 doi:10.20944/preprints202010.0234.v1

Perspective

The failed clinical story of myostatin inhibitors against Duchenne Muscular Dystrophy: Exploring the biology behind the battle

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Abstract: Myostatin inhibition therapy has held much promise for the treatment of muscle wasting disorders. This is particularly true for the fatal myopathy, Duchenne Muscular Dystrophy (DMD). Following on from promising pre-clinical data in dystrophin-deficient mice and dogs, several clinical trials were initiated in DMD patients using different modality myostatin inhibition therapies. All failed to show modification of disease course as dictated by the primary and secondary outcomes measures selected: the myostatin inhibition story thus far, is a failed clinical story. These trials have recently been extensively reviewed and reasons why pre-clinical data collected in animal models has failed to translate into clinical benefit to patients has been purported. However, the biological mechanisms underlying translational failure need to be examined to ensure future myostatin inhibitor development endeavors do not meet with the same fate. Here, we explore the biology which could explain the failed translation of myostatin inhibitors in the treatment of DMD.

Keywords: myostatin inhibition; Duchenne Muscular Dystrophy; skeletal muscle; muscle development; clinical trials; translation

1. Introduction

Since McPherron's initial discovery of the mighty mouse [1] and the subsequent clinical case report of an infant with uncharacteristic muscling and superhuman strength caused by mutations in the myostatin (growth differentiation factor 8 (GDF8) gene [2], researchers and drug companies have been in a race to develop drugs targeted against myostatin to treat muscle wasting conditions. Therapeutic myostatin inhibition has been purported for muscular dystrophy, cachexia, sarcopenia, disuse atrophy associated with osteoporosis, diabetes, amyotrophic lateral sclerosis (ALS) and multiple sclerosis [3]. While myostatin inhibition cannot correct the primary defect in many of these diseases, severe and progressive muscle wasting could, theoretically, be attenuated, halted or reversed to increase the longevity and quality of lives of patients and reduce burden on the healthcare system. Subsequent development of a range of myostatin inhibitors and promising pre-clinical results encouraged human trials. However, for Duchenne Muscular Dystrophy (DMD) (as well as several other diseases) human clinical trials have not progressed to an effective medicine.

Recently, Wagner (2020) reported on the failed clinical trials that have highlighted the futility of myostatin inhibitor drugs against DMD and provided several key reasons for unsuccessful

 doi:10.20944/preprints202010.0234.v1

translation between pre-clinical and clinical studies [4]. These were: (1) the distinct differences in native myostatin levels in mice compared to humans (by ~10 fold); (2) disparity in the proportional basal suppression of circulating myostatin between wild-type/healthy and *mdx*/DMD muscles (skeletal muscle myostatin is 25% of WT levels in *mdx* mice compared to 8% of healthy control levels in DMD patients); and (3) the confounding effects of standard of care corticosteroid treatment in DMD patients which was never extensively tested in pre-clinical animal trials. These factors are explored in detail herein, in context of the known cellular pathophysiological events which drive DMD. We also discuss other potential factors which might alternatively explain the failed translation of myostatin inhibitor drugs with important implications for future drug development programs.

2.0 Myostatin is differentially expressed in mice and humans

Myostatin negatively controls skeletal muscle growth and quality through multiple molecular mechanisms. A member of the transforming growth factor β superfamily, myostatin is important for the regulation of both pre- and post-natal muscle growth. There is evidence to suggest that through interplay with GDF11, myostatin coordinates muscle growth to ensure a proportionate ratio between skeletal muscle and bone growth rate and density (as reviewed recently in [5]), such that the skeleton is capable of supporting the musculature and the musculature capable of moving the skeleton. Myostatin is a well-established inhibitor of mRNA translation, i.e. protein synthesis, in part, via targeted suppression of mammalian/mechanistic target of rapamycin complex (mTORC), a highly conserved serine/threonine kinase widely considered to be a master regulator of cell growth. Additionally, myostatin drives atrophy through pro-degradative signal-transduction mechanisms in a Smad2/3-dependent manner, increasing FoxO transcriptional activity and upregulating the expression of E3-ubiquitin ligases [6]. Collectively, these mechanisms account for most of myostatin's activity against post-natal muscle growth. In this regard, myostatin may act as an environmental sensor/signaler of nutritional status (particularly in a low amino acid environment) [7] in synergy with the cellular energy sensor, adenosine monophosphate-activated protein kinase [8], promoting a negative feedback loop that inhibits ribosomal biogenesis and, subsequently, mTOR-dependent protein synthesis [9-11].

Myostatin is also a negative regulator of muscle stem "satellite" cell proliferation and differentiation at the G1 to S progression phase of mitosis, which maintains satellite cells in a quiescent state [12]. While strong repressor activity of satellite cell proliferation and differentiation through Smad 2/3 signaling may account for a proportion of myostatin's role in post-natal muscle growth inhibition, myostatin is probably most influential on the regulation of embryonic muscle progenitors during pre-natal muscle growth. Embryonic muscle growth is both hyperplastic and hypertrophic: that is, muscle tissue growth involves both increased myofibre number via the accretion of myoblasts > myotubes > myofibres, followed by their relative diametric and longitudinal growth, which is equally dependent upon motor neuron outgrowth and functional innervation [13]. Usually by 7 years of age, hyperplastic muscle growth ceases and thereafter, only hypertrophic growth is responsible for increased muscle size [14] – this is achieved through protein synthesis, which is dependent upon the genetic material donated through satellite-cell dependent myotube fusion [15]. Myostatin is strongly expressed in embryonic somite where it appears to modulate the balance between proliferation and differentiation of embryonic muscle progenitors during

 doi:10.20944/preprints202010.0234.v1

development [16], possibly by sensitizing progenitors to pro-differentiation signals (e.g. Notch signaling [17,18]). In this manner, myostatin helps to set both the finite myofibre number as well as the extent of the satellite cell pool, which dictates the capacity for post-natal growth. Thus, myostatin exerts very different effects on skeletal muscle growth in the embryonic versus the post-natal cellular environments.

The differential growth of skeletal muscle in the pre- and post-natal environment is important to this story because proof of concept efficacy of myostatin inhibition in animal models of DMD was initially proven through myostatin knockout (KO), which manipulates both pre- and post-natal hyperplastic and hypertrophic muscling [19]. This is in stark contrast to drug inhibitors of myostatin which can only manipulate the post-natal hypertrophic growth of muscle. It might be that myostatin inhibition is much more influential on global muscle mass when applied from the earliest muscle growth during embryogenesis – this explains the phenomenal impact of myostatin gene mutations in mice [1], larger order animals (dogs [20], sheep [21], cows [22], horses [23,24]) and humans [2]. Indeed, in myostatin KO mice, hyper-muscling is attributable to hyperplasia (i.e. >80% more muscle fibres) more so than hypertrophy (~30% larger cross-sectional area of the individual muscle fibres [1,25]). Nevertheless, each of the experimental drugs which failed in clinical testing were shown to modify the disease course of murine DMD in the gold standard *mdx* mouse model [26-29], albeit, only some of these studies used sexually mature mice [26,29] as opposed to juvenile mice [27,28] which are likely to be more amenable to myostatin inhibition [30] through hyperplasia. Wagner has reported that mice (*mdx*) maintain ~10-fold higher circulating myostatin levels than humans and that myostatin repression is ~3-fold higher [4]. This implies that more robust myostatin repressor activity is required to keep muscle mass checked in mice compared to humans. Thus, human muscle mass may, by comparison, be relatively less modifiable by drugs that interfere with myostatin activation than murine muscle mass. This begs the question: why?

Satellite cell regulation of muscle stemness is maintained through daughter cell (muscle progenitor) fate selection, which is under the influence of various factors, including inflammatory cytokines and myokines, growth factors, local cell (i.e. fibro-adipocyte progenitors, extracellular matrix and endothelial cells) and non-muscle stem cell signaling, the dystrophin-associated glycoprotein complex (DGC; especially syntrophins), micro and long non-encoding RNA's, and telomere activity (reviewed in [31]). During moderate-severe muscle damage, satellite cell cycling is amplified, and both daughter cells will commit to symmetric satellite pool expansion initially, before next undergoing asymmetric division where one daughter cell will commit to myogenic lineage and the other to self-renewal of the satellite cell pool to prevent the depletion of stem cell function [32]. This facilitates the rapid repair of lost muscle tissue to maintain mass and function, which is advantageous to the organism during acute muscle injury, but which adversely impacts telomere length. In contrast, during steady-state muscle turnover or minor injury, only asymmetric division occurs [32]. In this scenario, muscle repletion is slower, but telomere shortening is minimized. This is an important concept because muscle regenerative capacity is contingent not only on the number of satellite cells available but also on their proliferative capacity i.e. how many times they can re-enter the cell cycle. Satellite cells have a finite replicative life which is proportional to telomeric DNA length [33]. It is a well-established pathogenic feature of DMD that the satellite cell pool becomes exhausted (both in satellite cell content and proliferative capacity) due to unremitting cycles of chronic muscle injury and regeneration caused, fundamentally, by the absence of dystrophin protein and alterations to the DGC. In this setting, satellite cells are less able exit the cell cycle and revert to their homeostatic

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 October 2020 doi:10.20944/preprints202010.0234.v1

quiescent state, which is required for telomere maintenance and long-term regenerative capacity [32]. Compared to humans and for reasons unknown, mice have remarkably longer telomeres, which do not substantially shorten through replication and aging [34]. This suggests a greater comparative capacity for satellite cell activity-mediated regeneration and muscle growth and may explain why the *mdx* mouse recapitulates a milder phenotype than human DMD, particularly since knockdown of telomerase in the *mdx* mouse induces "human-like" DMD [35]. Thus, an exaggerated myostatin repressor function might be fundamentally important to restrain a naturally higher propensity for muscle growth in mice compared to humans because satellite cell activity is more robust and enduring. This effect seems to even endure the elevated demand for muscle regenerative activity caused by dystrophin-deficiency, as suggested by the higher relative suppression of circulating myostatin levels in *mdx* mice (25%) versus DMD patients (8%) compared to healthy controls [4]. For this reason alone, myostatin may be more amenable to inhibition in mice but less so in humans, simply because there is more of it to inhibit and thus the scope for biological modification of muscle growth is greater (as summarized in Figure 1). It would be interesting to determine the capacity for myostatin inhibitor drugs to modify murine DMD using the *mdx*/mTR$^{-/-}$ model, which has shortened telomeres, as a staple in pre-clinical trials, where the scope for modification is more comparable to human DMD [36,37].

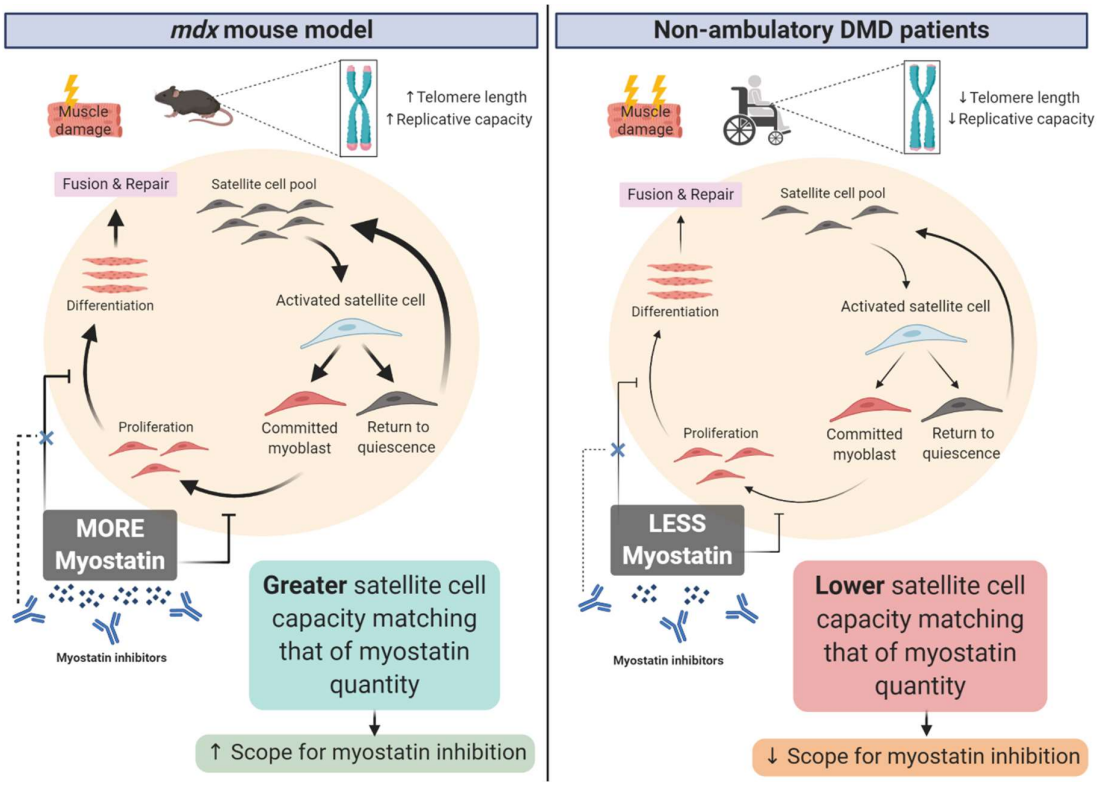

Figure 1. Summary of the contrast between satellite cell regulation in the murine *mdx* model of Duchenne Muscular Dystrophy (DMD) and human DMD patients. It is proposed that a larger and more enduring satellite cell replicative capacity in the *mdx* mouse model of DMD, which is associated with longer telomere length compared to humans, results in a greater scope for myostatin inhibition therapies to prevent myostatin-mediated repression of myoblast differentiation. Conversely, unremitting satellite cell cycling in non-ambulant DMD patients in response to chronic muscle degeneration compared to *mdx* mice, results in depletion of the satellite cell pool, reduced regenerative capacity and, thus, the scope for myostatin inhibition to be therapeutically effective.

 doi:10.20944/preprints202010.0234.v1

3.0 Myostatin inhibition enhances muscle mass but not necessarily function

Despite the lack of translational outcomes for myostatin inhibitors so far, it is important to note that the clinical trials investigating myostatin inhibitors against DMD did not all fail to elicit muscle mass increases. ACE-031 (NCT01099761) and ACE-083 (NCT02927080) both developed by Accleron Pharmaceuticals, domagrozumab developed by Pfizer Inc (NCT02310763) and RG6206/R07239361 (NCT03039686) developed by Hoffman La Roche, all produced mild (generally <5%), yet statistically significant increases in muscle/lean mass as measured through non-invasive imaging of DMD patients [4,5]. However, what these studies did fail to show were concomitant improvements in strength which should have accompanied these mass gains. Each of these trials failed to meet either their primary or secondary/surrogate outcome measures concerning muscle function [4,5]. This is important when considered in context of the endpoint and surrogate outcome measures selected through which to validate drug efficacy. The only successful outcome for patients is the attenuation of disease course sufficiently to maintain or improve quality of life. For most of these trials, functional tests to monitor the progression of DMD in the clinic (i.e. the 6 minute walk test) were used as outcome measures to evaluate the efficacy of myostatin inhibition against muscle wasting, and therefore strength decline. However, this works off the assumption that myostatin inhibition can induce both mass and function equally, or that mass increases always produce functional/strength improvements. It is well known that muscle strength and size are not increased in concert [38,39] and neither proportionate loss of mass can explain, nor gain of mass arrest, strength decline as humans age [40]. Indeed, studies of myostatin inhibitor drugs against age-related sarcopenic muscle wasting are consistent with data from the DMD clinical trials and support the lack of synergy between mass and strength, as well as the poor translation of murine myostatin inhibition in clinical trials.

In vivo, muscle fibre growth is initiated through an interplay governed by the net positive difference between protein synthesis and degradation, and satellite cell-dependent myotube fusion, but is functionally modulated by neuromotor activation patterns across the neuromuscular junction and mechanical loading [41]. Strength gains are most intensely observed when type II myosin heavy chain isoforms are preferentially expressed (i.e. over slow type I isoforms). Type II fibres have higher power due to faster contraction velocity and larger cross-sectional areas [42], thus greater type II fibre expression leads to bigger fibres capable of higher force outputs and therefore strength. Type II fibres are induced through specific stimuli, namely intense mechanical loading which induces rapid succession action potential transfer across the neuromuscular junction into the t-tubules, and comparable sarcoplasmic reticulum-mediated calcium (Ca^{2+}) transients which are typically longer and more concentrated than for temperate mechanical loading [43,44]. Myostatin inhibition induces slow (type I) to fast (type II) fibre transition [45], and thus should theoretically evoke strength gains in the clinic. To understand why this may not be possible for DMD patients, one must consider the pathogenesis of the disease, which preferentially drives the wasting of type II(x) fibres first [46-48]. Dystrophin stabilizes the sarcolemma during muscle contraction and is, thus, particularly important to type II fibres which bear the brunt of the mechanical load. Type II fibres are more prone to damage for this reason alone, but without dystrophin (i.e. in DMD), mechanical damage is intensely exacerbated. As such, converting fibres towards type II, yet maintaining the lack of dystrophin, may simply make them bigger and more susceptible to damage. Further, type II fibres also lack the magnitude of endogenous antioxidant and cytoprotective responsivity of type I fibres [49,50] because

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they rely less on oxidative metabolism by the mitochondria, which is a principle source of damaging free radicals in cells. Notwithstanding, type II fibres can produce significant amounts of reactive oxygen species (ROS) during explosive, high-intensity activation which drives xanthine oxidase activity through the degradation of purine nucleotides: the net result is a rapid and intense ROS production [51]. DMD mitochondria are notoriously dysfunctional and produce appreciably less adenosine triphosphate (ATP) [52-54] but more ROS [53,55,56], placing stress on the anaerobic energy systems, which further drives cytosolic ROS production (i.e. through xanthine oxidase, amongst other ROS producing enzymes). DMD muscles, particularly dystrophin-deficient type II fibres, are thus also more prone to oxidative stress-induced damage [48]. For this reason, drugs that mediate fibre type transformations from fast to slow have been suggested as a therapeutic treatment avenue for DMD [57]. Conversely, myostatin inhibition directly contests this idea by promoting expression of the very fibres that are prone to damage and wasting. This might explain why strength gains are less obvious than mass gains, since any muscle mass induced by higher proportions of larger type II fibres likely manifests as a more severe pathology, which limits functionality. It might also explain why, for domagrozumab, muscle volume gains were observed following the first few months of treatment [58], yet at ~12 months, there was no observable difference in muscle volume compared to placebo [59] i.e. the initial muscle gained had preferentially wasted.

Without proportionate synthesis of contractile proteins as part of the overall protein synthesis rate, muscle mass gains are futile. Indeed, for sarcopenia (the age-related loss of skeletal muscle mass and function), functional decline has been delineated as the most important indicator of this condition [60] i.e. without functional improvement, there is no point to mass increases. Exercise represents the definitive muscle growth stimulus (particularly eccentric resistance exercise) where intrinsic skeletal muscle molecular signaling of hypertrophy (e.g. ROS, metabolites, and mechanical signals) are synergistically activated alongside motor neuron stimulation. While myostatin inhibition may represent an important molecular signal for hypertrophy and hyperplasia, these events may in fact be futile without synergistic motor neuron signaling. To illustrate this point, it is important to highlight that muscle satellite cells represent the only means for post-natal muscle hyperplasia, except for the fact that developing myotubes have no means to access the neural input required to translate new muscle accretion into functional improvements. For this reason, myotubes must, in post-natal muscle, fuse with pre-existing fibres possessing established neuromuscular connections to gain access to neural motor input and become "functional" [15]. Muscle fibre "splitting" is characteristic of DMD muscles [61] and is purportedly caused by the incomplete fusion of satellite cell-mediated myotubes with existing skeletal muscle fibres [62]. The extent to which incomplete fusion might contribute to corresponding increases in function comparative to skeletal muscle accretion is unknown but can be logically interpreted. The less able a myotube is to fully fuse with a given skeletal muscle fibre, the less access its nucleus has to the molecular signals of skeletal muscle contraction e.g. the t-tubular action potential and the myoplasmic Ca^{2+} and ROS transients among others, which denote the magnitude of mechanical stress relative to neuronal input. A flow on effect of this is a reduction in myokine signals released from muscles during activation, which in turn, attract nerve outgrowth, an essential event that must accompany hypertrophic growth of fibres to maintain proportionate function [63-65]. In the muscles of *mdx* mice, aberrant pre- and post-synaptic neuromuscular junction changes have been described [66], but otherwise, *mdx* mice maintain robust contractile function and ambulation throughout life [67]. This might explain why in *mdx* mice, myostatin inhibition induces both muscle mass and function gains. In DMD patients, neuromuscular

 doi:10.20944/preprints202010.0234.v1

junction vulnerability is evident [66], which along with reduced neural input through developmental delay of weight-bearing activities such as standing and walking, and wheelchair confinement following loss of ambulation, could contribute significantly to the impaired translation of the neurological signal to skeletal muscles. Combined with myostatin inhibitor drugs, the likely outcome is muscular hypertrophy without the synergistic improvement of skeletal muscle function.

4.0 Corticosteroids interfere with myostatin inhibition

In pre-clinical animal studies, it is relatively simple to control for the many confounding variables that can inadvertently impact research outcomes. This becomes more difficult in clinical trials with humans, and ever more difficult again with rare diseases where there are so few patients to access. Corticosteroids (prednisolone/prednisone, or deflazacort) are standard of care for DMD treatment (which may also include angiotensin converting enzyme (ACE) inhibitors or angiotensin blockers) [68], but due to significant side-effects, not all DMD patients are amenable to them. This leaves a very small population of DMD boys who are steroid naïve and prime candidates for trialing candidate therapeutics. For the rest of the DMD patient population, standard of care cannot be ethically withdrawn, which has important implications for trial participant selection and stratification. Inevitably, there is a trade-off that must be made between having a sufficiently powered trial to establish efficacy of a therapeutic candidate, which can only be achieved with larger numbers of study participants; and introducing significant confounders to the research.

Paradoxical to their use against muscle wasting, corticosteroids are atrophic agents, but were introduced for the treatment of DMD due to their potent anti-inflammatory and immuno-modulatory action [68]. There has been only one myostatin inhibitor drug tested pre-clinically in combination with corticosteroids. Hammers *et al*. demonstrated that when administered with prednisone, the muscle mass increases induced by myostatin inhibition (with a pro-peptide) were abolished [69]. Importantly, the failed trials testing domagrozumab and RG6202 enlisted patients receiving the standard of care, giving scope for drug interactions which likely impacted myostatin inhibition capacity.

Genetic myostatin deletion can curb the muscular atrophy effects of corticosteroid treatment, suggesting that myostatin may modulate corticosteroid receptor signaling in skeletal muscle [25]. In healthy mouse muscle, glucocorticoids reduce IGF-1 mRNA leading to removal of IGF-R1 repression of atrogin-1, causing muscle atrophy. When administered to myostatin KO mice, the same skeletal muscles respond to corticosteroid treatment by upregulating IGF-2, which can alternatively interact with the IGF-R1 receptor [25] to repress atrogin-1 and muscular atrophy. While these data ostensibly suggest that myostatin inhibition could be useful to counteract the side effects of corticosteroid treatment, i.e. muscle atrophy, it is important to highlight that corticosteroids also increase the expression of myostatin mRNA and protein [70-72]. Since this effect cannot occur in genetically ablated KO mice, but can during drug-induced myostatin inhibition where the myostatin gene is functional, it stands to reason that corticosteroids may directly oppose myostatin inhibitor drug action through increasing myostatin levels to competitively antagonize the activinin receptor or myostatin activation. In this regard, whether a DMD clinical trial participant is steroid treated (as opposed to steroid naïve), and then specifically, the dosage of corticosteroid administered to individual participants, would be highly influential on the capacity of myostatin inhibitor drugs.

 doi:10.20944/preprints202010.0234.v1

5.0 Conclusions

Myostatin inhibitor drugs have the potential to be greatly beneficial against muscle wasting diseases and disorders yet have to date been highly ineffective. The dramatic impact of loss of function myostatin mutations on muscle mass and strength accretion, which are probably most profoundly influential during embryonic development, must be balanced against the capacity of drugs to resist skeletal muscle wasting driven by a plethora of stimuli in the post-natal environment. Clinical trials in DMD patients present a variety of challenges which make participant stratification, and the selection of primary and secondary outcomes measures difficult, and confounding variables numerous. We suggest that in addition to corticosteroid use as standard of care, the physical capacity of patients as well as their relative level (volume and intensity) of physical activity should be considered when testing myostatin inhibitors, since neural input is likely very impactful on eliciting functional improvement alongside mass gains. Emerging evidence suggests that myostatin not only regulates muscle mass, but also metabolism, adiposity and insulin-sensitivity [73]: targeting downstream molecular targets of myostatin rather than upstream activation and receptor binding, could thus represent an alternative druggable target against DMD.

Author Contributions: **C**onceptualization, E.R., C.T. and D.D.; writing—original draft preparation, E.R. and C.T.; writing—review and editing, E.R., C.T., R.B., D.D., D.C., and A.H.; visualization, E.R., C.T., R.B., D.D., and A.H.; supervision, E.R., C.T., and A.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: N/A.

Conflicts of Interest: The authors declare no conflict of interest.

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