit{mdx-hdf} mice. \textbf{F)} \textit{MyoD} mRNA transcript abundance was correlated with \textit{V0/V1 Vcan} gene expression in diaphragms from \textit{mdx} mice (*p = 0.0469), but not \textit{mdx-hdf} mice (p = 0.9795). \textbf{H)} \textit{Myogenin} mRNA transcript abundance tended to be positively correlated with \textit{V0/V1 Vcan} gene expression in diaphragms from \textit{mdx} mice (*p = 0.0673) but not \textit{mdx-hdf} mice (p = 0.6042). N = 9 \textit{mdx} and n = 11-12 \textit{mdx-hdf} mice.
4.3.10 Genetic reduction of versican and ECM gene markers

Upon the conclusion of regeneration, the transitional matrix is remodelled and replaced with a mature matrix rich in collagens and CS proteoglycans, such as decorin and biglycan (486). The differential expression of collagen isoforms distinguishes the interstitial matrix collagens (e.g. collagen I and III) from those in the basement membrane matrix (e.g. collagen IV and VI) (21, 36, 37). Col1a1 and Col4a1 gene expression did not differ between diaphragm muscles from mdx and mdx-hdf mice (Figure 4.10 A and E). However, a trend for reduced Col3a1 mRNA transcript abundance was observed in diaphragm muscles from mdx-hdf mice (p = 0.075; Figure 4.10 C).

As was done for the gene markers of inflammation and myogenesis, the potential correlation between versican gene expression and collagen mRNA transcript abundance was determined in the diaphragms. Col1a1 mRNA transcript abundance and V0/V1 Vcan gene expression were positively correlated in mdx mice (p = 0.0388; Figure 4.10 B), but not mdx-hdf mice. However Col3a1 and Col4a1 mRNA transcripts were not correlated with versican gene expression in either mdx or mdx-hdf diaphragm muscles (Figure 4.10 D-F). No gene correlations tested here were of any significance in the mdx-hdf diaphragms.
Figure 4.10 Collagen mRNA transcript abundance in diaphragm muscles from \textit{mdx} and \textit{mdx-hdf} mice.

A, C and E) \textit{Col3a1} gene expression tended to be lower in diaphragm muscles from \textit{mdx-hdf} diaphragms compared to \textit{mdx} littermates (\( p = 0.075 \); T-test), whereas \textit{Col1a1} and \textit{Col4a1} were similar between \textit{mdx} and \textit{mdx-hdf} diaphragms (\( p = 0.152 \) and \( p = 0.915 \), respectively; T-test). B) \textit{Col1a1} mRNA transcript abundance was positively correlated with \( V0/V1 \) \textit{Vcan} gene expression in diaphragms from \textit{mdx} mice (\(*p = 0.0388\)), but not \textit{mdx-hdf} mice (\( p = 0.1403 \)). D and F) \textit{Col3a1} and \textit{Col4a1} mRNA transcript abundance did not correlate with \( V0/V1 \) \textit{Vcan} gene expression in diaphragms from \textit{mdx} or \textit{mdx-hdf} mice. \( N = 9 \) \textit{mdx} and \( n = 11-12 \) \textit{mdx-hdf} mice.
The gene expression of *Decorin* and *Biglycan* was also investigated, as both are upregulated in dystrophic muscles (42, 487) and are highly relevant to the pathogenesis of the disease. Decorin has been shown to regulate the bioavailability of TGFβ (488) and biglycan is thought to be important in stabilising the sarcolemmal expression of utrophin, the functional homologue of dystrophin (62). The gene expression of smooth muscle alpha actin 2 (*Acta2*) was also measured, as *Acta2* is a marker for myofibroblasts (489), and because versican has been implicated in driving the differentiation of fibroblasts to myofibroblasts (265, 348). In dystrophic muscles, myofibroblasts are thought to significantly contribute to collagen synthesis and fibrosis (107).

The mRNA transcript abundance of *Decorin*, *Biglycan* and *Acta2* was similar in diaphragm muscles from *mdx* and *mdx*-hdf mice (Figure 4.11 A, C and E). The mRNA transcript abundance of *Decorin* was not correlated with versican gene expression in either *mdx* or *mdx*-hdf diaphragm muscles (Figure 4.11 B). Whereas, *Biglycan* mRNA transcript abundance and *V0/V1 Vcan* gene expression were positively correlated in diaphragm muscles from *mdx* mice (p = 0.0266; Figure 4.9 D), but not *mdx*-hdf mice. Interestingly, *Acta2* mRNA transcript abundance and *V0/V1 Vcan* gene expression were negatively correlated in diaphragm muscles from *mdx* mice (p = 0.0221; Figure 4.9 F), but not *mdx*-hdf mice. This was somewhat surprising given that versican may mediate the fibroblast to myofibroblast transition (265, 348). Although, as correlations do not indicate causality, given that myofibroblasts secrete a collagen rich matrix rather than a transitional matrix, it can be speculated that perhaps the muscles with the highest *Acta2* gene expression had the most severe pathology and fibrosis. No gene correlations tested here were of any significance in the *mdx*-hdf diaphragms.
Figure 4.11. Decorin, Biglycan and Acta2 mRNA transcript abundance in diaphragm muscles from mdx and mdx-hdf mice.

A, C and E) The mRNA transcript abundance for the small proteoglycans Decorin and Biglycan, and for Acta2, a myofibroblast marker, did not differ between diaphragm muscles from mdx and mdx-hdf mice (p = 0.251 p = 0.462 and p = 0.308, respectively; T-test). B) No correlations were present between Decorin or V0/V1 Vcan gene expression. D) Biglycan was positively correlated with V0/V1 Vcan gene expression in mdx diaphragms (*p = 0.0266), but not mdx-hdf mice (p = 0.5877). F) Acta2 was negatively correlated with V0/V1 Vcan gene expression in the mdx diaphragms (*p = 0.0221), but not mdx-hdf mice (p = 0.1407). N = 9 mdx and n = 11-12 mdx-hdf mice.
Previous studies investigating fibrosis have used a fluorescently labelled wheat germ agglutinin (WGA) to stain affected areas (415, 490). WGA is known to bind to a variety of ECM components (491), including proteoglycans and GAG rich regions of the matrix (492), along with collagen I (415). WGA is also routinely used to stain cell membranes (493, 494), where it binds to glycoproteins and glycolipids (495). These factors limit the sensitivity of WGA for analysis of fibrosis. The percentage of diaphragm muscle cross-section stained with WGA did not differ between \textit{mdx} and \textit{mdx-hdf} mice (Figure 4.12 C). However, given the limitations of WGA staining, this finding needs to be confirmed with a more quantitative marker of collagen deposition and fibrosis, such as muscle hydroxyproline content.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4_12}
\caption{Fibrosis in diaphragm muscles from \textit{mdx} and \textit{mdx-hdf} mice as determined by WGA staining.}
\end{figure}

Fibrosis (WGA positive tissue) is red, nuclei are stained blue (DAPI). A-B) Representative \textit{mdx} and \textit{mdx-hdf} diaphragm sections stained with WGA, taken at x20 magnification. C) The proportion of muscle cross-section stained with WGA did not differ between the diaphragm muscles from \textit{mdx} and \textit{mdx-hdf} mice (p = 0.251; T-test). Scale bar = 200 µm. n = 9 \textit{mdx} and n = 10 \textit{mdx-hdf} mice.
4.3.11 The genetic reduction of versican and mRNA transcript abundance of ECM proteases

Fibrosis encompasses not only aberrant ECM synthesis, but also remodelling through protease upregulation in DMD (496, 497). The versican-hyaluronan rich transitional matrix (74) is remodelled by ADAMTS versicanases, including ADAMTS1, -5 and -15 (315, 316, 319) and by hyaluronidases, with Hyal2 being the predominant isoform in muscle (446). Hyal2, Adamts1 and Adamts5 mRNA transcript abundance was similar in diaphragm muscles from mdx and mdx-hdf mice (Figure 4.13 A, C and E). Although, Adamts15 gene expression tended to increase in mdx-hdf mice (p = 0.061; Figure 4.13 G). The biological relevance of this modest difference in Adamts15 gene expression is likely minimal, as versikine levels (as determined from immunohistochemical analysis in Figure 4.1 F) did not differ between diaphragm muscles from mdx and mdx-hdf mice. Whereas Hyal2, Adamts1 and Adamts15 mRNA transcripts did not correlate with V0/V1 Vcan (Figure 4.13 B, D and H), the Adamts5 mRNA transcript tended to have a positive correlation with V0/V1 Vcan gene expression in mdx diaphragms (p = 0.0761; Figure 4.13 F), but not mdx-hdf diaphragms.
Figure 4.13. Gene markers of transitional matrix remodelling in diaphragm muscles from mdx and mdx-hdf mice.

A, C and E) Hyal2, Adamts1 or Adamts5 mRNA transcript abundance did not differ between mdx and mdx-hdf diaphragm muscles (p = 0.626, p = 0.844 and p = 0.337, respectively; T-test). G) Adamts15 gene expression tended to be increased in mdx-hdf diaphragm muscles (p = 0.061; T-test). B, D, and H) Hyal2, Adamts1, and Adamts15 mRNA transcript abundance did not correlate with V0/V1 Vcan gene expression in diaphragm muscles from mdx or mdx-hdf mice. F) Adamts5 mRNA transcript abundance tended to positively correlate with V0/V1 Vcan gene expression in diaphragms from mdx (p = 0.0761), but not mdx-hdf mice (p = 0.1554). N = 9 mdx and n = 12 mdx-hdf mice.
4.3.12 The improved fatigability of mdx-hdf diaphragm muscles is not mediated by a shift in the proportion of type IIa fibres

Diaphragm muscles from 3-4 month old mdx mice contain approximately 55% type IIa fibres and 10% type I fibres, and together these account for the endurance phenotype of the diaphragm muscle (498). The proportion of fast, glycolytic type IIx fibres is much lower, being less than 20% (498). To test whether a shift to a more oxidative phenotype accounted for the improvement in fatigability (see Figure 4.6) and the increase in TPT (see Table 4.3), the proportion of type IIa fibres was assessed. As previously reported (498), the proportion of type IIa fibres in mdx diaphragm muscles is approximately 55%. However, the proportion of type IIa fibres in diaphragm muscles did not differ here between diaphragms from mdx and mdx-hdf mice (Figure 4.14 C). To conclusively rule out a fibre type shift with the genetic reduction of versican, the proportion of type I fibres will also need to be assessed in future studies. Interestingly, type IIa fibres from mdx-hdf mice tended to have a lower proportion of centrally nucleated fibres (p = 0.087; Figure 4.14 D). This in contrast to analysis of the H&E stained diaphragm muscle cross-sections, where muscles from mdx-hdf mice tended to have an increase in in the percentage of centrally nucleated fibres when compared to mdx littermates (Figure 4.7 D). However, whether this is of functional significance with regards to endurance or strength remains to be determined.
Figure 4.14 The genetic reduction of versican does not affect proportion of type IIa fibres in diaphragm muscles. MyHC IIa staining is green. A-B) Representative cross-sections of MyHC IIa immunoreactivity along with corresponding bright field images for orientation. C) The percentage of type IIa fibres was similar in diaphragms from mdx and mdx-hdf mice (p = 0.713; T-test). D) In type IIa fibres, the genetic reduction of versican tended to reduce the proportion of centrally nucleated fibres in mdx-hdf mice (p = 0.087; T-test). N = 6 mdx and n = 10 mdx-hdf mice. Scale bar = 100 µm.
4.4 Discussion

In DMD, fibrosis is not just a disease end point, but rather precedes overt muscle degeneration and actively contributes to the progression of disease pathology (94, 124). The aberrant ECM synthesis and remodelling associated with fibrosis in dystrophic muscles dysregulates inflammation and regenerative myogenesis. This potentiates further fibrosis, which in turn compromises contractile function (207). The fibrosis in DMD is associated with an upregulation of mature proteins, such as collagen I and collagen IV, and transitional matrix proteins such as fibronectin and versican (90, 119, 221). It can therefore be proposed that that a novel approach to target fibrosis in dystrophic muscles is to modulate the transitional matrix. Of particular relevance to DMD pathology is versican, as it is overexpressed in dystrophic muscles and downregulated by glucocorticoids (90, 465), the only treatment with clinical efficacy in DMD (371). This hypothesis was tested in this Chapter by genetically reduced versican in mdx mice, thereby modulating the transitional matrix in order to characterise the effects on whole body physiology, and diaphragm muscle structure and function. It should also be noted that this is the first time this strain of mice has been bred, and is hence a novel strain of dystrophic mice. In dystrophic mdx mice, the diaphragm is the most severely affected muscle with regards to fibrosis and deficits in contractile function, and as such most closely resembles the human form of the disease (454, 455). V0/V1 versican expression was found upregulated in diaphragm and hindlimb muscles from mdx mice, compared to wild type muscles (465). Furthermore, it is the worsening respiratory function in patients with DMD which contributes to the loss of ambulation and reduction in life expectancy (111, 499).

Male heterozygous heart defect (hdf) mice were bred with female mdx mice to produce male F1 mdx or mdx-hdf pups. The successful genetic reduction of versican was initially confirmed through genotyping, but was further confirmed by qRT-PCR analysis and immunohistochemistry. Despite a genetic reduction in V0/V1 versican expression, versikine (the cleaved form of versican) immunoreactivity did not differ between muscles from mdx and mdx-hdf mice.
This observation is not entirely unexpected, as versikine is not the end product of versican remodelling and is perhaps further degraded, as shown by the decreased expression in the 3 week old hind limb compared to an embryonic hindlimb at E13.5 (17). Similarly, versikine immunoreactivity did not differ between diaphragm muscles from \(mdx\) and wild type mice, despite greater V0/V1 versican gene and protein expression in the dystrophic diaphragm muscles (465). These observations are further supported by findings in Chapter 3, where significantly increased amounts of versican are present in 12 week old \(mdx\) mice when compared to normal and healthy wild type mice of the same age.

Given that versican is a transitional matrix component (74), its synthesis and remodelling are key to regulating cellular processes relevant to inflammation and regeneration in dystrophic muscles. As previously reported in hindlimb muscles (465), versikine was also localised to regions of mononuclear infiltrate in diaphragm muscles in this thesis. Mononuclear infiltrate is comprised of cells that are known to secrete versican and ADAMTS versicanases, such as myoblasts (17, 20, 465), macrophages (228, 500, 501) and fibroblasts (265, 502). Nuclear localisation of versikine was also observed within nuclei of centrally nucleated fibres, as well as in nuclei of unidentified cells in the endomysium. Previously, it has been suggested that nuclear versikine may have an important role in mitotic spindle organisation during cell proliferation (336).

In \(mdx\) mice, the genetic reduction of versican had no effect on body composition, including relative and absolute lean mass and fat mass, and had a modest effect on heart size, as indicated by an increase in absolute and normalised heart mass and a more dilated left ventricle. Nonetheless, cardiac function was compensated for, as indicated by a similar stroke volume in \(mdx\) and \(mdx\)-hdf mice. As 26 weeks of age equals 6 months, the heart weight, heart weight to body weight ratio, and stroke volume in these \(mdx\) mice are therefore similar to the data published for 7 month old \(mdx\) mice (503). The ejection fraction however, was much higher in this Chapter than what was published for the 7 month mice (503), possibly because the 7 month mice have a more
developed cardiomyopathy than the younger mice in this thesis. At this point, it is unclear whether the effects of versican reduction on the left ventricle are due to versican expression in the dystrophic myocardium or due to development effects of versican heterozygosity, as versican is known to affect the hfd heart (331).

Understanding how targeting versican affects the heart is important, given that cardiac myopathies are first observed from approximately 10 years of age and progressively increase over the lifespan of the patient (406). Indeed, 98.7 % of DMD patients, aged 18 and above, presented with cardiomyopathy; 71.7 % of patients had the predominant dilated cardiomyopathy, while 26.1 % had a less common hypertrophic cardiomyopathy (406). Mdx mice also begin to acquire the characteristics of a dilated cardiomyopathy-between 29-42 weeks of age (133). Furthermore, it remains controversial as to whether the heart pathology is ameliorated (504), remains unchanged (505) or becomes worsened (506) after skeletal muscle was specifically targeted and treated in mdx mice. When exon skipping was used to partially restore dystrophin to skeletal muscle, although increasing the locomotor activities of mdx mice was beneficial, the increased activity put extra pressure on the dystrophic heart and thereby worsened cardiac pathology by damaging myocardiocytes and increasing fibrosis (507). A similar outcome was observed when mdx mice were treated for 6 months with streptomycin to block calcium channels, although improving the pathology in the TA muscle, the pathology of the heart became worsened, with increased necrosis and fibrosis (508). Additionally, there are also studies that highlight treatments which were beneficial in mice, but when trialled in humans, was found to have the opposite effect. For example, Sildenafil, a drug commonly used in treating erectile dysfunction and pulmonary hypertension, was found to have cardioprotective effects and improved the cardiac function of mdx mice (509). However when Sildenafil was trialled in dystrophy patients, with cardiomyopathy, the study was cancelled prematurely due to a number of patients having worsened heart function (510). It is known that therapeutic strategies that aim to reduce fibrosis, or improve the contractile function of
dystrophic skeletal muscles, can have deleterious effects on the dystrophic heart, and thus, for any future therapeutics, any potential cardiac side effects will need to be considered (511, 512). A limitation of this study is that the \textit{mdx} and \textit{mdx}-hdf hearts, although collected, were not examined further, therefore no morphological data is available.

The defects in contractile function in dystrophic limb and respiratory muscles from patients with DMD include a reduction in muscle strength, as well as a decrease in muscle endurance (513, 514). Abnormalities in mitochondrial function and substrate metabolism are thought to contribute to this defect in muscle endurance (473). Improved endurance and metabolism are important therapeutic outcomes for improving muscle health in DMD, as insulin resistance and metabolic abnormalities are common co-morbidities in DMD (515). Interestingly, the therapeutic benefits of glucocorticoids in patients with DMD are thought to include pro-ergogenic effects on metabolic programming, leading to increased muscle endurance and exercise capacity (516). Hence, \textit{mdx}-hdf mice and \textit{mdx} littermates were placed into metabolic cages for 24 h to assess spontaneous physical activity and whole body metabolism. \textit{Mdx}-hdf mice moved more than their \textit{mdx} littermates, irrespective of body weight. Whereas, in the \textit{mdx} littermates an increasing body weight was associated with a reduction in spontaneous physical activity. It is worth noting that the activity levels observed here for the \textit{mdx} and \textit{mdx}-hdf mice were slightly lower than those in a previously published study where, under similar conditions to the study in this thesis, 14 week old male \textit{mdx} mice exhibited a total of approximately 40,000 movements over a 24 h period (470). Although the \textit{mdx}-hdf mice match the heightened activity levels reported in these much younger mice, the \textit{mdx} mice in this thesis had 30,000 movements. This may be due to dystrophic pathology being more progressed and prevalent in the older \textit{mdx} mice used here in this thesis.

The genetic reduction of versican was associated with reduction in the RER, but not resting energy expenditure (REE), during both the light and dark periods, indicating a shift towards a more oxidative metabolism, and thus a greater
reliance on lipids as a substrate. Additional support of the observation that versican reduction might affect oxidative metabolism, is the reduction in fatigability and improved force recovery of diaphragm muscles from *mdx-hdf* mice following 4 m of intermittent, submaximal stimulation *ex-vivo*. This is a very novel observation, in that the downregulation of a single ECM protein can modulate whole body metabolism and *ex-vivo* diaphragm fatigability. It was hypothesised that in the context of insulin resistance and diabetes, the remodelling of the skeletal muscle ECM alters mechanosignal transduction, which disrupts the expression of genes relevant to oxidative metabolism and mitochondrial biogenesis (517). Indeed, defects in mitochondrial function and ATP synthesis have been well described in dystrophic muscle (473, 518). As such, improving the metabolic phenotype and endurance of dystrophic muscles is especially important.

In addition to the effects on diaphragm muscle endurance, there was an increase in *ex-vivo* force output, as indicated by an upward shift in the force frequency curve, in diaphragm muscles from *mdx-hdf* mice when compared to *mdx* littermates. Altogether, these data indicate that the genetic reduction of versican improves the contractile function of dystrophic diaphragm muscles. A reduction in proportion of very small muscle fibres (< 9.99 um in diameter), may mediate this increase in diaphragm muscle strength following the genetic reduction of versican. The force producing capacity of very small muscle fibres is likely quite limited, and the positive correlation between fibre size and strength is well established (519). There was also a trend for increased proportion of centrally nucleated fibres, along with a decreased proportion of centrally nucleated type IIa fibres, in *mdx-hdf* diaphragms when compared to *mdx* littermates. *In-vitro*, the clearance of a hyaluronan and versican rich transitional matrix potentiates myoblast fusion and myotube growth (17). In addition to the genetic reduction of versican, hyaluronan synthase 2 (*Has2*) mRNA transcript abundance tended to be lower in the diaphragms from *mdx-hdf* mice when compared to *mdx* littermates. Whether this correlated with reduced hyaluronan content remains to be determined. Perhaps, based on these findings, improved
fusion may have contributed to the observed changes in myofibre size and the proportion of centrally nucleated fibres through the reduction of versican and its chondroitin sulphate GAG chains (17, 457). Lending support to this theory, in Chapter 3 of this thesis, the treatment of C2C12 muscle cells was associated with the reduction of versican and hyaluronan gene expression, and improved myoblast fusion \textit{in-vivo}. The hypothesis therefore requires further analysis, and in future studies, the proportion of recently regenerated fibres expressing embryonic and developmental myosin heavy chain isoforms should be quantified in \textit{mdx} and \textit{mdx-hdf} diaphragms (520, 521).

To gain some insight into how versican reduction might affect muscle repair, the mRNA transcript abundance of gene markers associated with satellite cell proliferation and myoblast differentiation was determined. However, \textit{Pax7}, \textit{Desmin}, \textit{MyoD} and \textit{Myogenin} gene expression did not differ between diaphragms from \textit{mdx} and \textit{mdx-hdf} mice. However, since versican has been linked to myogenesis during development through satellite cell proliferation and differentiation (20), and given the inherent variability of dystrophic muscle pathology in \textit{mdx} mice (481), additional correlation analysis was therefore completed on the gene expression data. In diaphragm muscles from \textit{mdx} mice, but not \textit{mdx-hdf} mice, the mRNA transcript abundance of \textit{MyoD} was positively correlated, while \textit{Myogenin} tended to be positively correlated, with versican gene expression. Altogether, the gene expression and histological analyses suggest that altering versican levels in dystrophic muscles may have an effect on regenerative myogenesis. However, more detailed mechanistic studies are required. These may include studies upon single fibres, which could be immunohistochemically stained to investigate the effect of reduced versican on satellite cells (522), or investigations involving muscular injury, such as treadmill running or crushing the muscle, in order to observe the regenerative processes though immunohistochemical and histological staining.

The increased diaphragm muscle \textit{ex-vivo} force output and effects on muscle morphology may also be due to the effects versican reduction may have on inflammation. Indeed, attenuating inflammation has been shown to improve
muscle function in *mdx* mice due to a reduction in necrosis and an improvement in regeneration (377, 523). To begin to assess the effects of versican reduction on inflammation in diaphragm muscles, an analysis of H&E stained sections for area of mononuclear infiltrate was undertaken (413, 414). However, no differences between diaphragm muscles from *mdx* or *mdx*-hdf mice were observed. H&E analysis of mononuclear infiltrate is a rather non-specific marker of inflammation. It does not take into account any differences in cell types such as satellite cells, myoblasts, fibroblasts, nor any of the inflammatory cell populations, such as neutrophils, monocytes, or the differing subsets of M1 or M2 macrophages. Further analysis of neutrophil, monocyte and macrophage infiltration in dystrophic diaphragm muscles therefore needs to be undertaken via immunohistochemistry or flow cytometry, with markers for the different cell types.

Versican can modulate inflammation directly by binding leukocytes on its CS GAG chains (53), or through regulating the bioavailability of chemokines and cytokines, including MCP-1 (51). Additionally, versican can regulate inflammation indirectly through its interactions with hyaluronan, which in skeletal muscle is predominantly synthesised by HAS2 (446). To gain additional insight into the effect versican reduction on inflammation in dystrophic diaphragm muscles, *Has2*, *MCP-1*, *TGF-β1* and *F4/80* gene expression was assessed. However, it should be noted that *F4/80* is an unspecific marker for monocytes, M1 and M2 macrophages alike (524). Although *Has2* mRNA gene expression tended to be lower in diaphragm muscles from *mdx*-hdf mice, the other gene markers of inflammation were unaffected by versican reduction. When the gene expression data was further interrogated, a positive correlation between *F4/80* mRNA transcript abundance and *V0/V1 versican* was observed. This is interesting, because monocytes and macrophages both produce versican (290, 390, 391). Given that the *mdx*-hdf mice would have reduced versican in cells, due to being haploinsufficient for a versican allele, thereby being heterozygous for versican (331), perhaps there is less versican being made by monocytes and macrophages in the *mdx*-hdf mice. A reduction of versican would therefore have implications
on monocyte migration (389) and differentiation into macrophages (290). However, given that there was no difference in F4/80 gene expression, it could therefore be possible that the genetically reduced versican in mdx-hdf mice may result in an impaired differentiation of monocytes to macrophages, or may even affect the transition of the pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages.

The data acquired thus far suggests minimal effects of versican reduction on inflammation in mdx diaphragm muscles. However, the experimental model and analysis are limited to fully justify such a conclusion. Future experiments involving contraction induced injury protocol (e.g. voluntary wheel running or forced treadmill running) to heighten inflammation in dystrophic mdx muscles (525), should be used to assess whether versican does indeed modulate pro- and anti-inflammatory responses in dystrophic muscles.

Additionally, the increased force exhibited by the mdx-hdf diaphragm could be due to a decrease in fibrosis. It was hypothesised that reducing versican levels might attenuate fibrosis when the transitional matrix is remodelled and replaced with a mature matrix. As such the mRNA transcript abundance of interstitial and basement membrane collagen isoforms and mature matrix proteoglycans were measured. Only the basement membrane Col3a1 showed a trend for decreased gene expression in diaphragm muscles from mdx-hdf compared to mdx mice, whereas Col1a1 and Col4a1 were similar. Decorin, Biglycan and Acta2 gene expression were also not affected by the genetic reduction of versican. Interestingly, although Acta2 gene expression was negatively correlated, the mRNA transcript abundance of interstitial Col1a1 and Biglycan was positively correlated with V0/V1 versican gene expression in diaphragm muscles from mdx mice, but not mdx-hdf mice. These observations are suggestive that perhaps the genetic reduction of versican does affect ECM synthesis. However, the regions of fibrosis, as determined from WGA staining, remained similar between the mdx and mdx-hdf mice. Therefore, this hypothesis needs to be rigorously tested with specific measures of interstitial and basement membrane collagens, along with biglycan and Acta2 content in mdx and mdx-hdf diaphragm muscle, through, for
example, the use of a hydroxyproline assay, immunoblotting techniques or proteomic analysis. The genetic reduction of versican had minimal effects on the expression of genes associated with the remodelling of versican and hyaluronan rich transitional matrix, specifically *Hyal2, Adamts1* and *Adamts5* – a highly upregulated ADAMTS protease in dystrophic muscles (349, 465). This is in concordance with the observation of similar versikine immunoreactivity in *mdx* and *mdx*-hdf diaphragm muscles. Furthermore, although similar levels of *Hyal2* and *Adamts5* gene expression were also observed in the glucocorticoid treated C2C12 cells of chapter 3, *Adamts1* gene expression was found to be increased. This is suggestive that glucocorticoids may still influence pathways that are not affected by low levels of versican. However this is highly speculative and remains to be elucidated.

In conclusion, the genetic reduction of versican was associated with an increase in spontaneous physical activity, a shift to oxidative whole body metabolism, and improved *ex-vivo* diaphragm muscle strength and endurance in 21 week old *mdx*-hdf mice. An overview of data obtained in Chapter 4, and the hypothesized mechanisms, can be viewed in Figure 4.15. Although it is still unclear as to why the *mdx*-hdf mice display increased locomotion along with improved force and endurance, it can be speculated that in addition to the changes in ECM, it may have also led to an improvement in regeneration and metabolism – although, the underlying mechanisms require further analysis. Furthermore, to more comprehensively establish the benefits of versican reduction, the effects of muscle fibre types and growth must be considered, as the developmental matrix is extensively remodelled during growth to maturity. Therefore, further studies were then undertaken in fast and slow twitch hindlimb muscles from young, growing (6 weeks) and adult (26 weeks) *mdx* and *mdx*-hdf mice.
Figure 4.15 Overview of Chapter 4 diaphragm results and possible mechanisms.

This figure summarises the data obtained from the diaphragm in Chapter 4 and also outlines the possible mechanisms that were hypothesised.
Chapter 5: The genetic reduction of versican differentially modulates function and pathology of dystrophic hindlimb muscles depending on muscle type and postnatal growth

5.1 Introduction

DMD is caused by a mutation in the dystrophin gene leading to loss of expression of the functional protein (464). This results in sarcolemma fragility, causing skeletal muscles to become highly susceptible to contraction induced injury. Pathology is comprised of persistent and ongoing bouts of damage and regeneration, which progressively become dysregulated (285). This ultimately results in the loss of myofibres, which are replaced by adipose tissue and fibrosis (107), leading to decreased strength and weakness in the muscles (207). Fibrosis is first observed as an expansion of connective tissue in muscle biopsies from patients with DMD as young as 2.5 weeks of age (94). Fibrosis reaches its peak during a period of growth in DMD patients, at approximately 6-7 years of age (124). As glucocorticoids can reduce fibrosis (526), glucocorticoids should therefore be administered before patients reach 6-7 years of age, as fibrosis determines, and may impair, the efficiency of the treatment (124). There is also increasing evidence that a dysregulated and fibrotic matrix may contribute to the metabolic dysfunction observed in dystrophic muscles (see Chapter 4). Therefore, this highlights the need to better understand the fibrotic matrix, as any effective therapeutic intervention for DMD must consider the ECM.

In dystrophic muscles from patients with DMD and mdx mice, the expansion of the ECM encompasses interstitial and basement membrane proteins. These include collagens and fibronectin (221, 285). Additionally, there is an increase in proteoglycans, particularly those containing GAG side chains (116); specifically, decorin, biglycan (42, 487), and versican (90, 465).
Normally, following an injury and as part of the regenerative response, there is the carefully regulated synthesis and secretion of a transitional matrix rich in versican, hyaluronan, tenascin and fibronectin (73, 74). This transitional matrix is loose and hydrated, functioning as a specialised structural support for the infiltration and guidance of inflammatory cells, satellite cells and fibroblasts (74, 527). Successful regeneration also requires remodelling of this transitional matrix and the deposition of a mature matrix, rich in collagens (382, 528) and CS proteoglycans, such as decorin and biglycan (486). However, in DMD, where fibrosis is aberrant due to persistent injury and regeneration, repair is ineffective and transitional matrix proteins remain overexpressed, contributing to the continual increase in fibrosis.

This dysregulation of the transitional matrix is observed not only in patients with DMD, but also in *mdx* mice. Fibronectin and tenascin-C are increased in muscles from DMD patient and *mdx* mice (421, 529, 530). Likewise, hyaluronan content and synthesis, shown via increased *Has1* and *Has2* mRNA transcripts, is also increased in *mdx* mouse muscles (435), while versican is overexpressed in muscles from DMD patients (90) and *mdx* mice (465). Hence, the transitional matrix proteins are also aberrantly synthesized in DMD.

Versican is a developmental protein, its expression being low in mature muscle, unless there is damage or a disease, whereupon its expression becomes upregulated (311). Highly relevant to DMD pathology, is the fact that versican can be synthesised by inflammatory cells, fibroblasts and myoblasts (17, 265, 290). Through its synthesis and remodelling, versican may affect myoblast proliferation (20) and differentiation of myoblasts (17), monocytes and fibroblasts (265, 348). Carefully regulated ECM synthesis and remodelling is not just important for muscle regeneration and pathology in DMD, it is also likely quite relevant to normal postnatal growth. As such, the skeletal muscles of patients with DMD experience the stress of dystrophin deficiency alongside normal growth. This is rarely considered in experimental studies investigating ECM remodelling and fibrosis in *mdx* mice, yet it is absolutely crucial, as carefully regulated ECM synthesis by fibroblasts is needed for satellite cell function (531).
Furthermore, results from Chapter 4 hinted at a change in fibre type, which brings about another limitation in that fibre types were not well investigated. It is well known that fast and slow fibre types have differing matrices. Slow twitch fibres contain more dystrophin (64), utrophin (65) and collagen IV (11), leading to a more stabilised and flexible basement membrane and allowing for the dissipation of contractile produced force. However, the fast twitch fibres contain more laminin (11) which provides more adhesion to the matrix, and as such, fast twitch fibres not only produce the most force (66), but are also the most damaged, being prone to contraction induced injuries (68). Further differentiating the fibre types are their contractile differences and metabolic requirements (532).

In Chapter 4, it was observed that in adult (21 week old) mdx mice, the genetic reduction of versican improved the strength and endurance of diaphragm muscles and increased spontaneous physical activity. Here, these observations were followed up taking postnatal growth and fibre type into account when considering the effects the genetic reduction of versican might have on the function and pathology of mdx hindlimb muscles. The strength, endurance and pathology of fast twitch EDL muscles and slow twitch soleus muscles were assessed in young, growing (6 week old) and adult (26 week old) mdx and mdx-hdf mice.
5.2 Methods

5.2.1 Ethics statement and animal husbandry

All mouse experiments were approved by Deakin University’s Animal Ethics Committee, under projects numbers A79/2011 and G06/2015. Female \textit{mdx} mice obtained from the Animal Resource Centre (Canning Vale, Western Australia), were bred with male hdf (heart defect) mice obtained from Hoffman-La Roche Pharmaceuticals. The hdf mice are haploinsufficient for the versican allele. All male pups underwent an ear punch biopsy and the resulting F1 generation \textit{mdx} and \textit{mdx}-hdf male pups were confirmed through genotyping. For a more detailed description, please refer to section 2.3.2. All mice were maintained on an alternating 12 h light and 12 h dark cycle, with standard mouse chow and water provided \textit{ad libitum}. All experimental procedures in the Chapter were completed on mice at 6 or 26 weeks of age.

5.2.3 Contractile function

Mice at 6 and 26 weeks of age were anaesthetised with medetomidine (0.5mg/kg), midazolam (5mg/kg) and fentanyl (0.05mg/kg), administered via an IP injection in ~1 ml sterile saline, until unresponsive to tactile stimuli. The EDL and soleus were removed and prepared for contractile function testing. Using small increments in length and 1 Hz twitches, the optimal length (Lo) was determined. This allowed for the assessment of twitch force (Pt), twitch force normalised to muscle size (sPt), time to peak tension (TPT), ½ relaxation time (½ RT), and maximal rate of force production (max dx/dt). Following which, the maximal force producing capacity for the EDL and soleus muscle was determined from a force frequency curve (sPo), ranging from 1 to 120 Hz with 2 m rest in between each stimulation. Afterward, at a submaximal stimulation frequency of 60 Hz, the muscles were stimulated every 5 s for a total duration of 4 mins, in order to fatigue the muscle. Muscle recovery was then assessed by stimulating the muscles at 60 Hz at 2, 5 and 10 mins post fatigue. For a more detailed description, please refer to section 2.3.6.
After contractile testing had concluded, the 6 and 26 week EDL and soleus muscles were removed of tendon, weighed and snap frozen in liquid nitrogen for gene expression analysis. The remaining EDL and soleus from the opposing and untested hindlimb were frozen in thawing isopentane for histological and immunohistochemical analysis. Furthermore, before mice were humanely culled, blood was collected via cardiac puncture for use in the total creatine kinase assay.

5.2.4 Histology and immunohistochemistry

The 6 and 26 week EDL and soleus muscles frozen in OCT were sectioned on a cryostat at -20 °C at a thickness of 8 μm. Slides from mdx and mdx-hdf were allocated as follows: 1) Stained with H&E in order to observe muscle structure, including fibre area (minimal feret diameter), central nucleation and area of mononuclear infiltrate; 2) Were reacted with primary antibodies for versican or versikine.

5.2.4.1 H&E staining

For analysis of muscle morphology, two non-overlapping digital images were captured from H+E stained sections at x10 magnification for analysis, and x20 magnification for representative images. Image analysis was completed on the x10 images using ImageJ 1.47v, a program freely available on the internet (https://imagej.nih.gov/ij/), along with Image-Pro Plus (version 7). To assess muscle fibre size, all fibres within each the field of view were manually traced to obtain the fibre area (minimal feret diameter). In addition, the proportion of centrally nucleated fibres, a hallmark of recent damage and repair, was also determined. However, note that in the soleus, only the 6 week data is presented, as data could not be obtained for the 26 week soleus due to the singular large internalised holes within the majority of fibres. Furthermore, as a very general indicator of degeneration and inflammation, regions of mononuclear infiltrate and degeneration were traced and expressed as a percentage of the total muscle area for the given field of view. For a more detailed description, please refer to section 2.3.9.
5.2.4.2 Immunohistochemistry
Four non-overlapping images were captured of muscle cross-sections immunohistochemically stained with versican and versikine primary antibodies at x60 magnification. Using the program Image-Pro Plus (version 7), the areas stained were quantified by measuring the area of staining and expressing as a percentage of the total area of muscle in the field of view. For a more detailed description, please refer to section 2.3.10.1.

5.2.5 Serum creatine kinase activity
Blood was collected from 6 and 26 week old mdx and mdx-hdf mice via cardiac puncture. Immediately after collection, blood was allowed to clot at 4 °C and then spun in a benchtop centrifuge at 12,000 rpm to form a pellet. The serum was carefully collected into a new tube, and was stored at -80 °C until analysis of total creatine kinase activity, a marker of muscle damage, using a commercially available kit, according to manufacturer’s directions (Abcam). Note that the assay measures brain, heart and muscle isoforms of creatine kinase within the serum, and is not specific for the muscle isoform.

5.2.6 Gene expression analysis
Gene expression was performed using cDNA created from snap frozen 6 and 26 week EDL and soleus muscles that were function tested (section 2.3.7). All mRNA samples were tested in duplicate. All gene expression data was calculated using the $2\Delta\text{CT}$ method, and normalised to Oligreen (sections 2.2.4 and 2.2.5). For primer sequences, please refer to Table 2.2.

5.2.7 Statistics
An independent sample T-test was undertaken for all histology, immunohistochemistry and gene expression analyses. To assess muscle strength and fatigability, a 2-way general linear model (GLM) ANOVA was performed and followed by Tukey’s post hoc analysis where appropriate. All data are presented as Mean ± S.E. and were considered statistically significant when $p < 0.05$. Additionally, it should be noted that the mice ages in this Chapter were not considered for statistical analysis for a few of reasons. The first being that the 6
and 26 week mice were 2 separate experimental models, one looking purely at differences in postnatal growth and the acute bout of degeneration and regeneration. The 26 week mice have reached maturity and reached growth, with satellite cells subjected to recurring cycles of damage and repair. The other investigating differences once growth has ceased and maturity reached. Furthermore, it is well recognised that each age group has distinct differences in dystrophic pathology and therefore cannot accurately be compared.

5.3 Results for fast twitch EDL muscles from \textit{mdx} and \textit{mdx-hdf} mice at 6 and 26 weeks of age

5.3.1 Reduced versikine expression in EDL muscles from \textit{mdx-hdf} mice

Similar to observations in TA and diaphragm muscles (Chapters 3 (465), and 4), at 6 and 26 weeks of age, the versican staining was localised to areas of endomysial fibrosis and mononuclear infiltrate in EDL muscles from \textit{mdx} and \textit{mdx-hdf} mice (Figure 5.1 A-D). Unlike the diaphragm muscles from Chapter 4 (Figure 4.1 C), the immunoreactivity of versican was similar in EDL muscles from \textit{mdx} and \textit{mdx-hdf} mice at both 6 and 26 weeks of age (Figure 5.1 E). Again, unlike the diaphragm muscles in Chapter 4 (Figure 4.1 G), the decrease in \textit{V0/V1 Vcan} gene expression did not reach statistical significance in EDL muscles from \textit{mdx} and \textit{mdx-hdf} mice at either 6 or 26 weeks of age (Figure 5.1 F).
Figure 5.1 The genetic reduction of versican had no effect on EDL muscles from 6 and 26 week old mdx-hdf mice. V0/V1 versican is stained red and nuclei blue (DAPI). A-D) Representative versican images in mdx and mdx-hdf EDL muscles showed versican was localised to areas of endomysial fibrosis and mononuclear infiltrate at 6 and 26 weeks of age. E) Versican immunoreactivity were similar in EDL muscles from mdx and mdx-hdf mice at 6 and 26 weeks of age (p = 0.658 and p = 0.447, respectively; T-test). F) Quantitation of V0/V1 Vcan gene expression transcripts were also similar in mdx and mdx-hdf mice at both ages (p = 0.248 and p = 0.100, respectively; T-test). White asterisks denote areas of mononuclear infiltrate. To assess versican immunoreactivity, n = 5 mdx and n = 5 mdx-hdf mice at 6 weeks of age, and n = 10 mdx and n = 10 mdx-hdf at 26 weeks of age. To assess V0/V1 Vcan gene expression, n = 8 mdx and n = 8 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 13 mdx-hdf mice at 26 weeks of age. Scale bar = 100 µm.
In addition to the expected expression in regions of endomysial fibrosis, very strong versican immunoreactivity was also associated with muscle spindles in EDL muscles from 26 week old \textit{mdx} and \textit{mdx-hdf} mice (Figure 5.2 A-B and E-F). Whereas, the intensity of versican staining in nerves was much lower (Figure 5.2 C, D, E and F). The versican antibody used in this thesis is specific for the GAG$\beta$ region, specific to the V0/V1 versican variants (289, 302). V0/V1 versican has been observed internally within peripheral nerves, where it was thought to be due to capillaries or fibroblasts (533). The observation of reduced versican staining in nerves is therefore unsurprising, especially given that the V2 versican variant, which contains only the GAG$\alpha$ domain, is the predominate form within the central nervous system and neuronal tissues (289, 302, 304).

![Image of versican immunoreactivity in EDL muscle spindles and nerves.](image)

**Figure 5.2 V0/V1 versican immunoreactivity in EDL muscle spindles and nerves.**

Versican is stained red and nuclei are blue (DAPI). Representative versican stained and brightfield images of A-B) muscle spindle and C-D) nerve found within EDL muscles from a 26 week old \textit{mdx} mouse. E-F) Representative versican and brightfield images of a versican stained muscle spindle next to a nerve in an EDL muscle from a 26 week old \textit{mdx-hdf} mouse. White arrows indicate muscle spindles and white arrowheads indicate nerves. Scale bar = 100 µm.
The EDL muscles were also stained with versikine, the cleaved product of V1 versican (315). In EDL muscles from 6 week mdx mice, versikine immunoreactivity was especially intense around individual muscle fibres (Figure 5.3 A). Whilst the general pattern of versikine immunoreactivity was similar in EDL muscles at 6 weeks of age, the staining intensity was reduced in mdx-hdf EDLs compared to mdx littermates (Figure 5.3 A and B), and was confirmed through quantification (*p = 0.037; Figure 5.3 E). At 26 weeks of age, versikine immunoreactivity was localised to the endomysial matrix in EDLs from both mdx and mdx-hdf mice (Figure 5.3 C and D). Again, image analysis revealed decreased versikine levels in EDL muscles from 26 week old mdx-hdf mice compared age matched mdx littermates (*p = 0.028; Figure 5.3 E). This reduction in versikine, but not versican (Figure 5.1 E and F), immunoreactivity in mdx-hdf EDL muscles, highlights the importance of considering the synthesis and remodelling of versican, given its role as a transitional matrix protein.

Furthermore, it is also worth noting that muscles from 6 week old mdx mice are still undergoing postnatal growth, and are regenerating from an acute bout of muscle degeneration which typically occurs at 3-4 weeks of age (126). At 6 weeks of age, versikine immunoreactivity was higher than in EDL muscles from 26 week old mice, where growth has ceased and the pathology has stabilised. In concordance with previous observations within the diaphragm (Chapter 4), nuclear localisation of versikine in the EDL was also observed in centrally localised myonuclei nuclei of newly regenerated fibres, as well as myonuclei within the mononuclear infiltrate, of mdx and mdx-hdf EDLs at both 6 and 26 weeks of age.
Figure 5.3 Decreased versikine expression in EDL muscles from 6 and 26 week old mdx-hdf mice.

Versican is stained red and nuclei are blue (DAPI). A-B) Representative images of EDL muscles from 6 week old mice showing versikine immunoreactivity localised to the endomysial matrix surrounding muscles fibres, as well as reduced staining in EDL muscles from 6 week old mdx-hdf mice. C-D) Representative images of EDL muscles from 26 week old mice, where versikine staining was also localised to the endomysial matrix, and again immunoreactivity was reduced in mdx-hdf mice. Note, that nuclear localisation of versikine was especially prominent in EDL muscles from the 26 week old mdx and mdx-hdf mice. E) Quantitation of versikine immunoreactivity revealed reduced versikine expression in EDL muscles from the mdx-hdf mice compared to mdx littermates at 6 and 26 weeks of age (*p = 0.037 and *p = 0.028, respectively; T-test). White arrowheads denote nuclei with internalised versikine staining. N = 6 mdx and n = 5 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 10 mdx-hdf at 26 weeks of age. Scale bar = 100 µm.
In complete contrast to the strong versican staining observed in EDL muscle spindles from *mdx* and *mdx-hdf* mice, versikine immunoreactivity was very much reduced and only very faint staining was observed (Figure 5.4 A-D). Versikine immunoreactivity in the nerve however, was essentially absent (Figure 5.4 A-D), which was not surprising given the low staining of versican (Figure 5.2 C-F).

**Figure 5.4 Versikine immunoreactivity in EDL muscle spindles and nerves.**

Versican is stained red and nuclei are blue (DAPI). Representative versican and brightfield images of **A-B)** a muscle spindle and nerves in EDL muscles of a 26 week old *mdx* mouse. **C-D)** Representative image of a versican stained muscle spindle next to a nerve in an EDL muscle from a 26 week old *mdx-hdf* mouse. White arrows depict muscle spindles, and white arrowheads depict nerves. Scale bar = 100 µm.
5.3.2 The genetic reduction of versican improved the endurance of fast twitch EDL muscle at 6 weeks, but not 26 weeks of age

At 6 and 26 weeks of age, bodyweight, EDL muscle mass, body mass to muscle mass ratio (BM:MM), optimal muscle length (Lo) and muscle cross sectional area (CSA), were all similar between mdx and mdx-hdf mice (Table 5.1). Irrespective of age, the genetic reduction of versican had no effect on the twitch contractile properties of EDL muscle. Specifically, in response to a 1 Hz stimulation, which corresponds to a single action potential, twitch force (Pt), specific twitch force (sPt), time to peak tension (TPT), ½ relaxation time (½ RT) and maximal rate of force development (max dx/dt), did not differ between EDL muscles from mdx or mdx-hdf mice at either age (Table 5.1).
Table 5.1. Body weight, EDL mass and ex-vivo twitch contractile properties from mdx and mdx-hdf at 6 and 26 weeks of age

<table>
<thead>
<tr>
<th>Units</th>
<th>6 weeks</th>
<th>26 weeks</th>
<th>P value</th>
<th>6 weeks</th>
<th>26 weeks</th>
<th>P value</th>
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<td></td>
<td></td>
<td>g</td>
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<tr>
<td>mdx</td>
<td>22.00 ± 0.78</td>
<td>21.75 ± 0.49</td>
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<td>32.32 ± 0.64</td>
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<td>21.75 ± 0.49</td>
<td></td>
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<td>32.79 ± 0.58</td>
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<tr>
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<td>9.57 ± 0.41</td>
<td>0.75</td>
<td>16.94 ± 0.84</td>
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<td>18.27 ± 0.93</td>
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<tr>
<td>mdx</td>
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<td>12.58 ± 0.22</td>
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<td>mdx</td>
<td>1.79 ± 0.10</td>
<td>1.81 ± 0.06</td>
<td>0.89</td>
<td>2.84 ± 0.12</td>
<td>3.14 ± 0.19</td>
<td>0.24</td>
</tr>
<tr>
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<td>1.81 ± 0.06</td>
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<td>2.84 ± 0.12</td>
<td>3.14 ± 0.19</td>
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<tr>
<td>Pₓ</td>
<td>mN</td>
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<td>37.64 ± 3.00</td>
<td>37.48 ± 2.66</td>
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<tr>
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<td>s</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.188 ± 0.021</td>
<td>0.179 ± 0.026</td>
<td>0.77</td>
<td>0.021 ± 0.001</td>
<td>0.027 ± 0.008</td>
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<tr>
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<td>0.179 ± 0.026</td>
<td>0.179 ± 0.026</td>
<td></td>
<td>0.021 ± 0.001</td>
<td>0.027 ± 0.008</td>
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<tr>
<td>½ RT</td>
<td>s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.025 ± 0.0017</td>
<td>0.85</td>
<td>0.019 ± 0.0021</td>
<td>0.017 ± 0.0017</td>
<td>0.12</td>
</tr>
<tr>
<td>mdx-hdf</td>
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<td>0.025 ± 0.0017</td>
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<td>0.019 ± 0.0021</td>
<td>0.017 ± 0.0017</td>
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<tr>
<td>Max dx/dt</td>
<td>mN/s</td>
<td></td>
<td></td>
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<td>0.98</td>
<td>6,282 ± 471</td>
<td>7,006 ± 368</td>
<td>0.23</td>
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<td>11,553 ± 2,599</td>
<td></td>
<td>6,282 ± 471</td>
<td>7,006 ± 368</td>
<td></td>
</tr>
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</table>

Data are means ± S.E.

L₀ = optimum muscle length; BM:MM = body mass to muscle mass ratio; CSA = cross sectional area; Pₓ = peak twitch; sPₓ = specific twitch force; TPT = time to peak tension; ½ RT = half relaxation time; Max dx/dt = maximal rate of force development.

N = 13-14 mdx and n = 10-11 mdx-hdf at 6 weeks of age, and n = 9 mdx and an n = 12 mdx-hdf mice at 26 weeks of age.
The *ex-vivo* maximal force producing capacity of EDL muscles was determined from a force frequency curve (1 to 120 Hz stimulation). When normalised to muscle size (specific force; sPo), the strength of EDL muscles from *mdx*-hdf mice was similar to EDL muscles from *mdx* littermates at 6 and 26 weeks of age (Figure 5.5 A-B).

**Figure 5.5 Genetic reduction of versican did not affect EDL muscle strength.**
A force frequency curve was used to assess the specific force output (sPo) of EDL muscles from *mdx* and *mdx*-hdf mice. The genetic reduction of versican had no effect on EDL strength at **A)** 6 weeks of age (p = 0.169; main effect strain; 2-way GLM ANOVA with factors being frequency and strain), or at **B)** 26 weeks of age (p = 0.849; main effect strain; 2-way GLM ANOVA with factors being frequency and strain). N = 13 *mdx* and n = 10 *mdx*-hdf mice at 6 weeks of age, and n = 9 *mdx* and n = 12 *mdx*-hdf mice at 26 weeks of age.
Following the force frequency protocol, EDL muscles underwent 4 m of intermittent, submaximal 60 Hz stimulations followed by a single 60 Hz stimulation at 2, 5 and 10 m post fatigue to assess fatigability and force recovery. During the 4 m of intermittent, submaximal (60 Hz) stimulation, the EDL muscles from the 6 week old mdx-hdf mice fatigued less (*p = < 0.001; Figure 5.6 A) when compared to mdx littermates. Force recovery, at 2, 5 and 10 m post fatigue was also improved in EDL muscles from 6 week old mdx-hdf mice (*p = < 0.001; Figure 5.6 A). By 26 weeks of age, this improvement in muscle endurance was lost. Fatigability, in response to 4 m of intermittent, submaximal stimulation, was similar in EDL muscles from mdx and mdx-hdf mice at 26 weeks of age, whilst force recovery was significantly reduced in mdx-hdf EDL muscles (*p = 0.035; Figure 5.6 B). Therefore, in dystrophic EDL muscles, postnatal growth and regeneration following the acute bout of degeneration at 3-4 weeks of age, modulates the consequences of versican reduction on contractile function in fast twitch hindlimb muscles. Therefore, to gain more insight into how the genetic reduction of versican affects EDL contractile function, muscle morphology and gene markers for inflammation, regenerative myogenesis, and matrix remodelling were investigated.
Figure 5.6 Genetic reduction of versican improved the endurance of EDL muscles at 6 weeks of age.

A) During 4 m of intermittent, submaximal (60 Hz) stimulation, EDL muscles from mdx-hdf mice fatigued less (*p = < 0.001; main effect strain; 2-way GLM ANOVA with factors being time and strain) and produced more force during 2 to 10 m of recovery (*p = < 0.001; main effect strain; 2-way GLM ANOVA with factors being time and strain) when compared to EDLs from mdx mice. B) By 26 weeks of age, this improvement was lost, as the fatigability of EDL muscles was similar between mdx and mdx-hdf mice (p = 0.523; main effect strain; 2-way GLM ANOVA with factors being time and strain). Furthermore, force recovery was decreased in EDL muscles from mdx-hdf mice (*p = 0.035; main effect strain; 2-way GLM ANOVA with factors being time and strain). N = 12 mdx and n = 10 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 11 mdx-hdf mice at 26 weeks of age.
5.4.3 The genetic reduction of versican has a greater effect on the morphology of dystrophic EDL muscles at 26 weeks than at 6 weeks of age

Morphological analysis of H&E stained EDL muscle cross sections was completed at 6 and 26 weeks of age (Figure 5.7 A-D for representative images). As expected, EDL muscle cross sections from 6 week old *mdx* and *mdx*-hdf mice contained small isolated regions of mononuclear infiltrate, endomysial fibrosis was very limited, and approximately 40% of muscle fibres showed signs of recent regeneration, as indicated by one or two centrally localised nuclei (Figures 5.7 A-B). At 6 weeks of age, the total number of myofibres per mm\(^2\), the percentage of centrally nucleated myofibres, muscle fibre size and percent area of mononuclear infiltrate did not differ between EDL muscles from *mdx* and *mdx*-hdf mice (Figure 5.7 E-F, G and I).

However, by 26 weeks of age, subtle differences in the morphology of EDL muscles from *mdx* and *mdx*-hdf mice became apparent. EDL muscle cross-sections from *mdx* mice exhibited increased fibrosis, and mononuclear infiltrate and degeneration, including visibly necrotic fibres (Figure 5.7 C-D). The morphology of EDL muscles from *mdx*-hdf mice appeared to have less fibrosis and mononuclear infiltrate and perhaps fewer necrotic fibres.

Similar to 6 weeks, the total number of myofibres per mm\(^2\) did not differ between EDL muscles from 26 week old *mdx* and *mdx*-hdf mice (Figure 5.7 E). However, with age there was a progressive increase in the percentage of centrally nucleated fibres with 73% of fibres being centrally nucleated in 26 week EDL muscle cross-sections from *mdx* mice, and 68% of in the 26 week *mdx*-hdf mice (Figure 5.7 F).

The genetic reduction of versican tended to reduce the percentage of centrally nucleated fibres in the *mdx*-hdf mice (p = 0.060; Figure 5.7 F) compared to *mdx* mice. The percentage of mononuclear infiltrate and degeneration in EDL muscle cross-sections also tended to be lower in 26 week old *mdx*-hdf mice compared to age matched *mdx* littermates (p = 0.055; Figure 5.7 I). The genetic reduction of
versican appeared to have a subtle, yet beneficial effect on the pathology of dystrophic EDL muscles at 26 weeks of age; although, this difference in muscle morphology was not enough to result in a functional improvement in muscle strength. Nonetheless, given that areas of mononuclear infiltrate are comprised of inflammatory cells, muscle precursor cells and fibroblasts, these observations were investigated further by testing the effect of reduced versican upon gene expression markers of inflammation, regenerative myogenesis and fibrosis.
Figure 5.7 Morphological analysis of EDL muscles from mdx and mdx-hdf mice at 6 and 26 weeks of age.

A-D) Representative H&E images of EDL muscle cross-sections from 6 and 26 week old mdx and mdx-hdf mice. E) Total myofibre number per mm² was similar in EDL muscles from mdx and mdx-hdf mice at 6 and 26 weeks of age (p = 0.118 and 0.436, respectively; T-test). F) At 6 weeks of age, the genetic reduction of versican had no effect on the percentage of centrally nucleated fibres between mdx or mdx-hdf mice (p = 0.678; T-test). However, at 26 weeks of age, the EDL muscles from mdx-hdf mice tended to have a lower percentage of centrally nucleated fibres compared to mdx littermates (p = 0.060; T-test). G-H) Based on analysis of minimal feret diameter, the genetic reduction of versican had no effect on muscle fibre size at 6 or 26 weeks of age. I) The percent area of mononuclear infiltrate and degeneration was similar between mdx and mdx-hdf mice at 6 weeks of age (p = 0.193; T-test). By 26 weeks of age, EDL muscle cross-sections from mdx-hdf mice tended to have a reduced percentage mononuclear infiltrate and degeneration (p = 0.055; T-test) than their age matched mdx littermates. N = 8 mdx and n = 8 mdx-hdf mice at 6 weeks of age, and n = 10-11 mdx and n = 10-12 mdx-hdf at 26 weeks of age. Scale bar = 100 µm.
5.3.4 Genetic reduction of versican and gene markers of inflammation in EDL muscles

In order to gain insight as to how the reduction of versican affects gene markers of inflammation in the fast twitch EDL muscles, the mRNA transcript abundance of Has2, Mcp-1, Tgfβ1 and F4/80 was quantified using RT-PCR. As described in previous chapters, Has2 is the main hyaluronan synthase (HAS) in skeletal muscle, involved in the synthesis of hyaluronan (446). Hyaluronan forms a complex with versican, together binding inflammatory cells (74, 240). However, hyaluronan can also be degraded by hyaluronidase enzymes to a low-molecular weight pro-inflammatory form (236), which stimulates macrophages to produce pro-inflammatory cytokines (240, 241). Additionally, versican can bind MCP-1 (51), which is able to attract infiltrating monocytes and macrophages into the area of injury (478). TGFβ1 on the other hand, is a cytokine which can be both pro-inflammatory or anti-inflammatory depending on the context (534). In DMD, the elevated TGFβ1 levels contributes to failed regeneration and drives fibrosis (345). Finally, although F4/80 is a marker for macrophages (479), it does not differentiate between monocytes, or the M1 and M2 macrophages subsets (524). Furthermore, F4/80 can also be expressed by other inflammatory cells (479).

In EDL muscles, the mRNA transcript abundance of Has2, Mcp-1 and Tgfβ1 was similar between mdx and mdx-hdf mice, irrespective of age (Figure 5.8 A-C). At 6 weeks of age, F4/80 gene expression also did not differ between EDL muscles from mdx and mdx-hdf mice. However, at 26 weeks of age, F4/80 gene expression was significantly lower in EDL muscles from mdx-hdf mice compared to mdx littermates (*p = 0.040; Figure 5.8 D). In future studies, this observation should be followed up through an assessment of neutrophil, monocyte and macrophage infiltration in mdx and mdx-hdf muscle using immunohistochemistry or flow cytometry, using additional markers that are specific for monocytes and the pro-inflammatory M1 and anti-inflammatory M2 macrophage subsets.
Figure 5.8 The genetic reduction of versican was associated with a reduction in F4/80 gene expression in dystrophic EDL muscles at 26 weeks of age.

A-C) The mRNA transcript abundance of Has2 (p = 0.773 and p = 0.790, respectively; T-test), Mcp-1 (p = 0.932 and p = 0.385, respectively; T-test) and Tgfβ1 (p = 0.350 and p = 0.839, respectively; T-test) did not differ between EDL muscles from 6 or 26 week old mdx and mdx-hdf mice. D) At 6 weeks of age, the genetic reduction of versican did not alter F4/80 mRNA transcript abundance in EDL muscles (p = 0.179; T-test). By 26 weeks of age, EDL muscles from mdx-hdf mice displayed decreased F4/80 gene expression (*p = 0.040; T-test). N = 8 mdx and n = 8 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 13 mdx-hdf mice at 26 weeks of age.
5.3.5 Genetic reduction of versican and gene markers of regenerative myogenesis in EDL muscles

Ineffective regenerative myogenesis has an important role in the pathogenesis of DMD (285) and versican has been implicated in the proliferation and differentiation of satellite cells (17, 20). Hence, the mRNA transcript abundance of *MyoD* and *Myogenin* was quantified. Similar to previous observations in diaphragm muscles from chapter 4 (Figure 4.9 E and G), *MyoD* and *Myogenin* gene expression were also similar in EDL muscles from *mdx* and *mdx*-hdf mice, irrespective of age (Figure 5.9 A-B). These observations are suggestive that myogenic processes were unaffected in the EDL muscles of *mdx*-hdf mice.

![Figure 5.9 Genetic reduction of versican did not affect the expression of gene markers of myogenesis in dystrophic EDL muscles.](image)

**Figure 5.9 Genetic reduction of versican did not affect the expression of gene markers of myogenesis in dystrophic EDL muscles.**

**A-B)** At 6 and 26 weeks of age, mRNA transcript abundance of *MyoD* (p = 0.408 and 0.752, respectively; T-test) and *Myogenin* (p = 0.355 and 0.539, respectively; T-test) did not differ between EDL muscles from *mdx* and *mdx*-hdf mice. N = 8 *mdx* and n = 8 *mdx*-hdf mice at 6 weeks of age and n = 9 *mdx*, and n = 13 *mdx*-hdf mice at 26 weeks of age.
5.3.6 Genetic reduction of versican and ECM gene markers in EDL muscles

During development, a transitional matrix rich in versican is remodelled by ECM proteases, including ADAMTS5 (289). Upon maturity, the transitional matrix is replaced with a collagen rich mature matrix (382, 528). At 6 and 26 weeks of age, the genetic reduction of versican had no effect on the mRNA transcript abundance of *Adamts5* and *Col1a1* in dystrophic EDL muscles (Figure 5.10 A-B).

![Figure 5.10](image)

**Figure 5.10.** The genetic reduction of versican did not effect gene markers of ECM remodelling in dystrophic EDL muscles.

A-B) At 6 and 26 weeks of age, mRNA transcript abundance of *Adamts5* (p = 0.669 and 0.127, respectively; T-test) and *Col1a1* (p = 0.467 and 0.778, respectively; T-test) did not differ between EDL muscles from *mdx* and *mdx*-hdf mice. N = 8 *mdx* and n = 8 *mdx*-hdf mice at 6 weeks of age, and n = 9 *mdx* and n = 13 *mdx*-hdf mice at 26 weeks of age.
5.4 Results for slow twitch soleus muscles from *mdx* and *mdx*-hdf mice at 6 and 26 weeks of age

5.4.1 The genetic reduction of versican led to decreased versican expression in soleus muscles of *mdx*-hdf mice

In Chapters 3-5, versican and versikine immunoreactivity was localised to areas of endomysial fibrosis and mononuclear infiltrate in dystrophic diaphragm, and fast twitch EDL and TA muscles. However, versican or versikine expression, and their localisation, has not yet been characterised in dystrophic slow twitch soleus muscles. The characterisation of versican and versikine is interesting, as the soleus is a postural hindlimb muscle and is therefore more actively recruited than the EDL (535). This constant usage of the soleus muscle is perhaps why soleus muscles of *mdx* mice have such a severe pathology when compared to other dystrophic muscles (130).

In soleus muscles from 6 week old *mdx* mice, versican immunoreactivity was observed around individual muscle fibres, and was localised to regions of endomysial fibrosis and mononuclear infiltrate. The intensity of versican staining was lower in soleus muscles from 6 and 26 week old *mdx*-hdf mice compared to *mdx* littermates (Figure 5.11 A-D). The analysis of versican immunoreactivity in soleus muscles found that *mdx*-hdf mice expressed less versican than age matched *mdx* littersmates at both 6 and 26 weeks of age in the soleus (*p = 0.0001 and *p = 0.046, respectively; Figure 5.11 E). However, analysis of VO/V1 Vcan gene expression did not differ between soleus muscles from *mdx* and *mdx*-hdf-mice, irrespective of age (Figure 5.11 F).

Although muscle spindles and nerves were evident in dystrophic EDL muscles from 26 week old mice, none were observed in 26 week soleus. This is not unusual as muscle spindles follow nerves which are randomly dispersed, and therefore may not be located within the midbelly sections of soleus muscle tested within this Chapter (536).
Figure 5.11. V0/V1 versican expression was reduced in soleus muscles from 6 and 26 week old mdx-hdf mice. Versican is stained red and nuclei are blue (DAPI). A-D) Representative images of versican staining in soleus muscles from 6 and 26 week old mdx and mdx-hdf mice, with reduced versican immunoreactivity observed in soleus muscles from mdx-hdf mice at both ages. Soleus muscles at 26 weeks contained less versican staining than those at 6 weeks of age. E) Quantitation of versican immunoreactivity revealed that the mdx-hdf mice contained less versican than the soleus muscles of the mdx mice at 6 and 26 weeks of age (*p = 0.0001 and *p = 0.046, respectively; T-test). F) V0/V1 Vcan gene expression did not differ between mdx and mdx-hdf mice at both 6 and 26 weeks of age (p = 0.096 and 0.713, respectively; T-test). White asterisks denote areas of mononuclear infiltrate. To assess versican immunoreactivity, n = 7 mdx and n = 8 mdx-hdf mice at 6 weeks of age, and n = 8 mdx and n = 11 mdx-hdf mice at 26 weeks of age. To assess V0/V1 Vcan gene expression, n = 6 mdx and an n= 8 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and an n = 12 mdx-hdf mice at 26 weeks of age. Scale bar = 100 µm.
In soleus muscle cross-sections at 6 weeks of age, versikine immunoreactivity was localised to the endomysial matrix surrounding muscle fibres, and to regions of mononuclear infiltrate and fibrosis, with similar staining intensity in muscles from \textit{mdx} and \textit{mdx-hdf} mice (Figure 5.12 A-B). By 26 weeks of age, versikine immunoreactivity was reduced, with sparse endomysial staining observed in soleus muscle cross-sections from \textit{mdx} and \textit{mdx-hdf} mice (Figure 5.12 C-D). In contrast to EDL muscles (Figure 5.3 C-D), nuclear localisation of versikine was absent or very limited in soleus muscles from 26 week old \textit{mdx} and \textit{mdx-hdf} mice. Analysis of versikine reactivity in soleus muscle cross-section did not differ between \textit{mdx} and \textit{mdx-hdf} mice at 6 or 26 weeks of age (Figure 5.12 E).
**Figure 5.12** Versikine immunoreactivity was similar in soleus muscles from 6 or 26 week old *mdx* and *mdx*-hdf mice. Versican is stained red and nuclei are blue (DAPI). **A-B)** Representative images of versikine staining in soleus muscles from 6 week old *mdx* and *mdx*-hdf mice. **C-D)** Representative images of versikine staining in soleus muscles from 26 week old *mdx* and *mdx*-hdf mice. **E)** Quantitation of versikine immunoreactivity, indicated similar expression in *mdx* and *mdx*-hdf mice at 6 and 26 weeks of age (p = 0.386 and 0.249, respectively; T-test). White asterisks denote areas of mononuclear infiltrate, and white arrowheads denote nuclei with internalised versikine staining. N = 6 *mdx* and n = 7 *mdx*-hdf mice at 6 weeks of age, and n = 8 *mdx* and n = 12 *mdx*-hdf mice at 26 weeks of age. Scale bar = 100 µm
5.4.2 The genetic reduction of versican improved the strength and endurance of 6 week soleus muscles of mdx-hdf mice

Similar to EDL muscles (Table 5.1), the soleus muscle mass, muscle mass ratio (BM:MM), optimal muscle length ($L_o$) and muscle cross sectional area (CSA), did not differ between mdx and mdx-hdf mice at 6 or 26 weeks of age (Table 5.2). Furthermore, the genetic reduction of versican had modest effects on the twitch contractile properties of soleus muscles. At 6 weeks of age, in response to a 1 Hz twitch stimulation, the twitch force ($P_t$) and specific twitch force ($sP_t$) did not differ between muscles from mdx and mdx-hdf mice. At 26 weeks of age, $P_t$ was similar, however $sP_t$ was lower (*$p = 0.04$; Table 5.2), in soleus muscles from the mdx-hdf mice compared to age matched mdx littermates. The time to peak tension (TPT), $\frac{1}{2}$ relaxation time ($\frac{1}{2}$ RT) and the maximal rate of force development ($dx/dt$) were similar in soleus muscles from mdx and mdx-hdf mice at 6 and 26 weeks of age (Table 5.2).
Table 5.2. Body weight, soleus mass and ex-vivo twitch contractile properties from mdx and mdx-hdf at 6 and 26 weeks of age

<table>
<thead>
<tr>
<th>Units</th>
<th>6 weeks mdx</th>
<th>6 weeks mdx-hdf</th>
<th>P value</th>
<th>26 weeks mdx</th>
<th>26 weeks mdx-hdf</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight g</td>
<td>22.00 ±0.78</td>
<td>21.75 ± 0.49</td>
<td>0.80</td>
<td>32.32 ± 0.64</td>
<td>32.79 ± 0.58</td>
<td>0.60</td>
</tr>
<tr>
<td>Soleus mass mg</td>
<td>8.74 ± 0.94</td>
<td>8.47 ± 0.64</td>
<td>0.82</td>
<td>14.67 ± 0.88</td>
<td>15.26 ± 0.82</td>
<td>0.63</td>
</tr>
<tr>
<td>BM:MM</td>
<td>0.39 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>0.85</td>
<td>0.45 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.70</td>
</tr>
<tr>
<td>L0 mm</td>
<td>10.38 ± 0.22</td>
<td>11.51 ± 1.15</td>
<td>0.28</td>
<td>10.99 ± 0.19</td>
<td>10.73 ± 0.22</td>
<td>0.39</td>
</tr>
<tr>
<td>CSA mm²</td>
<td>1.11 ± 0.10</td>
<td>1.06 ± 0.09</td>
<td>0.72</td>
<td>1.77 ± 0.10</td>
<td>1.90 ± 0.11</td>
<td>0.41</td>
</tr>
<tr>
<td>Pt mN</td>
<td>22.91 ± 1.56</td>
<td>25.43 ± 2.05</td>
<td>0.33</td>
<td>50.65 ± 4.74</td>
<td>41.63 ± 4.28</td>
<td>0.18</td>
</tr>
<tr>
<td>sPt kN/m²</td>
<td>21.02 ± 1.13</td>
<td>24.44 ± 3.07</td>
<td>0.26</td>
<td>28.64 ± 2.01</td>
<td>21.99 ± 2.13</td>
<td>0.04 *</td>
</tr>
<tr>
<td>TPT s</td>
<td>0.200 ± 0.021</td>
<td>0.193 ± 0.027</td>
<td>0.83</td>
<td>0.033 ± 0.001</td>
<td>0.032 ± 0.001</td>
<td>0.64</td>
</tr>
<tr>
<td>½ RT s</td>
<td>0.045 ± 0.003</td>
<td>0.052 ± 0.005</td>
<td>0.28</td>
<td>0.054 ± 0.004</td>
<td>0.062 ± 0.006</td>
<td>0.30</td>
</tr>
<tr>
<td>Max dx/dt mN/s</td>
<td>9,764 ± 3,117</td>
<td>12,889 ± 3,941</td>
<td>0.53</td>
<td>2,335 ± 241</td>
<td>1,938 ± 201</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.

* Statistical significance at p < 0.05 for all measurements, analysed by 2-tailed T-test.

L0 = optimum muscle length; BM:MM = body mass to muscle mass ratio; CSA = cross sectional area; Pt = peak twitch; sPt = specific twitch force; TPT = time to peak tension; ½ RT = half relaxation time; Max dx/dt = maximal rate of force development

N = 11-14 mdx and n = 8-11 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 12 mdx-hdf mice at 26 weeks of age.
The ex-vivo maximal force producing capacity, the specific force ($s_{Po}$), of soleus muscles was determined from a force frequency curve (1 to 120 Hz). At 6 weeks of age, soleus muscles from $mdx$-hdf mice were stronger than those from $mdx$ littermates, as indicated by an upward shift in the force frequency curve (*p = 0.001; Figure 5.13 A). However, by 26 weeks of age, this improvement in muscle strength was lost and soleus muscles from $mdx$-hdf mice produced less force than those from age matched $mdx$ littermates (*p < 0.001; main effect strain; 2 way GLM-ANOVA; Figure 5.13 B).
Figure 5.13 At 6 and 26 weeks of age, the genetic reduction of versican has differential effects on the strength of dystrophic soleus muscles.

A force frequency curve was used to assess the strength of soleus muscles from \textit{mdx} and \textit{mdx-hdf} mice. \textbf{A)} At 6 weeks of age, soleus muscles from \textit{mdx-hdf} mice produced more force than soleus muscles from \textit{mdx} littermates (\textit{p} = 0.001; main effect strain; 2 way GLM-ANOVA with factors being frequency and strain). \textbf{B)} By 26 weeks of age, soleus muscles from \textit{mdx-hdf} mice produced less force than soleus muscles from \textit{mdx} littermates (\textit{p} < 0.001; main effect strain; 2 way GLM-ANOVA with factors being frequency and strain). \textit{N} = 11 \textit{mdx} and \textit{n} = 8 \textit{mdx-hdf} mice at 6 weeks of age, and \textit{n} = 9 \textit{mdx} and \textit{n} = 12 \textit{mdx-hdf} mice at 26 weeks of age.
Following the force frequency protocol, the fatigability of soleus muscles was assessed using 4 m intermittent, submaximal (60 Hz) stimulations, followed by a single 60 Hz stimulation at 2, 5 and 10 m post fatigue to assess force recovery. During the 4 m of intermittent submaximal stimulation, the soleus muscles from the 6 and 26 week old \textit{mdx}-hdf mice fatigued less (*p = 0.028 and *p = 0.021, respectively; Figure 5.14 A-B) when compared to \textit{mdx} littermates. However, irrespective of age, the genetic reduction of versican had no effect upon force recovery (Figure 5.14 A-B). As was done for EDL muscles, to gain more insight into how the genetic reduction affected the contractile function, muscle morphology and gene markers for inflammation, regenerative myogenesis and matrix remodelling were then investigated.
Figure 5.14 The genetic reduction of versican improved the fatigability of dystrophic soleus muscles.

A-B) During 4 m of intermittent, submaximal (60 Hz) stimulation, at both 6 and 26 weeks of age, soleus muscles from mdx-hdf mice fatigued less compared to soleus muscles from age matched mdx littermates (*p = 0.028 and *p = 0.021, respectively; main effect strain; 2-way GLM ANOVA with factors being time and strain). However, force recovery at 2, 5 and 10 m post fatigue, was similar in soleus muscles from aged matched mdx and mdx-hdf mice (p = 0.645 and p = 0.494, respectively; 2-way GLM ANOVA with factors being time and strain). N = 12 mdx and n = 10 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 11 mdx-hdf mice at 26 weeks of age.
5.4.3 The genetic reduction of versican had differential effects on the morphology of dystrophic soleus muscles depending upon mouse age

Morphological analysis of H&E stained soleus muscle cross sections was undertaken at 6 and 26 weeks of age (Figure 5.15 A-H for representative images). At 6 weeks of age, soleus muscles at both ages contained scattered, small regions of endomysial fibrosis and mononuclear infiltrate, as well as centrally nucleated fibres, indicative of recent damage and regeneration (Figure 5.15 A-B). At this time point, modest differences in the morphology of soleus muscles from *mdx* and *mdx*-hdf mice were observed. The number of fibres per mm² tended to be lower in soleus muscles from 6 week old *mdx*-hdf mice compared to age matched *mdx* littermates (*p* = 0.055; Figure 5.16 A). Whilst the percentage of centrally nucleated fibres was not significantly different in soleus muscle cross-sections from 6 week old *mdx* and *mdx*-hdf mice (Figure 5.16 B).

Minimal feret diameter was used to assess muscle fibre cross sectional area, and the resultant data were binned in order to better observe differences in fibre size. Soleus muscles of *mdx*-hdf mice tended to have more large fibres (>70 µm in diameter; *p* = 0.062; Figure 5.16 C) than mdx littermates at 6 weeks of age. The binned data of fibre size data indicates a rightward shift towards larger fibres in the *mdx*-hdf mice. This is in concordance with a reduction in the number of myofibres per mm² and may contribute to the increase in force production of *mdx*-hdf soleus muscles at 6 weeks of age (Figure 5.13 A), as larger muscle fibres produce more force (519). At 6 weeks of age, the percentage of soleus muscle cross-section comprised of mononuclear infiltrate and degeneration did not differ between *mdx* and *mdx*-hdf mice (Figure 5.16 E). Additionally, total serum creatine kinase activity, a non-specific marker of muscle damage, also did not differ *mdx* and *mdx*-hdf mice (Figure 5.16 F).
Figure 5.15 Representative H&E images of soleus muscle cross-sections from 6 and 26 week old *mdx* and *mdx-hdf* mice.

**A-B)** Representative H&E images from soleus muscle cross-sections from 6 week old *mdx* and *mdx-hdf* mice containing undamaged fibres, recently regenerated centrally nucleated fibres, occasional necrotic fibres and regions of mononuclear infiltrate and endomysial fibrosis. **C-H)** Representative H&E images from 26 week old *mdx* and *mdx-hdf* mice depicting the varying severity of dystrophic pathology. Specifically, **C-D)** depicts muscle cross-sections with a moderate dystrophic pathology; specifically, intact undamaged muscle fibres, centrally nucleated muscles fibres, and expansion of endomysial fibrosis and mononuclear infiltrate. **E-F)** Depicts soleus muscle cross-sections from *mdx* and *mdx-hdf* mice with a more severe dystrophic pathology; including fibres containing large internalised holes, degenerating fibres, large areas of ghost fibres (empty spaces surrounding by basement membrane) and mononuclear infiltrate. While **G-H)** shows soleus muscle cross-sections from *mdx* and *mdx-hdf* mice with the most severe dystrophic pathology, including extensive degeneration and mononuclear infiltrate and a further increase in ghost fibres. Note that a few intact undamaged and centrally nucleated fibres are still evident. Black arrows denote degenerating fibres, and black arrowheads denote ghost fibres. Scale bars = 100 µm.
Figure 5.16 H&E analysis of the 6 and 26 week mdx and mdx-hdf soleus.

A) The mdx-hdf mice had a trend for decreased fibre numbers at 6 weeks of age, whereas fibre numbers remained similar at 26 weeks, when compared to mdx littermates (p = 0.055 and p = 0.770, respectively; T-test). B) At 6 weeks of age, the percentage of centrally nucleated fibres did not significantly differ between soleus muscle cross-sections from mdx and mdx-hdf mice (p = 0.869; T-test). C) Analysis of minimal feret diameter at 6 weeks of age revealed that soleus muscle cross-sections from mdx-hdf mice tended to an increase in the percentage of large muscle fibres >70 µm in diameter compared to soleus muscle cross-sections from mdx littermates (T-test; p = 0.062). D) Analysis of minimal feret diameter at 26 weeks of age, revealed a shift towards a reduction in myofibre size in soleus muscles from mdx-hdf mice. Specifically, soleus muscles cross-sections from mdx-hdf mice had a greater percentage of small myofibres between 10-19.99 µm and 30-39.99 µm in diameter (*p = 0.023 and *p = 0.023, respectively; T-test), and fewer large myofibres between 60-69.99 µm and >70 µm in diameter (*p = 0.022 and *p = 0.006, respectively; T-test). E) The percentage area of soleus muscle cross-section comprised of mononuclear infiltrate and degeneration did not differ between mdx and mdx-hdf mice at 6 weeks of age (p = 0.753). Whilst, soleus muscle cross-sections from the 26 week old mdx-hdf contained significantly more mononuclear infiltrate and degeneration (*p = 0.018; T-test). F) Total serum creatine kinase activity, a non-specific marker of muscle damage, was similar in mdx and mdx-hdf mice irrespective of age (p = 0.513 and p = 0.598, respectively; T-test). To assess soleus muscle morphology, n = 8 mdx and n = 9 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 13 mdx-hdf mice at 26 weeks of age. To assess total serum creatine kinase activity, n = 8 mdx and n = 6 mdx-hdf mice at 6 weeks of age, and n = 8 mdx and n = 9 mdx-hdf at 26 weeks of age.
By 26 weeks of age, soleus muscles from *mdx* and *mdx*-hdf mice had undergone morphological changes of varying severity, indicative of increased degeneration and a more severe dystrophic pathology. Therefore, three representative muscle cross-sections from three different *mdx* and *mdx*-hdf mice were chosen to help depict the range of dystrophic pathology in the 26 week soleus (Figure 5.15 C-H). All *mdx* and *mdx*-hdf soleus sections at 26 weeks contained an increase in mononuclear infiltrate and endomysial fibrosis and muscle fibres of variable size and morphology, ranging from undamaged fibres, centrally nucleated fibres, fibres with centrally internalised holes, and fibres that were undergoing degeneration and necrosis.

Soleus muscle fibres were clustered together in groups separated by multiple gaps which appear to contain mononuclear infiltrate and remnants of ECM. It appears that fibres with a large internal hole were in the early stages of degeneration, and when the fibres are completely degraded, leave behind empty areas surrounded by ECM. These empty spaces are commonly called ghost fibres (537). Soleus muscles from 26 week old *mdx* and *mdx*-hdf mice were the only muscle type examined in this thesis to exhibit this morphology of internalised myofibre holes and the ghost fibres between groups of myofibres. This morphological phenotype is unlikely to be caused by freezer damage. Rather, these ghost fibres are formed after a previously occupying fibre has been removed, leaving behind a structural hollow matrix ‘tube’, which is not degraded, and thereby remains structurally intact (537).

At 26 weeks of age, the number of myofibres per mm² was similar in soleus muscle cross-sections from *mdx* and *mdx*-hdf mice (Figure 5.16 A). In contrast to soleus muscles from 6 week old *mdx*-hdf mice, where myofibre size tended to be increased compared to *mdx* litters (Figure 5.16 C), by 26 weeks of age soleus muscles from *mdx*-hdf mice had a noticeable shift towards a reduction in myofibre size when compared to *mdx* littermates (Figure 5.16 D). Specifically, soleus muscle cross-sections from *mdx*-hdf mice had a greater percentage of small sized fibres between 10-19.99 µm and 30-39.99 µm in diameter (*p = 0.023 and *p = 0.023, respectively; Figure 5.16 D), and fewer large fibres
between 60-69.99 µm and >70 µm in diameter (*p = 0.022 and *p = 0.006, respectively; Figure 5.16 D). Furthermore, soleus muscle cross-sections from 26 week old mdx-hdf had a greater percentage of mononuclear infiltrate and degeneration compared to age matched littermates (*p = 0.018; Figure 5.16 E). The shift in myofibre size towards more small fibres and fewer large fibres, along with the increase in mononuclear infiltrate and degeneration, likely accounts for the reduced force output of soleus muscles from 26 week old mdx-hdf mice (refer to Figure 5.13 B). At 26 weeks of age, and as observed in 6 week old mice, the genetic reduction of versican had no effect on total serum creatine kinase activity (Figure 5.16 F). This assessment of soleus muscle morphology was supported by analysis of gene markers for inflammation, regenerative myogenesis and ECM remodelling.

5.4.4 Genetic reduction of versican and gene markers of inflammation in soleus muscles

In order to gain insight as to how the genetic reduction of versican might affect inflammation in the slow twitch soleus muscles, the mRNA transcript abundance of Has2, Mcp-1, Tgfβ1 and F4/80 were quantified using RT-PCR. As described in section 5.3.4 of this chapter, all of these genes can be linked to inflammation, either directly binding or attracting inflammatory cells, or through the modulation of inflammatory processes. The mRNA transcript abundance for Has2, Mcp-1, Tgfβ1 and F4/80 were all similar between mdx and mdx-hdf mice at 6 and 26 weeks of age (Figure 5.17 A-D). This was somewhat surprising given the increase in mononuclear infiltrate and degeneration observed in soleus muscle cross-sections from 26 week old mdx-hdf mice. Therefore, given these results, it would be beneficial to confirm the gene expression data via an assessment of monocyte, pro-inflammatory M1 and anti-inflammatory M2 macrophage infiltration in mdx and mdx-hdf muscle using immunohistochemistry or flow cytometry.
Figure 5.17 Genetic reduction of versican did not affect the expression of gene markers of inflammation in dystrophic soleus muscles.

At 6 and 26 weeks of age, soleus muscles from mdx and mdx-hdf mice did not differ in the mRNA transcript abundance of A) *Has2* (p = 0.814 and 0.303, respectively; T-test); B) *Mcp-1* (p = 0.166 and 0.545, respectively; T-test); C) *Tgfβ1* (p = 0.468 and 0.110, respectively; T-test); and D) *F4/80* (p = 0.521 and 0.526, respectively; T-test). N = 6 mdx and n = 8 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 12 mdx-hdf at 26 weeks of age.
5.4.5 Genetic reduction of versican and gene markers of regenerative myogenesis in soleus muscles

As previously described in section 5.3.5 of this chapter, regenerative myogenesis has a large role in DMD pathology. Similar to observations in dystrophic EDL muscles (Figure 5.9 A-B), MyoD and Myogenin gene expression did not differ between soleus muscles from mdx and mdx-hdf mice, irrespective of age (Figure 5.18 A-B).

![Graph showing gene expression of MyoD and Myogenin in mdx and mdx-hdf mice at 6 and 26 weeks of age.]

Figure 5.18 Genetic reduction of versican did not affect the expression of gene markers of myogenesis in dystrophic soleus muscles.

At 6 and 26 weeks of age, soleus muscles from mdx and mdx-hdf mice did not differ in the mRNA transcript abundance of A) MyoD (p = 0.908 and 0.432, respectively; T-test) and B) Myogenin (p = 0.719 and 0.530, respectively; T-test). N = 6 mdx and n = 8 mdx-hdf mice at 6 weeks of age, and n = 9 mdx and n = 12 mdx-hdf at 26 weeks of age.
5.4.6 Genetic reduction of versican and gene markers of ECM remodelling in the soleus muscles

The genetic reduction of versican no differences in either *Adams5* or *collagen1a1* gene expression in mdx or mdx-hdf mice at 6 or 26 weeks of age (Figure 5.19 A-B). Although, it is worth noting that in line with the increase in dystrophic pathology, mRNA transcript abundance of *collagen1a1* was increased in 26 weeks soleus compared to those at 6 weeks. Similar to dystrophic EDL muscles, these results do not consider basement membrane proteins, therefore more detailed biochemical investigations into the ECM should be undertaken. This is especially important given the differences in the percentage of mononuclear infiltrate between soleus muscles from 26 week old *mdx* and *mdx-hdf* mice (Figure 5.16 E).

![Gene expression graph](image)

**Figure 5.19 Genetic reduction of versican did not effect ECM markers in dystrophic soleus muscles.**

At 6 and 26 weeks of age, soleus muscles from *mdx* and *mdx-hdf* mice did not differ in the mRNA transcript abundance of **A)** *Adams5* (p = 0.790 and 0.380, respectively; T-test) and **B)** *Col1a1* (p = 0.981 and 0.212, respectively; T-test). N = 6 *mdx* and n = 8 *mdx-hdf* mice at 6 weeks of age, and n = 9 *mdx* and n = 12 *mdx-hdf* at 26 weeks of age.
5.5 Discussion

DMD is characterised by skeletal muscle degeneration concurrent with the progressive deposition of extracellular matrix proteins. Expansion of the endomysial matrix is observed in patients with DMD as early as 2.5 weeks of age (94) and precedes any overt degenerative or regenerative changes. Fibrosis in dystrophic muscles is comprised of proteins from the interstitial and basement membrane matrices, such as collagen 1, collagen IV and fibronectin (119, 221); as well as transitional matrix proteins, with versican being overexpressed in DMD and therefore of particular interest (90, 465). From Chapter 3 of this thesis, versican expression was found to be downregulated by dexamethasone (465), significant as glucocorticoids are the only treatment with clinical efficiency in DMD. Furthermore, the breeding of female mdx mice with male hdf mice produced a novel strain of a dystrophic mdx-hdf pups with reduced versican. This genetic reduction of versican was associated with improved strength and endurance of mdx-hdf mouse diaphragms (Chapter 4), the muscle group with the most similar pathology to DMD (125, 454). Together, these data are suggestive that versican plays a role in the pathology of DMD.

The skeletal muscles of patients with DMD are subjected to the stress of dystrophin deficiency in addition to muscle growth, a process in which muscle fibres increase in length and cross-sectional area (538). The initial study on the effects of genetically reduced versican on diaphragm muscle function did not take into account the potentially confounding factors of postnatal growth and fibre type when investigating the potential benefits of versican reduction in dystrophic muscles. Hence, this Chapter describes the functional benefits of genetically reduced versican in fast twitch EDL and slow twitch soleus muscles, which were more evident in 6 week compared to 26 week old mdx mice. Similar to the diaphragm in Chapter 4, in soleus muscles from young mdx mice, the genetic reduction of versican was associated with improved ex-vivo strength and endurance, whilst only endurance was improved in EDL muscles. This greater improvement in contractile function in 6 week mdx-hdf mice compared to 26 week mdx-hdf mice may be mediated by age related differences in versican
expression. These findings highlight the dynamic nature of the dystrophic ECM and the importance of considering age and postnatal growth in pre-clinical studies investigating strategies that target aberrant synthesis and remodelling in dystrophy.

Immunohistochemical staining for versican and versikine was used to assess versican synthesis and remodelling in EDL and soleus muscles from \textit{mdx} and \textit{mdx}-hdf mice, and to confirm the efficacy of the versican reduction in the muscles from \textit{mdx}-hdf mice. In fast twitch EDL and slow twitch soleus muscles, versikine immunoreactivity was greater at 6 weeks compared to 26 weeks of age. Furthermore, V0/V1 versican immunoreactivity was also greater in soleus muscles at 6 weeks of age, indicative of fibre type differences in versican expression. At 6 weeks of age, versikine immunoreactivity was localised to the endomysium surrounding muscle fibres and to regions of mononuclear infiltrate, comprised of satellite cells, inflammatory cells and fibroblasts. These hindlimb muscles are also likely to be completing the regenerative process following the acute bout of degeneration and regeneration at approximately 3 weeks of age (126). This high level of versikine staining highlights the biological relevance of versican remodelling to growth and repair in dystrophic muscles. Given the improvements in EDL and soleus muscle \textit{ex-vivo} contractile function in 6 week old mice, and the difference in versican and versikine expression, it is likely that the remodelling of the endomysial matrix is quite different in these two hindlimb muscles. Aside from a well characterised increase in collagen deposition in patients and \textit{mdx} mice (119, 221), the effects of postnatal growth and differences in fibre type on the composition and remodelling of transitional matrix proteins in dystrophic muscles has not been well described. Although, the versicanase ADAMTS5, which is implicated in versican remodelling during myogenesis (17), is increased in muscles of \textit{mdx} mice (423).

As observed in dystrophic diaphragm and TA muscles in Chapters 3 and 4, nuclear localisation of versikine was observed in dystrophic EDL muscles. This observation was associated with centralised nuclei of recently regenerated myofibres and nuclei of cells localised to the endomysium, which could include...
inflammatory cells, fibroblasts and muscle precursor cells. The nuclear localisation of versikine was especially pronounced in EDL muscles from 26 week old \textit{mdx} and \textit{mdx}-hdf mice. It has previously been suggested that versican may play a role in mitotic spindle organisation during cell proliferation (336), however careful reading of the methods used revealed that the antibody used for the study was specific for versikine and was the exact same antibody used within this thesis. Nuclear localisation of versikine was very limited in dystrophic soleus muscles irrespective of age, an interesting observation given the more severe pathology of soleus compared to EDL muscles at 26 weeks of age. Indeed, the soleus is one of the most affected muscles in \textit{mdx} mice (130). Furthermore, these differences in the nuclear localisation of versikine between EDL and soleus further supports the hypothesis that the fibres and their matrices are quite different between fast and slow twitch dystrophic muscles.

An additional novel observation was the strong versican, and weak versikine, immunoreactivity in muscle spindles in EDL muscles from 26 week old \textit{mdx} mice. Located parallel to muscle fibres, muscle spindles are sensory receptors that provide information about muscle length to the central nervous system, ultimately preventing the muscle from overstretching (536, 539). They can contain anywhere between 2-10 intrafusal muscle fibres which are surrounded by a viscous fluid, with the entirety enclosed within a capsule comprised of connective tissue (536, 540). The capsule of muscle spindles contains components of the transitional matrix, specifically hyaluronan (435), and this Chapter provides the first known instance of both versican and versikine being present within the capsule, and also surrounding the intrafusal muscle fibres. During the advancement of DMD pathology, although fibres within the muscle undergo degeneration and are eventually lost, the muscle spindles and their intrafusal fibres are structurally preserved, although are enclosed by an abnormally thickened capsule (541, 542). This preservation may possibly be due to having greater regenerative capabilities. Intrafusal muscle fibres have been observed to contained desmin (541), a marker for newly regenerated muscle fibres (484), along with hyaluronan and versican, transitional matrix proteins.
which are associated with myogenesis and regeneration (17, 435). Perhaps the presence of hyaluronan and versican may provide a permissive extracellular environment for muscle spindle regeneration. As hyaluronan was present in muscle spindles of wild type mice (435), it would be beneficial, therefore, to assess versican and versikine expression in muscle spindles from a wild type mouse to determine whether the upregulation of versican is inherent to muscle spindles, or is indeed part of the dystrophic pathology.

The results indicate that in dystrophic hindlimb muscles from \textit{mdx} mice, age and fibre type can all differentially affect versican synthesis and remodelling. This was especially apparent when versican and versikine immunoreactivity was quantified in EDL and soleus muscle cross-sections from \textit{mdx} and \textit{mdx-hdf} mice to confirm the genetic knock-down of versican. At 6 and 26 weeks of age, versikine immunoreactivity was lower in EDL muscles from \textit{mdx-hdf} mice compared to age matched \textit{mdx} littermates, whereas versican immunoreactivity did not differ. Whereas, in soleus muscles from 6 and 26 week old mice, versican immunoreactivity was lower in \textit{mdx-hdf} mice compared to age matched \textit{mdx} littermates, however versikine was not. This mirrors findings of reduced versican, but not versikine, immunoreactivity in diaphragm muscles from 21 week old \textit{mdx} mice (Chapter 4). A reduction in either V0/V1 versican or versikine immunoreactivity is indicative of a successful genetic reduction of versican at the protein level in \textit{mdx-hdf} mice. The reduction of versikine, rather than full length versican, in fast twitch EDL compared to slow twitch soleus or diaphragm muscles indicates fibre type differences in versican remodelling in \textit{mdx} mice, and as such, the expression of ADAMTS versicanases should be further investigated in these muscles. Given versican and versikine have been implicated in regulating cellular processes all relevant to DMD pathology, the observations detailed within this chapter highlights the need to consider synthesis and remodelling when targeting versican to ameliorate the pathology of dystrophic muscles. It should be noted that versikine itself is also likely further degraded, as seen through its clearance between E13.5 and 3 weeks of age in wild type mice (17),
although the pathophysiological significance of this process has not yet been well described.

Given that age and fibre type modulated versican synthesis and remodelling in dystrophic hindlimb muscles, it is not surprising that the genetic reduction of versican had differential effects on the contractile function of dystrophic hindlimb muscles from 6 or 26 week old \textit{mdx} mice. At 6 weeks of age, the genetic reduction of versican attenuated the fatigability and improved the force recovery of isolated EDL muscles following 4 m of intermittent, submaximal stimulation. However, this improvement in EDL muscle endurance was lost by 26 weeks of age. This was in contrast to observations in the 21 week old \textit{mdx}-hdf diaphragm muscles, which continued to have improved fatigability and force recovery \textit{ex-vivo}, alongside data from metabolic cages that demonstrated increased spontaneous physical activity (Chapter 4).

Unlike the dystrophic diaphragm muscles in Chapter 4, the genetic reduction of versican did not improve the force producing capacity of EDL muscles from 6 or 26 week old \textit{mdx} mice. In line with these functional observations is the fact that the genetic reduction of versican had minimal effects on muscle morphology. The dystrophic pathology of EDL muscles when compared to diaphragm muscles or soleus muscles, especially in adult mice, was overall quite modest with minimal myofibre degeneration, endomysial fibrosis and mononuclear infiltrate observed. The most striking effect of versican reduction is the decrease in \textit{F4/80} gene expression in EDL muscles from 26 week \textit{mdx}-hdf mice, associated with a trending decrease in mononuclear infiltrate and degeneration. These data might be indicative of a suppressed inflammation in EDL muscles from \textit{mdx}-hdf mice, and the reduction of versican may therefore impair the differentiation of monocytes to macrophages (290). This may be a potential reason why \textit{mdx}-hdf muscle function was similar to that of the \textit{mdx} mice. Therefore, more detailed investigations of monocytes and both pro-inflammatory M1 and anti-inflammatory M2 macrophage subsets using immunohistochemical techniques or flow cytometry, are therefore required to gain further insight on whether versican does indeed modulate inflammation in \textit{mdx}-hdf mice. An excessive
inflammatory response can be potentially driven by a dysregulated ECM (285), and may contribute to muscle damage and degeneration in DMD. Alternatively, attenuating inflammation reduced muscle necrosis and degeneration in \textit{mdx} mice (377, 523). The proportion of centrally nucleated fibres tended to be lower in EDL muscles from 26 week old \textit{mdx-hdf} mice and could therefore be associated with a reduction in myofibre damage. To assess myofibre damage within EDL muscles, immunohistochemical staining for albumin should be used to positively label myofibres sarcolemmal damage (543).

Myogenesis requires the cleavage of versican in order for myoblasts to fuse together (17), however, versican was similar between the \textit{mdx} and \textit{mdx-hdf} EDLs at 26 weeks. This is in contrast with results from Chapter 3 from glucocorticoid treated C2C12 cells which were associated with a reduction in versican and hyaluronan and had an increase in myoblast fusion, and hence improved regeneration \textit{in-vitro}. In addition to similar V0/V1 versican, the myogenic genes tested, \textit{MyoD} and \textit{Myogenin}, also remained similar within the mice. This is suggestive that unlike the reduced versican in glucocorticoid treated C2C12 cells, a reduction of versican may not influence myogenic properties in the 26 week EDL. As myogenesis is a very complex process, containing a range of signalling pathways (246), a more in depth study into the \textit{mdx} and \textit{mdx-hdf} EDLs is required in future studies. This may be achieved through histological or immunohistological staining, as well as gene expression covering myogenic pathways in order to better understand the regenerative processes that are occurring in this muscle.

Similar to the EDL muscles at 6 weeks of age and diaphragm muscles at 21 weeks of age, the genetic reduction of versican was associated with improved fatigability \textit{ex vivo} in soleus muscles from 6 and 26 week old \textit{mdx} mice. Although, this improvement was more modest when compared to EDL and diaphragm muscles, and force recovery post fatigue was not affected. At 6 weeks of age, the genetic reduction in versican also increased in the strength of dystrophic soleus muscles, as indicated by an upward shift in the force frequency curve. Contributing to this increase in strength, might be an increase in muscle fibre
size, as soleus myofibres from mdx-hdf mice tended to an increase in the proportion of large muscle fibres (>70 µm in diameter) when compared to soleus myofibres from mdx littermates. This improvement in strength and slight increase in myofibre size was also observed in diaphragm muscles from mdx-hdf mice at 21 weeks age. However, by 26 weeks of age, soleus muscles from mdx-hdf mice produced less force than muscles from age matched mdx littermates. The increased mononuclear infiltrate and smaller fibre size are most likely the cause of the reduced soleus strength at 26 weeks, as both inflammation (544) and small fibre size (545), results in decreased force production. As previously discussed, this mononuclear infiltrate is comprised of inflammatory cells, satellite cells and fibroblasts. Although no differences were observed for the mRNA transcripts of MyoD, Myogenin, F4/80 and Collagen1α1 in the 26 week old soleus muscles, given the increase in mononuclear infiltrate, future studies would be required to investigate the populations of neutrophils, monocytes, macrophages, satellite cells, myoblasts and fibroblasts in soleus muscles from the 26 week old mdx and mdx-hdf mice.

It is generally thought that fast twitch muscle fibres are more susceptible to contraction induced injuries than slow twitch muscle fibres (68, 546). It was therefore surprising that the 6 week EDL had a lower amount of centrally nucleated fibres, and at 26 weeks had less mononuclear infiltrate and degeneration, when compared to the soleus at the same ages. Furthermore, H&E sections revealed the dystrophic pathology of soleus muscles was indeed more severe when compared to the EDL muscles from mdx and mdx-hdf mice at 26 weeks of age. This was similar to previous findings reporting the soleus of mdx mice to be the most damaged hindlimb muscle at 3-4 weeks of age (128), during the period of spontaneous degeneration and regeneration (126), and was still the most damaged hindlimb muscle at 26 months of age, displaying increased fibrosis and mononuclear infiltrate (130). This may be because the soleus is a postural muscle and is permanently in use, hence being more highly recruited than the EDL (127, 535).
Furthermore, the diaphragm is said to be the muscle in mdx mice that most closely resembles a limb muscle in the human form of the disease, due to its increased fibrosis, degeneration and functional decline (455), however in chapter 4, the diaphragms did not have differing severity levels of pathology as the soleus muscles did in this chapter. Although the diaphragm is in constant use, stated to be one of the most used muscles in sedentary laboratory mice (130), the postural soleus muscle also has constant use during ambulation (127, 535), but may be influenced to work harder by the weight of the mouse. Furthermore, it has also been suggested that the severity of dystrophic pathology is not directly related to muscle fibre types, given that the diaphragm, tongue and soleus muscles are all comprised of differing fibre types, yet all exhibit worsened pathology (130). Therefore, perhaps the soleus pathology is worsened upon ambulation, leading to an increase in degeneration and an apparent disruption of regenerative processes, potentially caused by satellite cell depletion (547).

The H&E stained soleus muscle cross-sections contained extensive regions of mononuclear infiltrate, with necrotic and degenerating myofibres observed alongside ghost fibres. Ghost fibres are formed when a damaged myofibre becomes necrotic and is removed by inflammatory cells, leaving behind the ECM which remains structurally intact (537, 548). This allows for the stability of the surrounding environment, including other myofibres, blood vessels and nerves (537). These hollow tubes of ECM also act as a guide for the migration of satellite cells and formation of new myofibres, ensuring their correct orientation within the old basement membrane (548, 549). The ECM surrounding the ghost fibres did not stain for versican or versikine, nor were any nuclei observed within ghost fibres during H&E analysis. As versican is known to be associated with the proliferation of satellite cells (20) and myogenesis (17), the results are therefore suggestive that the ghost fibre scaffolds may be incompatible for myogenesis or that regeneration may potentially be impaired in the 26 week old soleus muscles.
Indeed, it is known that the loss of dystrophin negatively affects satellite cells, leading to a dysregulated function and regenerative impairment in mdx mice (114). Additionally, satellite cell exhaustion has previously been reported in mdx mice, potentially arising through an exhausted supply due to dystrophic pathology with ongoing cycles of degeneration and regeneration, or through a natural decline with age (547). From the results detailed within this Chapter, it can be hypothesised that multiple bouts of degeneration and repair, such as the increased amounts of mononuclear degeneration in the 26 week mdx-hdf soleus, potentially contribute to the exhaustion of satellite cells within dystrophic muscles. Hence, further investigations into the effects of satellite cell proliferation in fast and slow twitch muscles, with and without the reduction of versican, should be undertaken. This could be achieved through single fibre studies in fast and slow twitch muscles of mdx and mdx-hdf mice, by immunohistochemically staining single muscle fibres in order to view the activation, proliferation, migration and differentiation of the attached satellite cells (522).

Modulating the transitional matrix in mdx mice by genetically reducing versican tended to improve the pathology of the 26 week EDL, as seen through the trending decrease in mononuclear infiltrate; however it worsened the pathology of the 26 week soleus by increasing the amount of mononuclear infiltrate. This further highlights the importance of the ECM, being muscle and fibre type specific. The downstream effects on muscle structure and function, as well as versican synthesis and remodelling, are all modulated by muscle fibre type and mouse age, and altogether determine postnatal growth and regenerative capacity of skeletal muscle. In hindlimb muscles, the greatest functional benefits of versican reduction were observed in the 6 week mdx mice. Specifically, in the mdx-hdf mice, the genetic reduction of versican resulted in a modest and transient improvement in ex-vivo force producing capacity in slow twitch soleus muscles at 6 weeks, but not 26 weeks, of age. The association between versican reduction, improved muscle endurance and a shift towards a more oxidative whole body metabolism has been a consistent observation of this thesis,
although the underlying mechanisms remain to be elucidated. Similar to the diaphragm muscles observed in Chapter 4, the genetic reduction of versican also did not affect time to peak tension (TPT) or \( \frac{1}{2} \) relaxation time (\( \frac{1}{2} \) RT) in response to a single twitch stimulation in EDL and soleus muscles at 6 or 26 weeks of age. This is suggestive that a fibre type shift towards a more slower phenotype is less likely and perhaps the improvement in ex-vivo endurance was mediated by an increase in mitochondrial oxidative capacity. Future studies should therefore investigate this hypothesis by using gene expression markers for glucose and fatty acid oxidation to gain a better insight into the metabolic properties of the mice. Furthermore, the functional significance of these findings should be investigated in more detail by using exercise protocols to assess maximal exercise capacity and endurance training adaptations (550). In \( \textit{mdx} \) mice treated with tranilast, an antiallergic drug, an association between improved endurance and a reduction in fibrosis was observed in the diaphragm and TA muscles (551). Furthermore, glucocorticoids prolonged ambulation in patients with DMD (552) and reduced fibrosis in \( \textit{mdx} \) mice (526), perhaps due to a reduction of versican. Indeed, versican is associated with the transition of fibroblasts to myofibroblasts (265, 348). Myofibroblasts are constantly stimulated in DMD (107) and hence secrete copious amounts of collagens I, III and IV, thereby expanding fibrosis in dystrophic muscles (271). The effect of reduced versican upon fibroblasts and myofibroblasts will need to be investigated in future studies. This could be undertaken by immunohistochemically staining muscle sections with antibodies specific for fibroblasts and myofibroblasts. Furthermore, fibrosis would also need to be investigate in more detail, and a hydroxyproline assay which allows for total collagen content (553) would be beneficial. However as versican is an abnormally overexpressed transitional matrix protein in fibrotic muscle of DMD patients and \( \textit{mdx} \) mice (90, 465), future studies detailing the composition of the fibrotic ECM would also be required. Additionally, it remains unknown as to whether fast and slow twitch muscles elicit any fibre type differences in the composition of fibrotic tissue. Therefore, future studies would require detailed studies involving immunohistochemically stained muscle sections and qRT-PCR
techniques, in both fast and slow twitch muscles, in order to investigate reduced versican on the composition of fibrotic ECM in differing fibre types.

Altogether, the results detailed within this Chapter demonstrate significant differences in the synthesis and remodelling of a versican-rich transitional matrix in fast and slow twitch hindlimb muscles from young 6 week, and adult 26 week *mdx* and *mdx*-hdf mice. An overview of EDL and soleus data obtained in Chapter 5 and the associated mechanisms that were hypothesized, can be viewed in Figure 5.20 for the EDL, and Figure 5.21 for the soleus. To effectively target fibrosis in dystrophy, the transitional matrix, and the factors which modulate its synthesis and remodelling, need to be better understood. The data detailed within this Chapter has important implications for the design of pre-clinical studies that target fibrosis in mouse models of DMD disease treatment, highlighting the growing and urgent need to consider age, growth and fibre types when designing and administering therapeutics to patients with DMD.

**Figure 5.20 Overview of Chapter 5 EDL results and possible mechanisms.**
This figure summarises the data obtained from the soleus in Chapter 5 and also outline the possible mechanisms that were hypothesized.
Figure 5.21 Overview of Chapter 5 soleus results and possible mechanisms.
This figure summarises the data obtained from the soleus in Chapter 5 and also outline the possible mechanisms that were hypothesized.
Chapter 6: Discussion and future directions

6.1 Research problem

It has been thirty-one years since dystrophin was identified as the genetic cause of DMD (464). There is no cure for the disease, although advancements in therapeutics have led to improvements in the quality of life of patients with DMD. Medical interventions targeting cardiac (554) and respiratory function (555) have increased the life expectancy of patients with DMD, and survival into the third decade of life is becoming more common (556, 557). To further improve health outcomes for patients with DMD, there is an urgent need for more effective therapeutic strategies, which target fibrosis in skeletal muscles and the heart. In DMD, a dysregulated ECM is not just a consequence of the disease, but actively contributes to skeletal muscle degeneration and loss of function (119). Therefore, the ECM should be a target for novel therapeutics.

Despite intensive research efforts, glucocorticoids remain the only pharmacological intervention with clinical efficacy in DMD. Indeed, ambulation is prolonged in patients treated with glucocorticoids (552), and fibrosis is attenuated (526). However, glucocorticoids are not without significant side effects (365, 376), and to reduce these, intermittent administration is used (526). In dystrophic muscles from patients with DMD and in mdx mice, glucocorticoids attenuate fibrosis (526), enhance regenerative myogenesis (397, 430) and improve skeletal muscle function (366). A better understanding of underlying cellular mechanisms by which glucocorticoids exert their effects upon dystrophic muscles, particularly during postnatal growth, should lead to the development of more effective therapeutic strategies for DMD.

6.2 Relevance of versican in DMD

Chapter 3 of this thesis identified versican as an important downstream target of dexamethasone during myogenic differentiation, which builds on previous studies describing enhanced myogenesis (397, 430), and in other models, pleiotropic effects on the extracellular matrix (123, 373, 375). As expected,
treating differentiating C2C12 myoblasts with a low dosage (25 nM or 100 nM) dexamethasone enhanced fusion and myotube formation. An important contributing mechanism to this enhancement in myogenesis was the decreased expression of genes associated with the synthesis of a transitional matrix; in particular, V0/V1 versican and Has2, Has2 being a key enzyme in hyaluronan synthesis within muscle that synthesises hyaluronan (435). Both hyaluronan and versican are removed during myogenesis (17). Furthermore, hyaluronan forms a complex with versican, and together are an important part of the transitional matrix (73, 74). This transitional matrix is crucial for successful development and tissue repair, being secreted into an injured area where it directs cellular processes such as inflammation and regeneration. There is significant interest in the transitional matrix because of its association with various diseases, such as idiopathic pulmonary fibrosis (558) and myocardial infarction (527). The transitional matrix is dysregulated in DMD, as indicated by the increased expression of versican (90), hyaluronan (420) and tenascin-C (421). As described in Chapter 3, versican is also upregulated in DMD, being observed in the TA muscle, and to much larger extent, in the more severely affect diaphragm muscle (465). In skeletal muscle, the carefully regulated synthesis and remodelling of versican is important for the proliferation of satellite cells and myogenic differentiation (17, 20). Versican has also been implicated in regulating inflammation and fibrosis, by directly binding chemoattractant proteins and leukocytes (51, 53), and being associated with the differentiation of monocytes into macrophages, and fibroblasts into myofibroblasts (265, 290). These observations strengthen the relevance of versican as an ECM target for DMD.

The signalling pathways that regulate the promoter and expression of versican are well known (306, 559). Quite interestingly, some of the pathways that modulate the expression of versican are also pathways regulated by glucocorticoids, which are the only treatment with clinical relevance for DMD. For instance, the canonical wingless and sonic hedgehog pathways are known to modulate versican expression (306). Glucocorticoids have been shown to supress the canonical wingless pathway in human osteoblast cell cultures (560), and
inhibited the sonic hedgehog pathway which led to the inhibition of cerebellar growth and proliferation of cerebellar granule neuron precursor cells in 7 day old postnatal mice (561). Furthermore, based on in-vitro data from fibroblasts, another modulator of versican is TGFβ (346). Glucocorticoids can downregulate TGFβ (285), which in turn can then down regulate versican expression. These observations are suggestive that some of the signalling pathways that regulate versican expression are also modulated by glucocorticoids. This further strengthens the relationship between versican and glucocorticoids, a major theme of Chapter 3 in this thesis.

The most common glucocorticoids administered to patients with DMD are prednisone, prednisolone and deflazacort (562). There is also increasing interest in dissociative glucocorticoids, modified versions created to have fewer side effects, yet still retaining all the benefits such as muscle membrane stability and reduced inflammation (371). One such dissociative glucocorticoid is VBP15 (377), more recently known as vamorolone (563). In mdx mice, vamorolone increased muscle strength and reduced inflammation, with a reduced side effect profile (377). Vamorolone is currently in phase II clinical trials in human patients with DMD (564).

Given that versican is suppressed by glucocorticoids in-vitro, and is upregulated in dystrophic diaphragm and hindlimb muscles, a genetic approach was taken to reduce versican expression in mdx mice and to establish its relevance as a target in DMD. Specifically, female mdx mice were mated with male hdf mice, which have a transgene insertional mutation in the versican gene, to generate F1 male mdx and mdx-hdf pups. It should be noted that this breeding has never before been undertaken, and the creation of the mdx-hdf mice, a dystrophic mouse with reduced versican, represents a new novel strain of dystrophic mice. Chapters 4 and 5 of this thesis detailed the characterisation on whole body physiology, muscle morphology and contractile function in diaphragm and hindlimb muscles from young 6 week, and adult 26 week mdx and mdx-hdf mice.
The results in Chapter 3 (12 week diaphragm), Chapter 4 (21 week diaphragm) and Chapter 5 (6 and 26 week EDL and soleus) all show that versican is differentially expressed diaphragm and hindlimb muscles in the mdx and mdx-hdf muscles at different ages. Of particular note was the expression of versican in the 12 week mdx diaphragm, which remained at a similar level of expression in the 21 week mdx diaphragm. This was despite an expected worsening of pathology and fibrotic progression at an older age (107). Furthermore the mdx diaphragm at 12 weeks and the mdx soleus at 6 weeks had similar levels of versican. This is of great interest as the soleus was the most damaged hindlimb muscle in mdx mice during the period of spontaneous degeneration and regeneration, at approximately 3-4 weeks of age (126, 128). As expected, the 21 week old mdx diaphragm still had the highest amount of versican out of any muscle tested. However the 26 week mdx soleus had the lowest amount, which was unexpected considering the soleus is most damaged hindlimb muscle at 26 months of age (130). Also unexpected was the versikine level for the 6 week mdx EDL, which was at a much higher level than any muscle tested at any age, however, by 26 weeks the levels had reduced to that similar to the 21 week old diaphragm. Furthermore, it wasn’t just the levels of versican and versican fluctuating between the mdx muscles, but also the levels of versican and versikine expressed by the mdx-hdf mice. In the 6 and 26 week EDL, mdx-hdf mice were found to have similar levels of versican, with significantly reduced versikine expression, however in the 6 week soleus and 21 week diaphragm muscles of mdx-hdf mice, the opposite occurred with significantly reduced versican and similar expression levels of versikine when compared to the control mdx mice. This signifies that the differential expression patterns of both versican and versikine in these muscles are highly dependent upon muscle type and age.

Muscle function was also differentially expressed amongst the different ages and muscles tested. The reduced versican in the diaphragm was found to be very beneficial, being observed to increase muscle strength, and resistance to fatigue. Additionally, the relationship between reduced versican in the hindlimb and muscle function appeared to be more complex, however the majority of benefits
were observed in the younger 6 week old mice. It was originally hypothesized that the reduction of versican would attenuate inflammation and fibrosis, and increase regeneration. However, this was proven to be incorrect. When the mononuclear infiltrate and collagen gene expression was investigated, neither the diaphragm (Chapter 4), nor the 6 or 26 week EDL, or 6 week soleus (Chapter 5) had differences in inflammation, fibrosis or pathology. However, in the 26 week soleus however, the \textit{mdx}-hdf mice had significantly increased mononuclear degeneration, yet gene expression markers for MCP-1 and F4/80 remained unchanged. This is suggestive that the soleus was undergoing degeneration but with possible limited inflammation, however this remains to be tested in future studies. In contrast the other hindlimb muscle tested, the 26 week \textit{mdx}-hdf EDL, although only a trending decrease in the mononuclear infiltrate was observed, the gene expression of the macrophage marker F4/80 was reduced.

Furthermore, in Chapter 3, the \textit{in-vitro} treatment of C2C12 muscle cells with glucocorticoids was associated with both the reduction of \textit{Vcan} and \textit{Has2} gene expression, and with an increase in myogenesis. Myogenesis was not affected in the \textit{mdx}-hdf EDL or soleus, as no differences in fibre numbers were observed, while the gene expression for \textit{Vcan} and \textit{Has2}, and the myogenic markers \textit{MyoD} and \textit{myogenin}, all remained similar in both muscles. However a trend existed in the \textit{mdx}-hdf diaphragm for increased centrally nucleated fibres, an indicator of recently regeneration. Additionally, the \textit{mdx}-hdf diaphragm also had a reduction in \textit{Vcan} and a trending reduction in \textit{Has2} gene expression (Chapter 4). This is in accordance not only with the findings in Chapter 3, but also with a study which found both versican and hyaluronan is cleaved prior to muscle fusion (17). Furthermore, the \textit{mdx}-hdf diaphragm also was similar to the glucocorticoid treated C2C12 cells of Chapter 3, as expression of both \textit{Hyal2} and \textit{Adams5} remained similar in both models. Interestingly, unlike the \textit{mdx}-hdf diaphragm, the glucocorticoid treated cell culture model had increased gene expression levels of \textit{Adams1}. However, a cell culture model is very simple, and does not have the complex interplay of processes, such as inflammation and regeneration, like a mouse model would have. This may account for the differences observed.
The observations that versican and versikine were differentially expressed in the diaphragm, EDL and soleus, and at different ages suggested that the matrix was mostly likely fibre type specific in the different muscles tested. However, many of the conclusions in this thesis were limited by the experimental procedures performed, and therefore more comprehensive studies are required to fully elucidate the effects that genetically reduced versican may have on dystrophic pathology, and what differences may exist between the mechanism or signalling pathways of reduced versican and glucocorticoids. Hence there are a number of limitations in the next section that are listed accordingly for this thesis.

**6.3 Limitations of this thesis**

**6.3.1 Limitation of chapter 3**

A limitation for Chapter 3 is that only C2C12 mouse myoblasts were used for the experiments with dexamethasone. It would be interesting to use a DMD cell line, which would have been valuable in order to gain an understanding of how glucocorticoids worked in dystrophic cells, such as myoblasts and fibroblasts. It also would have allowed the identification of any dystrophic specific results.

Another limitation of Chapter 3 is that glucocorticoid induced reduction of versican was only shown in an *in-vitro* cell culture model using C2C12 myoblasts. This cell culture model recapitulates signalling pathways relevant to myogenesis, but does not model the complexities of muscle repair in dystrophic muscles *in-vivo*, where there is a complex interplay between cell populations involved in inflammatory, myogenic and fibrotic processes. Hence future experiments involving *mdx* mice being treated with dexamethasone would be required to see if it does indeed reduce versican *in-vivo*. Additionally *mdx*-hdf mice could also be treated to elucidate if there would be any effects of glucocorticoid action even with reduced versican. These experimental mouse models would help better understand the role of versican and glucocorticoids in dystrophy.

**6.3.2 Limitations of chapters 4 and 5**

There are a number of limitations that can be listed for Chapter 4 and 5 of this thesis. Versican is a transitional matrix protein with low levels expressed in wild
type mice, however versican becomes largely upregulated in dystrophic mdx mice (Chapter 3). In this thesis, hdf mice were bred with mdx mice to produce dystrophic offspring with reduced versican, which were characterised in Chapter 4 and 5 of this thesis. A limitation therefore arises in that no C57BL/10 wild type mice, the background strain of the mdx mice, or any C57BL/6 wild type mice, the background strain for the hdf mice, were utilised in these chapters. The inclusion of the C57BL/10 wild type mice would have allowed for a more comprehensive analysis, allowing for a comparison on how effective the reduced versican was at restoring normal function when compared to mdx mice. Additionally by including the C57BL/6 wild type mice into the study, it would have allowed a comparison to be made between all strains, allowing for the determination of what effects were specific to genetically reduced versican in the dystrophic mdx phenotype, and which were specific to the hdf phenotype.

Furthermore, as the mdx-hdf mice have never before been bred, and as inflammation and regeneration were not thoroughly investigated in this thesis, it represents a lost opportunity to characterise these processes during known points in DMD where degeneration and regeneration occur. Hence, future studies should be performed on mdx and mdx-hdf mice during the known bout of spontaneous degeneration at 4 weeks of age, where inflammation and regeneration could be thoroughly investigated. This would determine whether or not genetically reduced versican could affect the pathology of DMD.

Additionally, regarding the heart data in Chapter 4, it remains unclear if the effects upon the increased left ventricle in the mdx-hdf mice was due to the reduced versican expression in the dystrophic heart, or if it was due to possible developmental hearts defects in the heterozygous hdf mice, as versican is crucial for heart development. A limitation here is that although hearts were collected, they were not examined further than what was documented in this chapter, hence the morphological differences remain unknown.

An unexpected yet exciting result was that the reduction of versican was found to affect fatigability. Hence there is a need to better understand the endurance
phenotype by investigating the fibre types and mitochondrial markers. Chapter 4 studied only focused upon type IIa fibres, which found no differences and hence highlights the importance of having a complete and thorough analysis of fibre types in not only the mdx and mdx-hdf diaphragm, but also in the EDL and soleus muscles. The muscle and age specific differences could also have been analysed more deeply by including studies upon mitochondria, and is hence another limitation for Chapters 4 and 5 of this thesis. Here it could have been beneficial to include gene expression analyses, or perform an oxidative stain, to observe any mitochondrial defects in the mdx and mdx-hdf mice. This would have complimented the fibre type staining, and together may have explained the changes in muscle strength and function of the various muscles at 6 and 26 weeks of age.

Another limitation for these chapters is that there are no specific stains for fibrosis in the mdx or mdx-hdf muscles. In chapter 4, WGA was used to view the area of fibrosis, which was quantified. However WGA is known to stain the cell membrane and is not a specific fibrotic marker, hence this is a limitation for fibrotic quantification. Another limitation is that no specific staining or analysis for fibrosis was undertaken for the 6 or 26 week old EDL or soleus muscles. It would have been beneficial to include specific fibrotic staining, such as Van Geissons stain, or even performed a hydroxyproline assay, in order to accurately investigate fibrotic content in all of the muscles tested in Chapters 4 and 5.

6.4 Key findings and future directions

Overall, this thesis found that the genetic reduction of versican had various differential effects on the 3 muscles types tested in this theses, namely the diaphragm, EDL and soleus muscles. The reduction of versican resulted in a major improvement in the diaphragm, whereas the majority of benefits in the hindlimb were observed in the young and growing 6 week mice. Interestingly, the improvements seen in these mice was found not to be due to the original hypotheses that reduced versican would attenuate inflammation, fibrosis and improve myogenesis. Instead as the experiments in this thesis progressed, the
originally proposed hypothesis were proven wrong and new mechanisms of action were proposed along with suggested experimental procedures and future directions.

At 26 weeks of age, the genetic reduction of versican was overall beneficial. Indeed, \textit{mdx}-hdf mice had increased spontaneous physical activity when compared to their \textit{mdx} littermates. This was associated with a shift towards a more oxidative, whole body metabolism, along with increased diaphragm muscle strength and endurance \textit{ex-vivo}. The diaphragm is one of the most severely affected muscle with regards to deficits in contractile function and expansion of the extracellular matrix (125). Follow-up studies should investigate whether these changes in contractile function and whole body metabolism in the diaphragm lead to an increase in exercise capacity and endurance in the \textit{mdx} mice. The cellular mechanisms by which whole body metabolism shifted to more oxidative phenotype, as observed by the decrease in RER during the day and night periods, shown in Chapter 4, also requires further investigation. Specifically, mitochondria would need to be isolated and tested with multiple functional assays that measure the rate of ATP production, mitochondrial yield and size (473), followed by analyses using gene and protein markers of oxidative metabolism.

The fact that the downregulation of a single transitional matrix protein was able to modulate spontaneous physical activity, whole body metabolism, and diaphragm contractile function, is a novel and important finding. It builds upon previous studies in \textit{mdx} mice which targeted fibrosis in general, rather than a single ECM protein, and which also found improved muscle endurance and metabolism (551, 565). It’s worth remembering, that pathology of dystrophic muscles is also associated with profound metabolic abnormalities (473), and that the association between the ECM and mitochondria is gaining interest (517, 566).

Growth in skeletal muscle is associated with extensive ECM remodelling. Furthermore, there are differences in the matrix structure of fast and slow
twitch fibres. The fast twitch fibres contain more laminin (11). However, the slow twitch fibres contain more dystrophin, utrophin and collagen IV in their matrices (11, 64, 65). Therefore, Chapter 5 characterised the effects of reduced versican on the contractile function and pathology of fast twitch EDL muscles and slow twitch soleus muscles in young (6 week) and adult (26 week) mdx mice.

At 6 weeks of age, the genetic reduction of versican increased the strength and endurance of soleus muscles ex-vivo. However, this improvement in strength was transient, as by 26 weeks of age, soleus muscles from mdx-hdf mice produced less force than those from age matched mdx littermates. Interestingly, the genetic reduction of versican in EDL muscles was associated with a transient improvement in muscle endurance at 6 weeks age. In contrast to observations in slow twitch soleus and diaphragm muscles, the ex-vivo strength did not differ between EDL muscles of mdx and mdx-hdf mice.

The functional benefits of versican appear to be quite complex. Versican was found to be at higher levels in the 12 week old mdx diaphragm than wild type mice (Chapter 3). However, in an interestingly and unexpected observation, the 21 week old mice with reduced versican (Chapter 4) had similar versican levels to those of the 12 week diaphragms (Chapter 3), despite the age difference and despite the worsening progressive fibrotic pathology at a later age (107). Furthermore, the reduction of versican becomes much more complicated in the hindlimb muscles, which appear to contain less fibrosis than that of the diaphragm. Interestingly through, the pathology of the soleus was found to be much more affected than the EDL. This could be because the soleus is a postural muscle and is hence used more often than the EDL muscle. This is highly suggestive that there are clear fibre type differences in DMD, although this is currently not well recognised in the literature. Furthermore, this observation is suggestive that any fibrotic therapies will most likely effect muscles differently, and therefore fibre type should be considered upon the administration of therapeutic treatments.
This increases the relevance of the model to DMD, where fibrosis is more extensive in the diaphragm than in hindlimb muscles from *mdx* mice (125). However, these conclusions need to be tempered with caution, as in soleus muscles from 26 week old *mdx* mice, versican reduction was associated with a loss of muscle strength. The underlying cellular mechanisms remain to be determined, but likely involve downstream effects on satellite cells and infiltrating inflammatory cells. Nonetheless, the findings from Chapter 5 highlight the importance of considering the effects of postnatal growth, fibre type, ECM expansion, and remodelling when testing therapeutic strategies to ameliorate fibrosis in dystrophic muscles. Future studies are required to investigate the cellular mechanism by which versican reduction affects regenerative myogenesis, inflammation and metabolism, to produce the observed functional phenotype *in-vivo*.

Additionally, the lack of comprehensive fibre typing and no specific measure of fibrosis are obvious limitations and hence will be required in future experiments to better characterise the *mdx* and *mdx*-hdf diaphragm, EDL and soleus muscles tested within this thesis. Additional fibre type staining for all MyHC variants are hence required. A specific marker for fibrotic tissue, such as a hydroxyproline assay, as per TREAT-NMD guidelines (SOP ID number: DMD_M.1.2.006) would also be necessary. Once the fibre types and fibrosis content are characterised, a better understanding can be had of the impact of reduced versican on the different muscle types tested, and can also be used to further interpret the data obtained in chapters 4 and 5 of this thesis, particularly upon the alterations in muscle strength and resistance in fatigue.

Exercise interventions studies should be also undertaken to assess the effects of versican reduction on endurance and aerobic capacity. Exercise also results in the exacerbation of dystrophic muscle damage and degeneration (550), and hence can be used as a stimulus to investigate the effects of reduced versican upon inflammation and regeneration in *mdx* mice. The resultant inflammatory response within the muscle could be characterised through flow cytometry or immunohistochemical staining of inflammatory cells such as neutrophils.
monocytes and macrophages. Additionally, macrophages may be studied in further detail by investigating the populations of pro-inflammatory M1 and the anti-inflammatory, regenerative M2 a-c subsets.

Similar experimental techniques could also be used to assess the effects of versican reduction upon myogenic processes, such as satellite cell activation, along with myoblast proliferation and differentiation. Perhaps the decrease in soleus muscle strength was mediated by a decrease in the proliferative potential of satellite cells, through a reduction in versican (20). Indeed, this theory is supported by in-vitro observations from Chapter 3, where glucocorticoid treated C2C12 cells were associated with a reduction in versican expression and increased cell fusion, and hence resulted in increased regeneration. An alternate approach to assess the effects of versican reduction on the proliferation and differentiation of satellite cells would be single fibre cell culture. For this, single fibres would need to be carefully harvested from muscles of interest and placed into culture. As satellite cells remain under the basement membrane, this would allow for myogenic processes such as satellite cell activation, proliferation, migration and differentiation, to be observed by immunohistochemically staining and live imaging the cells (522).

Furthermore, the question arises whether it the effects of versican in this thesis are due to the entire versican protein, the core protein, or the presence or cleavage of the GAG regions. Any change in the form of the versican protein, such as a reduction in expression, an increase in GAG chain size or sulfation pattern could result in the modification of inflammation, myogenesis or ECM remodelling pathways. Hence it would be of great interest to investigate the synthesis, post-translation modifications and remodelling of versican (formation of versikine or cleavage of GAG chains) in the mdx and mdx-hdf mice in order to see if dystrophy, or the decrease of versican would change the form of the versican protein and greater results similar to what is described in this thesis. This would also help to further determine the role of versican in different muscle types and ages in a dystrophic model.
In addition, as the reduction in versican was only characterised in a dystrophic model in this thesis, it would therefore be beneficial to breed female C57BL/6 wild type mice with male hdf mice to generate normal healthy male mice with genetically reduced versican. Experiments performed in thesis will need to be done on muscles from these mice to characterise the effects of versican in a non-dystrophic model. Additionally, these mice could be subjected to a muscle injury, allowing the processes of degeneration, inflammation and regeneration to be studied in order to elucidate any effects of reduced versican in a normal mouse model. This would be a major help in understanding whether the effects in this thesis were because of an improvement in dystrophic pathology or due to a reduction in versican.

Furthermore, in Chapter 3 of this thesis, glucocorticoids were found to suppress versican gene and protein expression in C2C12 cells, a simplified and uncomplicated model of muscle regeneration. However it remains unknown if glucocorticoids also reduce versican within dystrophic muscles in-vivo, a highly intricate model which has the added complexity of cellular process such as inflammation, regeneration and fibrosis, and hence will need to be studied in the future. For this to occur, the period of glucocorticoid treatment would require careful consideration in an in-vivo model. For instance, a daily administration of glucocorticoids to mdx mice initially improved muscular strength, however the continuous treatment ultimately resulted in side effects, such as loss of muscle strength, increased cardiac fibrosis after a period of 100 days (366), and upregulated atrophic pathways (526). However, weekly treatment with glucocorticoids resulted in mdx mice having better repair of muscle membranes, improved the regenerative capacity of muscle, and had fewer side effects, as atrophic pathways were downregulated (526). Furthermore, the effects of glucocorticoids upon mice with genetically reduced versican currently remains unknown. It can be speculated that mdx-hdf mice treated with glucocorticoids could have minimal benefits from glucocorticoids as versican is already reduced. Or perhaps glucocorticoid treatments would still benefit these mice in areas where the reduction of versican has no effect, especially as the exact
mechanisms and cell signalling pathways regulated by glucocorticoids, such as the downstream consequences on inflammation, fibrosis and muscle function in DMD, are still relatively unknown. Therefore, in order to confirm versican downregulation in an in-vivo study, and to see if glucocorticoids have any benefits in mice with reduced versican, the glucocorticoid treatment of mdx and mdx-hdf mice must be undertaken by using a clinically relevant, intermittent dosing protocol. Based on findings from Chapters 4 and 5 of this thesis, most benefits of reduced versican were observed in mice at 6 weeks of age, therefore it is important to start glucocorticoid administration early, during the period of growth and before fibrosis becomes a problem (124). The diaphragm and the soleus muscle were found to be the most badly affected muscles both within this thesis, a finding supported by published literature (130, 567). Therefore, to confirm the functional benefits of glucocorticoid treatment in dystrophic mice, whole body metabolism, exercise capacity and skeletal muscle contractile function would need to be undertaken on the diaphragm and soleus muscles of 6 week old mdx and mdx-hdf mice. This should be followed with biochemical analysis of transitional matrix synthesis and remodelling, including versican and versikine expression, and markers of regenerative myogenesis and inflammation.

6.5 Novel pharmaceutical strategies to target excess versican in DMD

Using cell culture models and mdx mice, this thesis identifies versican as a transitional matrix protein overexpressed in dystrophic muscles. It presents a comprehensive physiological characterisation of the effects of versican reduction on the pathology and function of diaphragm and fast and slow twitch hindlimb muscles from young growing, and adult mdx mice, which results being overall are quite favourable. Glucocorticoids may not be the only pharmaceutical strategy to target versican upregulation and a dysregulated transitional matrix in dystrophic muscles. Versican is highly decorated with chondroitin sulphate GAG side chains, far more than decorin and biglycan – two other highly expressed CS GAG proteins in dystrophic muscles (40). It should be therefore considered whether it is an excess of versican, or its CS GAG side chains, which are driving
the pathology of dystrophic muscles. If an accumulation of versican is driving muscle pathology, then downregulating versican expression with dexamethasone (Chapter 3) may be of benefit. There is an upregulation of CS GAG chains in muscle biopsies from patients with DMD (116). These can be degraded via an intramuscular injection of chondroitinase ABC, which ameliorated the pathology of hindlimb muscles from *mdx* mice, presumably through enhanced regenerative myogenesis (457). Furthermore, there are variations of versican that do not contain the CS GAG rich domain, such as the V3 form of versican. In rabbit carotid arteries injected with cells transduced to express V3 versican, the V3 form was found to be beneficial in preventing atherosclerosis by structurally remodelling the matrix of the artery, thereby inhibiting macrophage infiltration and lipid deposition that bind to the GAG chains of versican (568). As V3 versican is found in healthy human skeletal muscle (569), an upregulation of the V3 form might also reduce the inflammation and fibrosis in dystrophic skeletal muscles, although this remains to be tested within future studies.

Another important factor that could be targeted for future pharmaceutical therapies is versikine, the cleavage product of V1 versican. Versikine has only just become of great interest as a bioactive protein, and has only recently been identified as a DAMP (342, 343), a protein produced upon muscular injury which stimulates inflammatory processes. Furthermore, versikine has also been associated with apoptosis during the formation of the digits (334). As detailed within various *mdx* mouse muscles in Chapters 3-5 of this thesis, versikine may potentially drive inflammation and apoptosis in dystrophic muscles. Therefore, future research is required to investigate the roles and signalling pathways of versikine. Additionally, facilitating the faster removal of versikine may also have beneficial effects, potentially reducing inflammation and apoptosis within dystrophic muscles. As such, a better understanding of the ECM proteases which degrade versikine is needed.
6.6 Final conclusions

Encapsulated in this thesis are data that show glucocorticoids increase myogenisis by reducing the ECM, and most importantly, components of the transitional matrix, which when modified through the reduction of versican in-vivo in mdx mice, exerted differential effects on various muscle types, with the majority of benefits being in the younger 6 week old mice. Furthermore, it also details the importance of acknowledging patient growth, muscle type and progression of disease when considering the right age and type of therapeutic when administering treatments for DMD, and in other inflammatory diseases. Indeed, the ECM, once overlooked, is now rapidly becoming a major field of interest. However, only recently has a dysregulated transitional matrix been associated with disease (527, 558). Modulating the transitional matrix components, such as hyaluronan and versican, may therefore be of great benefit for regenerative medicine applications. The data contained within this thesis are the first to link a dysregulated and overexpressed transitional matrix with dystrophic pathology, and hence signifies not only a major addition to the field of DMD, but also to the emerging area of the transitional matrix in regeneration and diseased states.
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