Investigating sex differences in resistance training-induced skeletal muscle adaptations in middle-aged adults

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General Abstract

Introduction: Resistance training improves muscle strength and induces myofiber hypertrophy in young males and females with blunted responses occurring in older adults. These adaptations are partially due to the function of muscle stem cells (MuSCs) and fibro-adipogenic progenitors (FAPs). It remains unknown whether middle-aged males and females respond similarly to resistance training with protein supplementation, specifically at the cellular level. **Purpose:** The purpose of this study is to investigate the potential sex-specific responses of middle-aged males and females to whole-body resistance training. **Methods:** Middle-aged adults (N=28), 40-64 years, participated in a 10-week progressive, whole-body resistance training intervention coupled with protein supplementation. Muscle biopsies were collected from the vastus lateralis and stained for fibre morphology, MuSCs, and FAPs. **Results:** Both sexes increased type II fibre cross-sectional area with training. Myonuclear content, myonuclear domain size, and MuSC content were not altered with training in either sex. Both males and females altered FAP content with training. Interestingly, the change in MuSCs and both FAPs were correlated in males but not females (both $P<0.05$). It was concluded that there were no sex-specific responses to resistance training in middle-aged males and females; however, MuSCs and FAPs appear to be correlated in males but not females.

List of Abbreviations

Abbreviation	Definition
CSA	Cross-sectional area
MHC	Myosin heavy chain
MuSC(s)	Muscle stem cell(s)
FAP(s)	Fibro-adipogenic progenitor(s)
Supp.	Supplementary
MRF(s)	Myogenic regulatory factor(s)
Pax7	Paired box transcription factor 7
MyoD	Myogenic determination protein 1
Myf5	Myogenic factor 5
MyoG	Myogenin
MRF4	Myogenic regulatory factor 4
MSC(s)	Mesenchymal stromal cell(s)
RM	Repetition maximum
CD90	Cluster of differentiation 90
PDGFRα	Platelet derived growth factor receptor alpha
SEM	Standard error of the mean
N	Sample size
MOD	Moderate protein intake group
HIGH	High protein intake group

Note. Abbreviations are listed in order of appearance in the text starting from Chapter I-General Introduction. Reagents used in methods are excluded from this table.

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Chapter I-General Introduction

Skeletal muscle is a contractile organ, and its main functions include body stabilization, support, metabolic regulation, and movement production (1)**.** Skeletal muscle is comprised of groups of highly organized, multinucleated, post-mitotic muscle fibres (1)**.** Human skeletal muscle is heterogeneous, and it is composed of three main fibre types: type I, type IIa, and type IIx (1–4)**.** Skeletal muscle is highly adaptable to biological factors, such as aging, and lifestyle factors, such as resistance training. Aging results in sarcopenia which is defined as the progressive and irreversible loss of muscle mass and function which is exacerbated with declines in physical activity (5, 6). Specifically, sarcopenia induces reductions in fibre size and number which are most apparent in type II fibres (7). Interestingly, menopausal females appear to be more prone to sarcopenia as demonstrated by a greater loss in muscle mass compared to males (8). Sarcopenic symptoms can be observed as early as the third decade of life, while being most prevalent in the fifth decade of life (8). This age range encompasses middle-aged adulthood which is loosely defined as individuals between the ages of 40-60 years (9). The onset of sarcopenia should be delayed since there is currently no treatment to cure the irreversible muscle loss. A proposed method to improve muscle mass involves resistance training.

Resistance training improves muscle mass and strength by fibre hypertrophy (10), which results in overall health benefits such as improving body composition and completion of activities of daily living (11–13). The majority of resistance training studies have examined the adaptive responses of males (10, 14–21), while rarely studying females (22). Present findings involving sex differences in young and older untrained males and females in response to resistance training are equivocal. Furthermore, there are a limited number of studies assessing the adaptations to

resistance training in middle-aged adults (23, 24), with the majority of studies being conducted in young and older adults above 80 years of age (22, 25–39). Thus, the overall objective of this thesis is to address the gap regarding how middle-aged adults respond to resistance training and if sex-differences exist in these adaptations. The effects of resistance training and protein supplementation on the skeletal muscle cellular adaptations occurring in middle-aged adults will be examined in detail.

1.1. Skeletal muscle adaptations to resistance training in males and females

Resistance training induces multiple physiological adaptations in both males and females. It is important to understand the potential sex-specific responses to resistance training to tailor training interventions and optimize the adaptive responses experienced. Strength and hypertrophic adaptations in response to resistance training have been studied extensively. The initial measurable response to resistance training involves changes in strength followed by fibre hypertrophy as determined by increases in fibre cross-sectional area (CSA) (40).

Both sexes have similar changes in strength following resistance training. Males have a larger absolute strength, but the relative percent increases in strength in response to lower- (24) and whole-body (23, 26, 28, 29, 39, 41) resistance training appears to be similar between males and females of all ages. The strength gains following resistance training could be explained by a shift in type II fibre proportion within skeletal muscle. Fibres expressing the type II myosin heavy chain (MHC) isoforms, particularly type IIx, have a greater capacity for force production compared to fibres expressing MHC type I. Evidence has shown the presence of fibre type shifting from type IIx to type IIa fibres in sexes of all ages performing resistance training (27, 30, 34, 37). Specifically, this shift in fibre type is explained by decreases in the size and proportion of type IIx

fibres and a corresponding increase in the size and proportion of type IIa fibres. These data indicate that both sexes are experiencing in a shift from type IIx fibres to type IIa fibres in response to resistance training. Therefore, there is a consensus in the literature that resistance training induces similar relative percent strength gains in males and females.

Conversely, the sex-specific hypertrophic responses to resistance training are unclear. Some studies have shown similar increases in CSA of both type I and II fibresin response to upper- (35) and lower-body (36) resistance training in young and older males and females. Alternatively, other work has indicated that males of all ages have greater increases in CSA, in both type I and II fibres, in response to lower- (27) and whole-body (31–33) resistance training compared to females. Furthermore, middle-aged and older females have greater increases in CSA in both fibre types compared to males (42). These ambiguous findings present a gap in the understanding of how fibres change size in response to resistance training. To begin to interpret these results it is important to understand the mechanisms driving fibre hypertrophy.

Resistance training induced fibre hypertrophy occurs by two primary mechanisms: 1) increases in muscle protein synthesis; which increases contractile proteins in fibres, and 2) muscle stem/progenitor cell-mediated mechanisms; which increase the number of myonuclei per fibre to provide greater transcriptional capacity (43)**.** Protein consumption is a known stimulator of muscle protein synthesis; however, the studies investigating sex-based differences in fibre adaptations to resistance training fail to consider protein intake in their study designs. In acute studies, it has been shown that resistance training supplemented with increased dietary protein intake improves muscle mass and strength in both sexes (44), while both males and females of all ages have similar rates of muscle protein synthesis (44–46) . Alternatively, chronic

resistance training studies lasting over six weeks have differing results. Protein supplementation has been shown to increase muscle mass and strength in young and older adults (47). However, resistance training with creatine and protein supplementation (48, 49) does not contribute to additional muscle mass and strength in older males. These resistance exercise/training, protein intake studies did not consider sex differences with most only considering the physiological responses of males (48, 49). Therefore, there appears to be no significant sex differences in the responses to acute resistance exercise; however, the effects on chronic resistance training are unclear. The contribution of protein supplementation to muscle protein synthesis and the relation to hypertrophy may be impacted by resident cell populations.

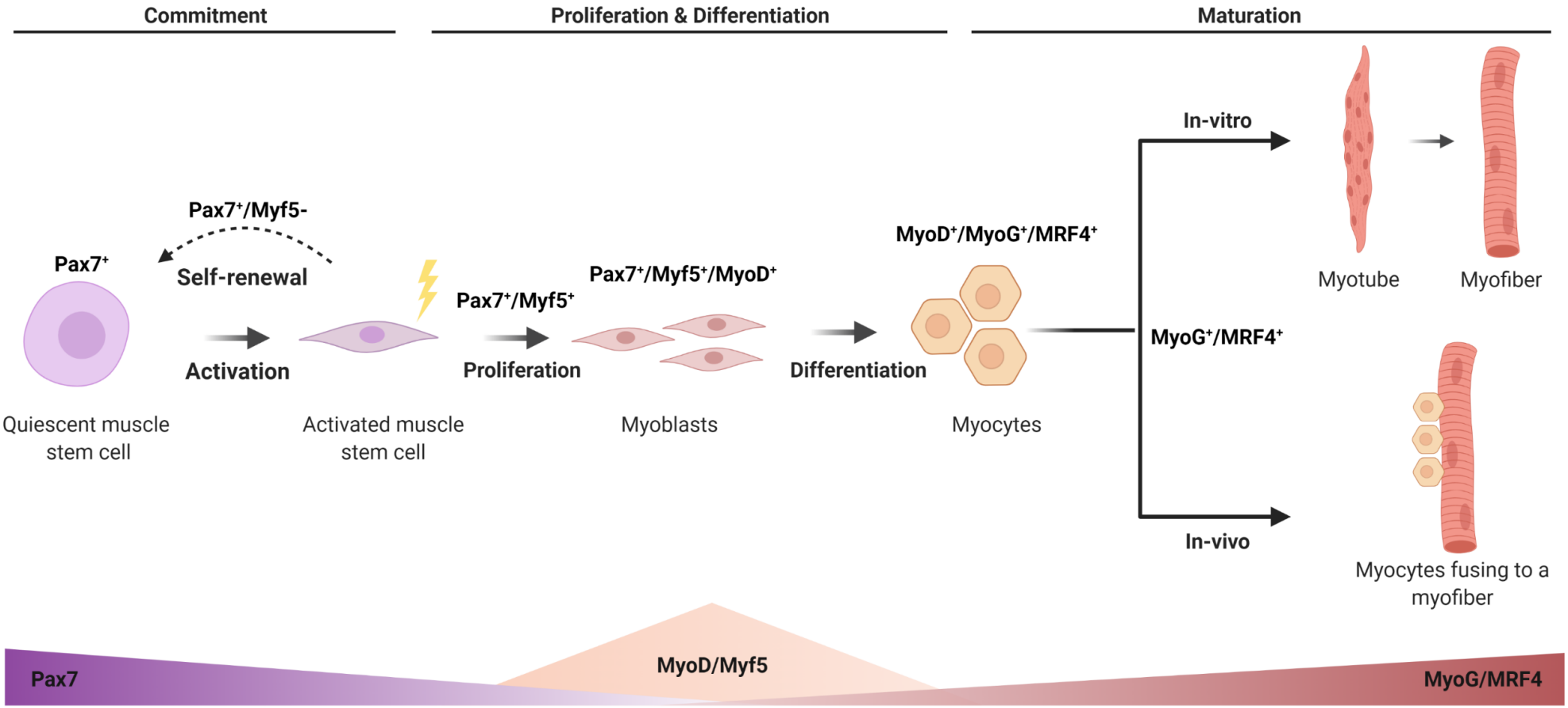
Muscle stem cell (MuSC)-mediated mechanisms can also support muscle hypertrophy via myonuclear addition. The myonuclear domain theory is a proposed explanation to described myonuclear accretion that occurs during fibre hypertrophy. The myonuclear domain theory suggests that each myonuclei is responsible for regulating transcription within a pre-determined area within each fibre (36, 50–52). Therefore, resident myonuclei can support myonuclear domain expansion during the early hypertrophic response, until a theoretical threshold is surpassed in which the addition of myonuclei is required to support the transcriptional capacity of the growing fibre (37, 52). As aforementioned, skeletal muscle is post-mitotic; thus, myonuclei can only be derived from the fusion of MuSCs to existing fibres. Myonuclear addition is not essential for early hypertrophy, but it is required once the transcriptional threshold is reached (36, 37). Currently, a few studies have compared the MuSC response to resistance training in males and females (25, 38, 53); however, these studies did not investigate the responses in

middle-aged adults. As such, there exists a significant gap in our understanding of the cellular responses to resistance training in middle-aged males and females.

1.2. Muscle-resident stem/progenitor cells and the myogenic program

Skeletal muscle is composed of two distinct stem progenitor cell populations: MuSCs and fibro-adipogenic progenitor cells (FAPs) (54). MuSCs are mononucleated, myogenic (i.e., muscle-forming) stem cells that reside between the basal lamina and sarcolemma of fibres (55–60). During steady state, MuSCs are in a state of reversible mitotic inactivity termed quiescence (56, 60). The specialized microenvironment where MuSCs are found is called the niche, which helps regulate MuSC fate (55–57, 60–62). The interactions between MuSCs and their niche contribute to maintaining the proper function of fibres.

Fibre repair and maintenance are regulated by the action of MuSCs and their niche through a process termed myogenesis. Myogenesis constitutes four main phases: 1) quiescence; 2) activation/self-renewal; 3) proliferation; and 4) differentiation (60, 63). Progression through these stages is collectively referred to as the myogenic program (Supp. Fig. 1). Quiescence is defined by MuSCs that are mitotically inactive (56, 60). MuSCs can exit quiescence when they are activated by a range of stimuli some of which include fibre damage, mechanical strain, growth factors, and cytokines (55, 57, 64). Activated MuSCs enter the cell cycle, migrate to the site requiring repair, and either self-renew or proliferate via symmetric or asymmetric divisions (55–58, 60, 63). Symmetric divisions produce identical daughter cells with the same cell fates while asymmetric divisions produce two daughter cells with differing cell fates. MuSCs have two potential fates: 1) self-renewal or 2) proliferation and differentiation into myoblasts (56–58, 60, 65). Self-renewal helps replenish the MuSC pool while committed MuSCs mature into myoblasts.



Supplementary Figure 1. The myogenic program.
Quiescent muscle stem cells are activated in response to stimuli and either self-renew or proliferate and differentiate.
Pax7, Paired box transcription factor 7; Myf5, myogenic regulatory factor 5; MyoD, myogenic determination protein 1; MRF4, myogenic regulatory factor 4; MyoG, myogenin.
Created with BioRender.com.

Myoblasts are committed myogenic progenitors that possess the capacity to differentiate and progress through the myogenic program (63). Myoblasts expand by proliferation, differentiate into myocytes, then either mature into new myotubes *in vitro*, or fuse with existing fibres, *in vivo* (55–58, 60–63, 66). Myogenesis provides a mechanism by which MuSCs regulate fibre repair, regeneration, and adaptation (60, 62).

Progression through the myogenic program is regulated by a family of transcription factors collectively referred to as myogenic regulatory factors (MRFs). MRFs are expressed at different stages of myogenesis and are defined as follows: paired box transcription factor 7 (Pax7), myogenic determination protein 1 (MyoD), myogenic factor 5 (Myf5), myogenin (MyoG), and myogenic regulatory factor 4 (MRF4) (55–58, 60–62, 66, 67). Pax7 is required for myogenesis, and it is expressed in both quiescent, activated, and early differentiated MuSCs (56, 60, 62, 63, 66, 68). Pax7 expression is upregulated when MuSCs are activated which signifies re-entry into the cell cycle and initiation of mitotic divisions (68). MyoD and Myf5 initiate the myogenic program, and their expression promotes MuSC commitment and myoblast differentiation (62). Both quiescent and activated MuSCs express Pax7, while only committed MuSCs express Myf5 and MyoD (56–58, 62, 69). The absence of My5 expression results in MuSC self-renewal to replenish the SC pool (55, 60, 66, 70). Conversely, Myf5 expressing MuSCs commit to the myogenic program and mature into myoblasts, followed by the upregulation of MyoD to form myocytes (56, 60, 62). The early stages of myogenesis are similar *in vitro* and *in vivo,* but the later phases differ slightly. *In vitro,* proliferating myoblasts express MyoG and MRF4, and these MRFs regulate differentiation into myocytes (62). Myocytes express MyoG and differentiate into multinucleated myotubes that mature into fibres (56, 58, 60, 66). *In vivo*, proliferating myoblasts

upregulate MyoG and MRF4 during differentiation and fuse with existing fibres with a concomitant downregulation of Pax7, Myf5, and MyoD (64). A combination of the upregulation and downregulation of these MRFs determines the unique expression patterns of the MuSCs and committed myogenic cells in the myogenic program.

The myogenic program is dynamic and highly regulated by cell-intrinsic factors, but its action is also affected by non-myogenic cells present within the niche (55, 57, 60, 61). MuSC progression through the myogenic program is facilitated indirectly by the action of non-myogenic, muscle-resident mesenchymal stromal cells (MSCs) called FAPs (71–73). MSCs and FAPs can be identified by the expression of specific cell surface markers. A classic MSC phenotypic marker is the cluster of differentiation 90 (CD90) (74) which has been previously used to identify human MSCs *in vitro* (75). FAPs, like MuSCs, can be identified by the expression of specific cell-surface markers. Platelet derived growth factor receptor alpha (PDGFRα) is expressed on the surface of FAPs in skeletal muscle of both mice (76) and humans (77); thus, it is considered a universal marker of FAPs. Since FAPs are a type of MSC they can also express CD90 (74, 75).

FAPs possess many functions, such as facilitating MuSC progression through the myogenic program via paracrine mechanisms (71, 73, 78). Importantly, preclinical studies have shown that FAPs are absolutely necessary for muscle maintenance with age (73). FAPs also contribute to the MuSC niche by differentiating into fibroblasts or adipocytes (79). FAP differentiation into fibroblasts forms fibrotic tissue (72), while FAP differentiation into adipocytes contributes to intercellular adipose tissue accumulation within the niche (76). Fibro-fatty tissue accumulation is usually associated with FAP expansion (80) and contributes to impaired regeneration and maintenance, which is commonly observed in atrophic conditions such as sarcopenia (81). These

functions infer that FAPs have the capacity to alter the niche to regulate myogenesis. In summary, the synergistic action of MuSCs and FAPs are necessary to support muscle fibre repair, regeneration, and adaptation.

1.3. Sex-based differences in resistance training-induced adaptations

There exists a consensus regarding the strength changes in males and females following resistance training. Specifically, the relative percent increases in 1-repetition maximum (1-RM) strength to lower- (24) and whole-body (23, 28, 29, 39, 41) resistance training are similar between males and females of all ages. However, the sex specific hypertrophic adaptations to resistance training are unclear. Some studies have shown similar increases in CSA of both fibre types in response to upper- (35) and lower-body (36) resistance training in young and older males and females. Conversely, other work has indicated that males of all ages have greater increases in CSA, in both type I and II fibres, in response to lower- (27) and whole-body (31–33) resistance training compared to females. Furthermore, middle-aged and older females have greater increases in CSA in both fibre types compared to males (42). These ambiguous findings present a gap in the understanding of how fibre hypertrophy may differ between the sexes following resistance training.

As aforementioned, fibre hypertrophy occurs by the following mechanisms: muscle protein synthesis, and the actions of MuSCs and FAPs. Elevated protein intake has been shown to contribute to improved muscle protein synthesis (82). The proposed optimal recommended daily protein intake falls within the range of 1.2-1.6g/kg/d for healthy, adults (83)**.** Evidence suggests that resistance training supported with protein intake provides many benefits including: improvements in lean body mass, strength, and muscle hypertrophy (44)**.** These effects are

attenuated with age and intake above ~1.6g/kg/d provides no added benefits (44)**.** The effects of protein intake on resistance training-induced adaptations have been evaluated using the relationship between muscle protein synthesis and muscle protein breakdown. In order to gain lean body mass and promote hypertrophy, muscle protein synthesis must exceed muscle protein breakdown; thus, additional protein intake aids in maintaining that balance to support these adaptations (84)**.** Furthermore, results presented in a recent review indicate that protein intake between 1.2-1.6g/kg/d increase lean body mass and strength (85) and supports muscle hypertrophy (86).

The second mechanism contributing to fibre hypertrophy is the actions of MuSCs and FAPs. The involvement of MuSCs in driving resistance training adaptations has been well described; however, the influential effects of FAPs are unclear. The studies exploring MuSC content in response to resistance training have been conducted mainly in the lower-body (33, 36, 38, 87) with only a single study implementing a whole-body resistance training program (25). There exists a variation in the findings of MuSC content with no clear explanation. For instance, it has been shown that there are no differences in MuSC content of all fibre types between both sexes irrespective of age and training status (36, 37). In contrast, it has been shown that MuSC content is increased in response to resistance training in young and older males and females, with this response being amplified in older females (38). Not only have sex-based differences been detected in terms of combined fibre type, but there have been fibre type specific responses reported. For example, older males had a greater MuSC content for type I fibres post resistance training than older females; however, there were no detectable sex-based differences for type II fibres (33). The ambiguity in these results could potentially be explained by changes in

myonuclear content in skeletal muscle. MuSCs facilitate myonuclear addition which appears to correlate in sex specific responses. Initially, it was revealed that males and females of all ages had similar myonuclear content (36, 37) which was reflected in similar myonuclear domain size (37). Alternatively, the reverse result has been shown in which older males had a greater myonuclear content and larger myonuclear domain size than older females (33).

Despite, multiple studies examining MuSCs in relation to training adaptations, FAPs are under-studied. Only one study to date has examined FAP populations in response to lower-body resistance training in young males (15). Young males increased PDGFRα^+ and CD90$^+$ FAP content following 12-weeks of unilateral, leg extensions (15). Further research is required to speculate the potential involvement of FAPs in supporting resistance training-induced adaptations.

In summary, there is confounding evidence in the literature on the potential sex-based responses resistance training. There appears to be no significant effect of age, health status, training type, or resistance intervention intensity or duration that can adequately explain the discrepancies in the findings. A potential explanation could be due to how the resistance training adaptations are measured: macroscopically or microscopically. It appears that the majority of resistance training studies examining hypertrophy at the macroscopic level indicate no sex differences (23, 24, 26, 28–30, 34, 35, 37, 41). Alternatively, most studies examining myonuclear content and MuSCs appear to show some sex differences (31, 33, 34, 38). This evidence suggests that the sex differences may not be detectable at the macroscopic level but instead at the microscopic level. The limited evidence on FAP contribution to resistance training adaptations requires further investigation. Therefore, there remains a gap in the understanding of how cell populations contribute to resistance training adaptations in males and females.

1.4. Problem and study rationale

Currently, there is conflicting evidence on resistance training-induced adaptations and the potential sex specific responses. It appears that the majority of resistance training studies indicate no sex differences in the hypertrophic response to resistance training (23, 24, 26, 28–30, 34, 35, 37, 41). Alternatively, most studies examining the responses of MuSC and myonuclei seem to show some sex differences (31, 33, 34, 38). It is unclear if males and females respond similarly to resistance training with protein supplementation, specifically at the cellular level. There are a lack of studies concerning the adaptations in middle-aged males and females, and this demographic is severely understudied. Furthermore, it is unconfirmed whether MuSCs respond similarly to resistance training in males and females, and the role of FAPs has yet to be examined in detail.

1.5. Study purpose, aims, and hypotheses

The purpose of this study was to investigate if middle-aged males and females respond similarly, at the cellular level, to resistance training-induced adaptations following 10-weeks of progressive, whole-body resistance training. In view of the literature presented above, the overall hypothesis is that middle-aged males and females would have similar resistance training-induced adaptations in fibre morphology and fibre type proportion. In addition, it was hypothesized that both sexes would have different responses with respect to MuSC and FAP content. The specific aims and hypotheses are presented in detail as follows:

- Aim #1: Determine if sex-differences are present for fibre morphology (CSA, myonuclear content, myonuclear domain, and fibre type proportion) following resistance training.

- Hypothesis #1: We hypothesized that no sex differences would be present in fibre morphology such that males and females would have similar increases in CSA, myonuclear addition, myonuclear domain size, and similar fibre type proportions.

- Aim #2: Determine if sex-differences are present for MuSC content following resistance training.
  - Hypothesis #2: We hypothesized that males would have higher MuSC content following training compared to females.
- Aim #3: Determine if sex-differences are present for FAP content following resistance training.
  - Hypothesis #3: We hypothesized that males would have higher FAP content following training compared to females.

1.6. Significance

A limited number of studies have investigated resistance training-induced adaptations in middle-aged adults (24, 29). It is important to study this population because it has been indicated that the initial onset of sarcopenia can be observed as early as 30 years of age (88). In addition, females begin menopause between the ages of 49-52 years (89), which could further exacerbate the sarcopenic response (90). There are currently no effective therapies to prevent sarcopenia but implementing early interventions such as protein supplemented resistance training could delay its progression. This study provides novel information on the potential sex-differences present in fibre and cell population adaptations to whole-body resistance training in middle-aged adults. These results provide significant insight on the adaptive responses which could inform resistance training interventions.

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Chapter II-Research Article: Sex based comparisons of muscle cellular adaptations after 10-weeks of progressive resistance training in middle-aged adults

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Running head: SEX-SPECIFIC RESPONSES TO TRAINING IN MIDDLE-AGED ADULTS

Keywords: Muscle stem cells, satellite cells, fibro-adipogenic progenitors, exercise, aging

New & noteworthy: We demonstrate that resistance training-induced hypertrophy and increased FAP content are similar between middle-aged males and females; however, MuSC and FAP content are correlated in males but not females.

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2.1. Abstract

Resistance training combined with protein intake supports skeletal muscle strength and hypertrophy. These adaptations are driven by the action of muscle stem cells (MuSCs) which are regulated, in part, by fibro-adipogenic progenitors (FAPs). It is unclear if middle-aged males and females have similar adaptations to resistance training at the cellular level. To address this gap, 28 (13 males, 15 females) middle-aged (40-64 years) adults participated in 10-weeks of whole-body resistance training with dietary counselling. Muscle biopsies were collected from the vastus lateralis pre- and post-training. Type II fibre cross-sectional area increased similarly with training in both sexes ($P = 0.014$). MuSC content was not altered with training; however, training increased $CD90^+$ FAP content ($P < 0.0001$) and reduced $PDGFR\alpha^+$ FAP content ($P = 0.044$), independent of sex. The change in MuSCs and $PDGFR\alpha^+$ ($P = 0.016$) and $CD90^+$ ($P = 0.005$) FAPs were correlated in males but not females. These results suggest that muscle fibre hypertrophy and stem/progenitor cell adaptations are similar between middle-aged males and females in response to whole-body resistance training; however, relationships between MuSCs and FAPs may differ between sexes.

2.2. Introduction

Skeletal muscle is comprised of highly organized, multinucleated, post-mitotic fibres that adapt to resistance training with the aid of resident mononuclear cell populations, such as muscle stem cells (MuSCs) and fibro-adipogenic progenitors (FAPs) (1). MuSCs are identified by paired-box transcription factor 7 (Pax7), and they are myogenic progenitors that have the capacity to differentiate into myocytes and fuse with mature muscle fibres (2). FAPs are non-myogenic cells present within the interstitial space that secrete paracrine factors creating a pro-myogenic environment to support MuSC activation and differentiation during repair and regeneration (3–5). In humans, FAPs are identified by platelet-derived growth factor receptor alpha (PDGFRα) (6) and cluster of differentiation 90 (CD90) (7). MuSCs and FAPs play an integral role in maintaining muscle mass throughout the lifespan (2, 8), as well as contributing to muscle hypertrophy in response to resistance training (9).

Progressive and irreversible fibre atrophy experienced with age is termed sarcopenia (10). The onset of sarcopenia can occur as early as the third decade of life with an increased prevalence in the fifth decade of life (11). While older females have lower muscle mass on average (12), the rate of absolute skeletal muscle mass loss is greater in males (13, 14). Information regarding the early stages of sarcopenia is lacking as most work has compared young adults to older adults with limited information on middle-aged adults (15, 16). The rate of the onset of sarcopenia may be attenuated by resistance training (17) combined with protein intake above 0.8 g/kg/d by promoting improvements in muscle mass and strength (18–21). Despite the improvements in muscle mass and strength observed in older adults who partake in resistance training interventions combined with appropriate nutritional support, the sarcopenic effects are

irreversible. Thus, it is important to develop a better understanding of how middle-aged adults respond to resistance training with protein intake to potentially implement interventions prior to the onset of sarcopenia.

The cellular responses to resistance training have been extensively examined in both young and older males (22–26); however, females (27, 28) and middle-aged adults (29, 30) are understudied. Males and females of all ages have similar increases in relative percent strength and hypertrophy as measured by fibre cross sectional area (CSA) (31, 32). Conversely, the results regarding MuSCs and FAPs are equivocal. Sex differences have been detected in MuSC content in which young males have been shown to have more MuSCs than young females and older adults (33), while other work has detected no sex differences in young and older adults (34, 35). FAPs have been found to increase with resistance training in young males (24), and are elevated in untrained individuals with chronic conditions (7); however, these studies have not examined the potential sex differences. It is unclear if middle-aged males and females have similar cellular adaptations to resistance training, especially when combined with appropriate nutritional support. Given the important role of MuSCs and FAPs in maintaining muscle mass with age and supporting fibre hypertrophy in response to resistance training, it is necessary to develop a better understanding of their actions in middle-aged adults.

The purpose of this study was to investigate the cellular adaptations of middle-aged males and females in response to a dietary controlled whole-body resistance training intervention. The overall hypothesis is that middle-aged males and females would have similar resistance training-induced adaptations in fibre morphology and MuSC content. Since previous work in young males

has shown that MuSCs and FAPs respond similarly to exercise training (24), we also hypothesized that FAP content would increase similarly in males and females following resistance training.

2.3. Materials and Methods

2.3.1. Ethical approval

Ethical approval was obtained from the University of Illinois Urbana-Champaign and the University of Ottawa research ethics boards. All protocols were in accordance with the Declaration of Helsinki. All participants provided written, informed consent prior to being enrolled in the study. This study was registered at ClinicalTrials.gov as NCT03029975.

2.3.2. Participants

The present work represents sub-analyses of a larger parent study (36). In brief, twenty-eight, healthy, untrained, middle-aged adults participated in this randomized, parallel-group study. Participant characteristics are presented in Table 1. Participants were between 40-64 years which encompasses the timeframe of early onset sarcopenia (11). Eligible participants had a body mass index (BMI) ≥ 18.5 and < 35 kg/m^2 and were not engaging in structured resistance or endurance exercise (≥ 1 year). Exclusion criteria included any one of the following factors: uncontrolled hypertension, musculoskeletal conditions or injuries sustained ≤ 1 year, dietary or exercise restrictions, consumed > 10 alcoholic beverages/week, consumed abnormal amounts of protein < 0.66 or > 1.80 g/kg/day, and/or history of regular tobacco and/or marijuana usage (< 6 months). Participants were instructed to discontinue the use of non-prescription medications, nutritional supplements, and cease alcohol consumption for 4 weeks before and during the study.

Table 1. Participant anthropometrics and physical characteristics

	Males (N=13)		Females (N=15)	
Measure	**Pre**	**Post**	**Pre**	**Post**
Sample size	MOD n=5; HIGH n=8		MOD n=9 [a]; HIGH n=6 [a]	
Age (years)	51.0 ± 2.2		48.7 ± 1.8	
Height (cm)	174.8 ± 0.03		169.3 ± 0.02	
Relative protein intake (g/kg/d) [e]	1.1 ± 0.1	1.4 ± 0.1 [d]	1.0 ± 0.1	1.3 ± 0.1 [d]
Total body mass (kg)	88.8 ± 4.1	90.2 ± 4.0 [d]	76.1 ± 2.3 [c]	77.8 ± 2.1 [c,d]
Body mass index (kg/m^2)	29.1 ± 1.1	29.6 ± 1.2 [d]	26.7 ± 0.9	27.3 ± 0.9 [d]
Lean body mass (kg)	60.6 ± 2.8	62.1 ± 2.4 [d]	42.9 ± 1.0 [c]	43.7 ± 0.9 [c,d]
Fat mass (kg)	25.0 ± 2.1	25.3 ± 2.0	31.0 ± 1.9 [c]	31.4 ± 1.8 [c]
Body fat (%)	27.7 ± 1.6	27.7 ± 1.4	40.3 ± 1.4 [c]	40.3 ± 1.3 [c]
1-RM Leg press (kg) [b]	137.5 ± 9.4	232.7 ± 18.3	86.5 ± 7.5	133.7 ± 10.5
1-RM Leg curl (kg)	74.3 ± 4.6	99.4 ± 4.8 [d]	52.5 ± 3.0 [c]	64.4 ± 2.2 [c,d]
1-RM Leg extension (kg)	78.9 ± 2.9	126.6 ± 6.6 [d]	57.5 ± 2.1 [c]	80.4 ± 3.6 [c,d]

Note. Data presented as mean ± SEM. Participant age and height were assessed using an unpaired, t-test with Welch's correction. Remaining variables were analysed using a 2-way RM ANOVA. Leg press revealed a significant interaction effect (Sex × Time) which was assessed using the Šidák's correction. N, total sample size; n, sample size subset; MOD, moderate protein group; HIGH, high protein group; 1-RM, one-repetition maximum.

[a] Three female participants per group, totaling six, were pre-menopausal.
[b] Interaction effect (sex × time). P = 0.026.
[c] Main effect of sex, P < 0.05.
[d] Main effect of time, P < 0.05.
[e] Post protein intake was an aggregate of the diet intervention records.

2.3.3. Experimental design

An overview of the experimental design is presented in Figure 1. Briefly, participants completed a preliminary screening session, 1-week of dietary habituation, followed by 10-weeks of progressive, whole-body resistance training, with a protein supplemented dietary intervention with dietary tracking and counselling to support targeted protein intake. Specifically, the initial screening session involved written, informed consent, anthropometric measurements, and strength testing familiarization. Participants were randomized in the parent study (36) to either a moderate (MOD) 0.8-1.0 g/kg/d, or high (HIGH) 1.6-1.8 g/kg/d, protein consuming group. Participant randomization to the protein groups was achieved by using an automated spreadsheet organized by participant age, sex, BMI, and baseline strength. However, there were no differences in strength and lean body mass adaptations between the MOD/HIGH protein groups (36); thus, we collapsed the protein groups and conducted sex-based analyses. The resistance training was supervised and performed weekly on three non-consecutive days. Participants were asked to avoid engaging in any additional structured resistance or endurance exercise throughout the study duration.

2.3.4. Resistance training intervention

Participants engaged in a 10-week supervised, progressive, whole-body resistance training intervention in which training occurred at the same time of the day on three, non-consecutive days of the week. The intervention was comprised of five alternating exercises: three bilateral lower-body and two upper-body exercises. The lower body exercises: leg press, leg extension, and leg curl, were repeated each training day. The upper body exercises were split into two categories: push (chest press and shoulder press) and pull (seated row and bicep curls

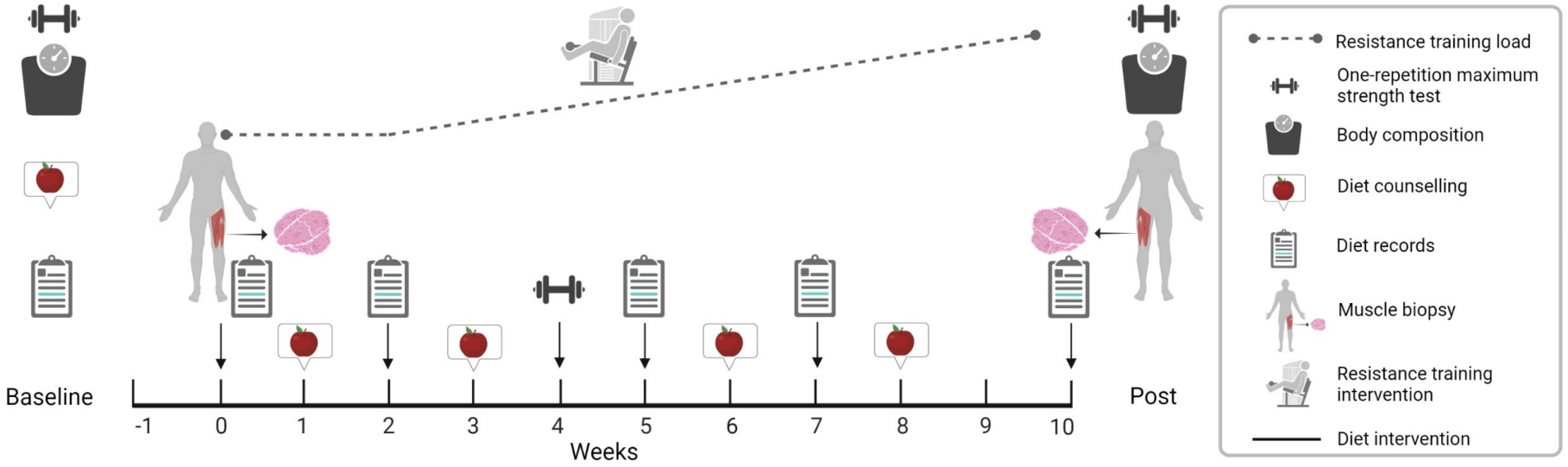


Figure 1. Experimental design. Baseline measures were tested before diet randomization. Diet habituation commenced at week -1 and resistance training commenced at week 0, and both interventions lasted until week 10. Post-measures were conducted 72 hours following the last training session of the 10th week. Muscle biopsies were collected at pre- (week 0) and post-timepoints. Diet counselling and tracking occurred on alternating weeks. Created with BioRender.com.

The upper body exercises were alternated each training session based on category (e.g., push, pull, push). All exercises were performed on guided motion machines except for barbell bicep curls. Each session commenced with a 5-minute warm-up on a cycle ergometer, followed by a specific warm-up (2 sets × 10 repetitions, 30-75% of the training load) for each exercise. The exercise intensity for the initial two weeks of training was set at 65% of 1-repetition maximum (RM). The training load increased linearly once participants could complete 3 sets × 10 repetitions with proper form.

2.3.5. Protein supplementation intervention, dietary counselling, and nutrient tracking.

The dietary intervention was commenced one week prior (-1 week) to the resistance training program to ensure dietary habituation. Protein requirements were met by providing participants with isocaloric meals during the following anabolic periods: immediately after each training session (3× week) and daily, 1-2 hours before sleep (37). Provided meals were isocaloric with differential protein doses: 15 (MOD) or 30 g (HIGH), sourced from lean beef, with carbohydrate doses to match energy intake.

Participants received dietary counselling from a research dietitian to promote dietary intervention adherence. Diet was tracked on alternating weeks (pre, 0, 2, 5, 7, 10) for three consecutive days, including one weekend day, one training day, and one rest day (e.g., Thursday-Saturday). The counselling sessions (weeks: pre, 1, 3, 6, 8) were evaluations of the previous diet record and consisted of recommendations on how to attain the daily protein intake goal, the timing of protein intake, and which nutrient-dense foods to consume. Macronutrient intake was recorded using the automated self-administered 24-hour dietary assessment tool (ASA24) (National Cancer Institute; Bethesda, MD, USA; version (v) 2016). Reported dietary intake during

the intervention was aggregated to evaluate dietary habits during training. All dietary logs were analysed for diet composition by a registered dietitian.

2.3.6. Body composition, strength assessments, and muscle biopsies

Body composition was measured at baseline and week 10 using dual-energy X-ray absorptiometry scans (Hologic QDR 4500 A; Bedford, MA, USA). Body mass and fat mass were directly quantified, while lean body mass was indirectly calculated from soft tissue mass using APEX software (Hologic, Bedford, MA, USA).

Strength was assessed at baseline, week 4, and post-intervention pre-established procedures: 1-RM for lower-body exercises (38) and 10-RM for upper body exercises (39).

Muscle biopsies were collected at week 0 (pre) and after 10-weeks (post) of training and were randomized between the dominant versus non-dominant legs among participants. Prior to biopsy collection participants completed an overnight fast and refrained from consuming caffeine for 24 hours and exercising for 72 hours, as previously *described* (40). In brief, samples were taken from the middle region of the vastus lateralis using a modified *Bergström needle. Samples were freed from* visible blood, adipose, and connective tissues and were embedded in optimal cutting temperature compound. Samples were frozen by immersion in isopentane, pre-cooled in liquid nitrogen, then stored at -80°C for cryo-sectioning.

2.3.7. Immunofluorescence Staining, Image Acquisition, and Analysis

Samples were cryo-sectioned (Thermo Fisher Scientific HM525 NX; Waltham, MA) to a thickness of 8 µm, mounted onto glass slides, then stored at -80°C until staining. All products used are presented in Table 2, and all antibodies and their dilutions are presented in Table 3. Unless otherwise stated, staining was conducted at room temperature (~21°C) in the dark.

Table 2. Products used for sample collection and immunofluorescence

Product	Cat. No.	Manufacturer; Location
Bovine serum albumin	BP1600-100	Thermo Fisher Scientific; Waltham, MA
DAKO protein block, serum-free	X0909	Agilent; Mississauga, TO
Fetal bovine serum	12483-020	Gibco; Amarillo, TX
Goat serum	16210-064	Gibco; Amarillo, TX
Glycerol, >99%	17904	Thermo Fisher Scientific; Waltham, MA
Fisherbrand feather microtome blades-S35	12-634-1C	Thermo Fisher Scientific; Waltham, MA
Fisherbrand cover glass (24x50mm; 1.5)	12-544E	Thermo Fisher Scientific; Waltham, MA
Fisherfinest premium cover glass (24x50mm; 1.0)	12-548-5M	Thermo Fisher Scientific; Waltham, MA
Fluoromount aqueous mounting medium	F4680-25ML	Sigma Aldrich; Oakville, ON
ImmEdge hydrophobic barrier PAP pen	H-4000	Vector Laboratories; Burlingame, CA
Paraformaldehyde, reagent grade, crystalline	P6148-1KG	Sigma Aldrich; Oakville, ON
Sodium Azide, ReagentPlus, ≥99.5%	S2002-100G	Sigma Aldrich; Oakville, ON
Tissue-Plus optimal cutting temperature compound	23-730-571	Thermo Fisher Scientific; Waltham, MA
Triton X-100, surfact-amps detergent	85112	Thermo Fisher Scientific; Waltham, MA
Tween 20	BP337-500	Thermo Fisher Scientific; Waltham, MA
UltraPure Tris Buffer (Powder)	15504020	Thermo Fisher Scientific; Waltham, MA

Note. Products used for experiments. Cat. No., manufacturer's category product number.

Table 3. Immunofluorescence antibodies and dye

Antibodies	Isotype	Species	Dilution	Concentration	Cat. No.	RRID	Manufacturer; Location	Associated Secondary
Primary Antibodies								
Anti-human CD90 (Thy-1, Clone: 5E10)	IgG1, κ	Mouse	1:100	[5µg/mL]	14-0909-80	AB_763535	Thermo Fisher Scientific; Waltham, MA	AF647 (A-21240)
Anti-Laminin (β-2/γ-1, A5)	IgG, 2a	Rat	1:200	[5µg/mL]	MA1-06100	AB_559896	Thermo Fisher Scientific, Waltham, MA	AF594 (A-11007)
Anti-MHC I	IgG, 2b	Mouse	1:100	[0.18µg/mL]	BA-D5 [a,c]	AB_2235587	DSHB, Iowa, IA	AF647 (115-605-207)
Anti-MHC IIa	IgG1	Mouse	1:100	[0.2µg/mL]	SC-71 [a,d]	AB_2147165	DSHB, Iowa, IA	AF488 (115-545-205)
Anti-Pax7, Supernatant	IgG1, κ	Mouse	1:12.5	[2µg/mL]	PAX7 [b]	AB_528428	DSHB, Iowa, IA	AF488 (A-21121)
Anti-PDGFRα (D1E1E) XP	IgG	Rabbit	1:100	[0.23µg/mL]	3174S	AB_2162345	Cell Signaling Technology; Danvers, MA	AF488 (A-11034)
Secondary Antibodies								
AF488 goat, anti-mouse	IgG1	Goat	1:500	[4µg/mL]	A-21121	AB_2535764	Thermo Fisher Scientific, Waltham, MA	n.a
AF488 goat, anti-mouse	IgG, Fcγ1	Goat	1:100	[8µg/mL]	115-545-205 [e]	AB_2338854	Jackson ImmunoResearch Laboratories Inc., Baltimore, MD	n.a
AF488 goat, anti-rabbit	IgG	Goat	1:100	[20µg/mL]	A-11034	AB_2576217	Thermo Fisher Scientific, Waltham, MA	n.a
AF594 goat, anti-rat	IgG	Goat	1:300	[6.67µg/mL]	A-11007	AB_10561522	Thermo Fisher Scientific, Waltham, MA	n.a
AF647 goat, anti-mouse	IgG1	Goat	1:500	[4µg/mL]	A-21240	AB_2535809	Thermo Fisher Scientific, Waltham, MA	n.a
AF647 goat, anti-mouse	IgG, Fcγ2b	Goat	1:100	[8.5µg/mL]	115-605-207 [f]	AB_2338918	Jackson ImmunoResearch Laboratories Inc., Baltimore, MD	n.a
Dye								
DAPI	n.a	n.a	1:20,000	[0.025µg/mL]	D9542	n.a	Sigma Aldrich; Oakville, ON	n.a

Note. Primary and secondary antibodies and nuclear dye used for immunofluorescence. Cat. No., manufacturer's category product number; RRID, research resource identifiers; IgG, immunoglobulin type/subclass; AB, antibody; MHC, myosin heavy chain; DSHB, Developmental Studies Hybridoma Bank; AF, Alexa Fluor; n.a, not applicable; DAPI, 4',6-Diamidino-2-phenylindole.

[a] Deposited to the DSHB by Shiaffino, S.

[b] Deposited to the DSHB by Kawakami, A.

[c] BA-D5 initial [36ug/mL], [d] SC-75 initial [40ug/mL], [e] AF488 initial [1.6mg/mL] and [f] AF647 initial [1.7mg/mL] aliquoted 1:1 in sterile glycerol.

For each stain the primary antibody was omitted from three control sections to confirm secondary antibody binding specificity. Prior to staining all samples were air dried for 30 minutes and outlined with ImmEdge PAP pen. Mounted slides were stored at 4°C until visualization using fluorescence microscopy. One female participant was excluded from all immunofluorescence analyses due to poor biopsy composition (< 20 fibres).

***Identification of myosin heavy chain isoforms and quantification of cross-sectional area, myonuclear content, and myonuclear domain size*.** Immunofluorescence for MHC isoforms was conducted as previously described with modifications (41). Sections were blocked in 5% BSA for 1 hour then incubated overnight at 4°C in the primary antibody rat, anti-laminin in 5% normal goat serum (G/S). Sections were washed for 3 × 5 minutes in 1 × PBS, then incubated for 1 hour with the secondary antibody AF594 goat, anti-rat in 1% BSA. Sections were washed, 3 × 5 minutes, then incubated for 45 minutes at 37°C in the following primary antibody mixture: mouse, anti-MHC-I and mouse, anti-MHC-IIa in 1% BSA. Sections were washed, 3 × 5 minutes, then incubated for 1 hour in the following secondary antibody mixture: AF488 goat, anti-mouse (IgG, Fcγ1) and AF647 goat, anti-mouse (IgG, Fcγ2b) in 0.5% BSA, 5% G/S. Sections were washed, 3 x 5 minutes, incubated for 5 minutes with 4', 6-diamidino-2-phenylindole (DAPI), washed for 3 x 2 minutes, mounted, and imaged. Fibre morphology and fibre types were assessed as previously described (42). Briefly, CSA was measured by tracing the perimeter of each fibre as indicated by laminin. Myonuclear content was determined by counting the number of $DAPI^+$ myonuclei with at least 50% of the nucleus located within the basal lamina of each fibre, while myonuclear domain was estimated by dividing the CSA by the number of myonuclei per fibre. Fibre type

proportions were expressed as a ratio of type I or II MHC isoform, to the total number of fibres analysed.

Muscle stem cell quantification. Immunofluorescence of $Pax7^+$ MuSCs was conducted as previously described, with modifications (43). Sections were fixed in 4% paraformaldehyde (PFA) for 10 minutes and washed for 3 × 2 minutes, in 1 x tris-buffered saline with 0.2% Tween (TBST). Sections were permeabilized in 0.2% triton X-100 for 10 minutes then washed, 3 × 2 minutes. Sections were blocked for 1 hour in 5% fetal bovine serum (FBS), 5% G/S, 0.2% triton X-100, and 0.1% sodium-azide, then washed, 1 x 5 minutes. Sections were incubated overnight at 4°C in the primary antibody anti-Pax7 diluted in 1 × PBS. Sections were washed, 3 × 5 minutes, then incubated for 2 hours in the secondary antibody AF488 goat, anti-mouse (IgG1) in 5% G/S. Sections were washed, 3 × 5 minutes, then incubated for 2 hours in the primary antibody, rat, anti-laminin in 5% G/S, 0.2% triton X-100. Sections were washed, 3 × 5 minutes, then incubated for 1 hour with the secondary antibody AF594 goat, anti-rat in 5% G/S. Sections were washed, 3 x 5 minutes, incubated for 5 minutes with DAPI, washed for 3 x 5 minutes, mounted, and imaged. MuSCs were identified as sublaminar $Pax7^+$ cells, colocalizing with $DAPI^+$ myonuclei, and they were expressed relative to 100 fibres as previously described (43).

Fibro-adipogenic progenitor cell quantification. Immunofluorescence of $PDGFR\alpha^+$ and $CD90^+$ FAPs were conducted as previously described, with modifications (6, 7). Sections were fixed in 4% PFA for 5 minutes and washed for 3 × 2 minutes in 1 × TBST. Sections were permeabilized in 0.2% triton X-100 for 10 minutes, washed, 3 × 2 minutes. Sections were blocked with DAKO protein block for 1 hour, then washed for 1 x 5 minutes. Sections were incubated overnight at 4°C in the primary antibody rabbit, anti-PDGFRα in 5% G/S, 0.2% triton X-100.

Sections were washed, 3 × 5 minutes, then incubated for 2 hours in the secondary antibody AF488 goat, anti-rabbit in 5% G/S. Sections were washed, 3 × 5 minutes, re-fixed in 2% PFA for 5 minutes, and washed, 3 x 5 minutes. Sections were blocked for 1 hour in 5% FBS, 5% G/S, 0.2% triton X-100, 0.1% sodium azide, then washed for 5 minutes. Sections were incubated overnight at 4°C in the primary antibody mouse, anti-CD90 in 5% G/S, 0.2% triton X-100. Sections were washed, 3 × 5 minutes, then incubated for 2 hours in the secondary antibody AF647 goat, anti-mouse (IgG1) in 5% G/S. Sections were washed, 3 × 5 minutes, then incubated for 2 hours in the primary antibody, rat, anti-laminin in 5% G/S, 0.2% triton X-100. Sections were washed, 3 × 5 minutes, then incubated for 1 hour with the secondary antibody AF594 goat, anti-rat in 5% G/S. Sections were washed, 3 x 5 minutes, incubated for 5 minutes with DAPI, washed for 3 x 5 minutes, mounted, and imaged. FAPs were quantified if they were located within the interstitial space and were PDGFRa^{+} only (6), CD90+ only, and/or PDGFRa^{+}CD90^{+} (7), and they were expressed relative to 100 fibres.

MHC images were captured at 5×, 0.25 numerical aperture (NA) (1× Optovar), using an AxioCam 506-Monochrome camera installed on the CellDiscoverer 7 microscope (Zeiss; North York, TO), equipped with Zen Blue (v. 2.6) software. MuSC and FAP images were captured at 20×, 0.80 NA, using an AxioCam mRm CCD monochrome camera installed on the Axio Imager 2 microscope (Zeiss; North York, TO; Research Resource Identifier (RRID): SCR_018876) equipped with Zen Blue (v. 2.3) software. Each stain had distinct imaging parameters that remained identical for all slides.

Images were converted to an 8-bit tag image file format and were analysed using Fiji, ImageJ (National Institutes of Health; USA; v. 1.53c). Image brightness and contrast were adjusted

to the same degree based on stain type. All intact fibres were analysed, while longitudinally cut regions, folded, overlapping, damaged, or incomplete fibres were excluded. As previously described, a minimum of 150 fibres is recommended to quantify CSA (44), 50 fibres is recommended to quantify myonuclei, and 125 fibres is recommended to quantify MuSCs (45). Thus, a mean ± standard deviation of 223.6 ± 133.8 fibres were analysed per section. All image analyses were performed manually, by the same investigator blinded to the experimental condition.

2.3.8. Statistical analyses and data presentation

Statistical analyses were conducted using GraphPad Prism (GraphPad Software; San-Diego, CA, USA; v. 8.0.1). Data were tested for normality using the Shapiro-Wilk test. If data were not normally distributed, they were log transformed prior to analyses. Sex differences in participant age and height were analysed using an unpaired, two-tailed Welch's t-test. Fibre distribution was assessed using a paired, two-tailed Wilcoxon signed-rank test with Pratt's correction. All remaining outcomes were analysed using a two-factor (sex × time), repeated measures ANOVA. Significant interactions were further evaluated using Šidák's multiple-comparisons post-hoc test. Correlations were assessed using either a two-tailed Pearson's or Spearman's test. Data are expressed as means ± standard error of the mean (SEM), unless otherwise stated, with significance set at $P < 0.05$.

2.9. Results

Both sexes improved body composition, leg strength, and relative protein intake. Males had a larger total body mass than females (Table 1) (sex effect, $P = 0.009$). Total body mass (time effect, $P < 0.006$) and BMI (time effect, $P = 0.004$) increased in both sexes. Males had a greater lean body mass (sex effect, $P < 0.0001$) than females. Lean body mass increased in both sexes (time effect, $P = 0.024$). Females had greater fat mass (sex effect, $P = 0.032$) and percent body fat (sex effect, $P < 0.0001$) than males. An interaction effect was observed for 1-RM leg press strength (sex × time interaction: $P = 0.026$) in which strength increased in both sexes (males: $P < 0.0001$, females: $P = 0.004$) with males having greater increases in leg strength with training (Pre: $P = 0.007$, Post: $P < 0.0001$). Males had greater 1-RM leg curl and 1-RM leg extension strength (sex effect, both $P < 0.0001$) than females, but both measures increased in both sexes (time effect, both $P < 0.0001$). Relative protein intake significantly increased (time effect, $P < 0.0001$), independent of sex.

Both sexes increased fibre cross-sectional area. Representative MHC images are presented in Figure 2A. Males had larger type I (Fig. 2B) (sex effect, $P = 0.001$) and II (Fig. 2C) (sex effect, $P < 0.0001$) fibre CSA. Type II fibre CSA increased with training in both sexes (Fig. 2C) (time effect, $P = 0.014$). Males had a trend for greater myonuclear content in type I fibres (Fig. 2D) ($P = 0.080$) and had significantly greater myonuclear content in type II fibres (Fig. 2E) (sex effect, $P < 0.001$). There were no significant sex or time effects on myonuclear domain in type I fibres (Fig. 2F); however, males had a significantly larger myonuclear domain in type II fibres (Fig. 2G) (sex effect, $P = 0.012$). There were no significant sex or time effects on type I (Fig. 2H) and type II (Fig. 2I) fibre proportion.

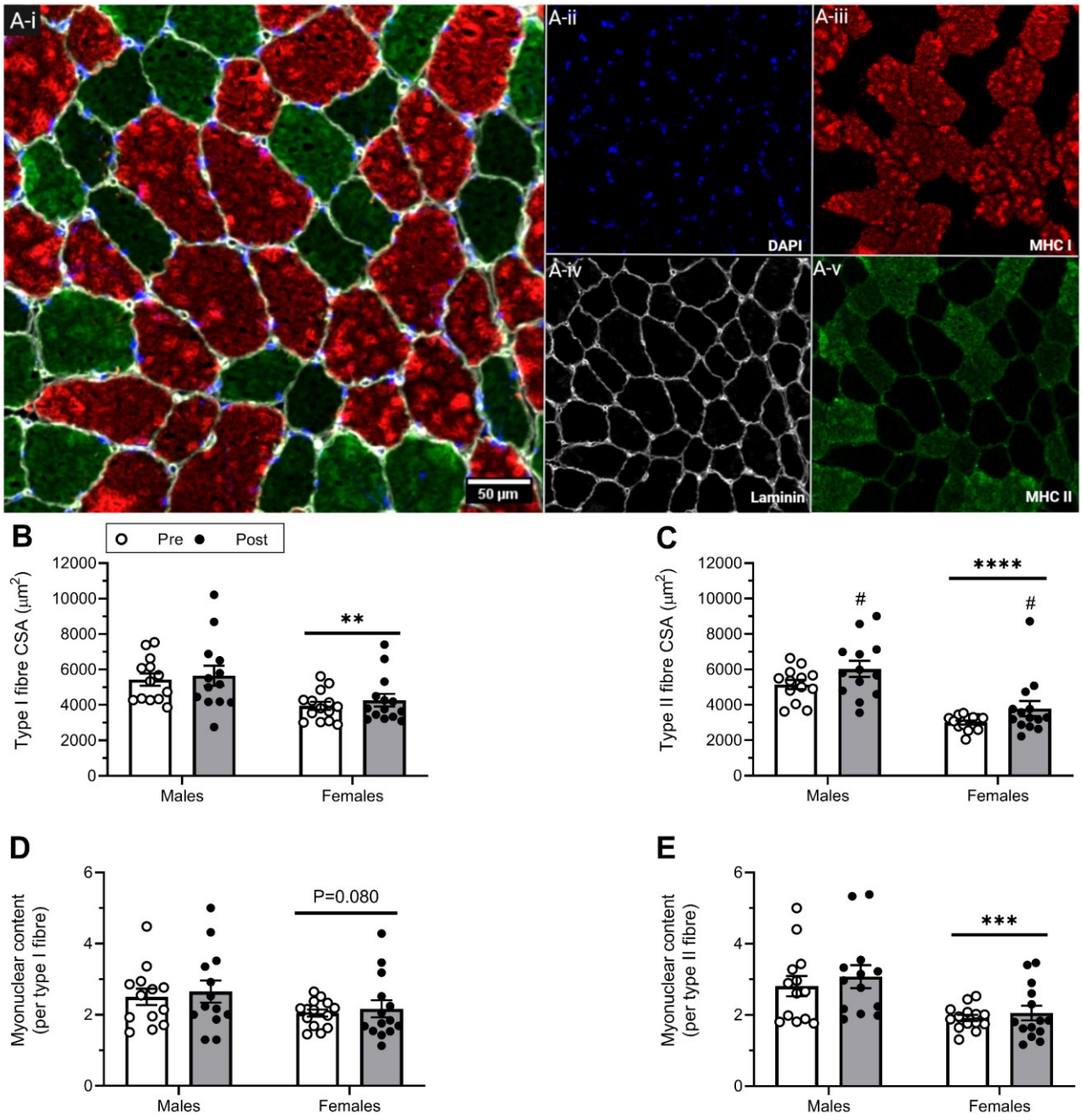


Figure 2. Influence of sex on fibre morphology and fibre type proportion. (A) Representative images of myosin-heavy chain isoform immunofluorescence captured at 5×, 0.25 NA. Created with BioRender.com. **(A-i)** Merged image. Scale bar represents 50 µm. **(A-ii)** DAPI-blue=nuclei. **(A-iii)** MHC I-red=type I fibres. **(A-iv)** Laminin-grey=fibre margins. **(A-v)** MHC II-green=type II fibres. **(B)** Type I and **(C)** II fibre cross-sectional area (μm^2). **(D)** Type I and **(E)** II fibre myonuclear content. ***(Fig. 2 continued on the following page).***

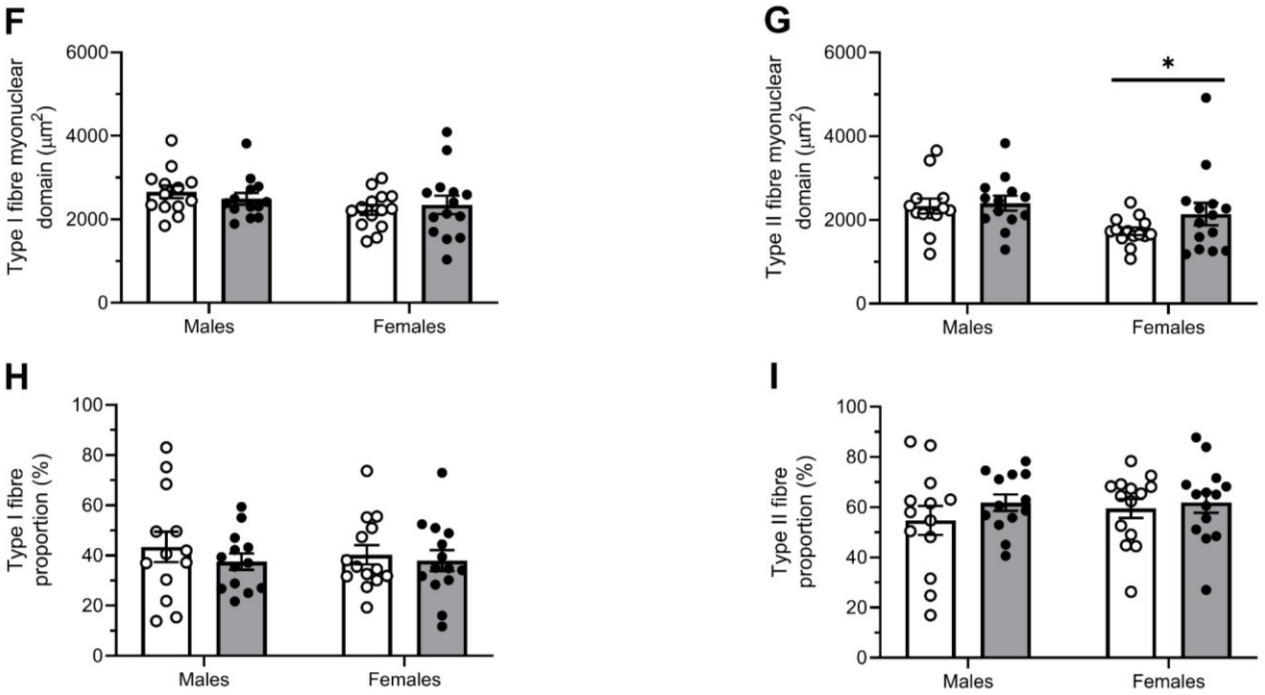


Figure 2. Continued. (F) Type I and **(G)** II fibre myonuclear domain (μm^2). **(H)** Type I and **(I)** II fibre proportions. **(B-I)** Data were analysed using a two-factor, repeated measures ANOVA. Data are expressed as mean ± SEM of N = 27 (13 males, 14 females), with individual data points corresponding to a participant. Sex effect: * P = 0.012, ** P = 0.001, *** P < 0.001, **** P < 0.0001. Time effect: # P = 0.014. Trend for sex effect: P = 0.080. (%).

Fibre CSA distribution did not change with training in males (Fig. 3A). Females had a trend (P = 0.091) for a reduction in fibres sized 2000-2999 μm^2 from ~ 27.9% to 22.1%, but had a significant increase (time effect, P = 0.022) in CSA distribution for fibres sized 7000-7999 μm^2, from ~1.1% to ~2.7% (Fig. 3B). CSA was not correlated with protein intake, MuSC content, or FAP content (data not shown).

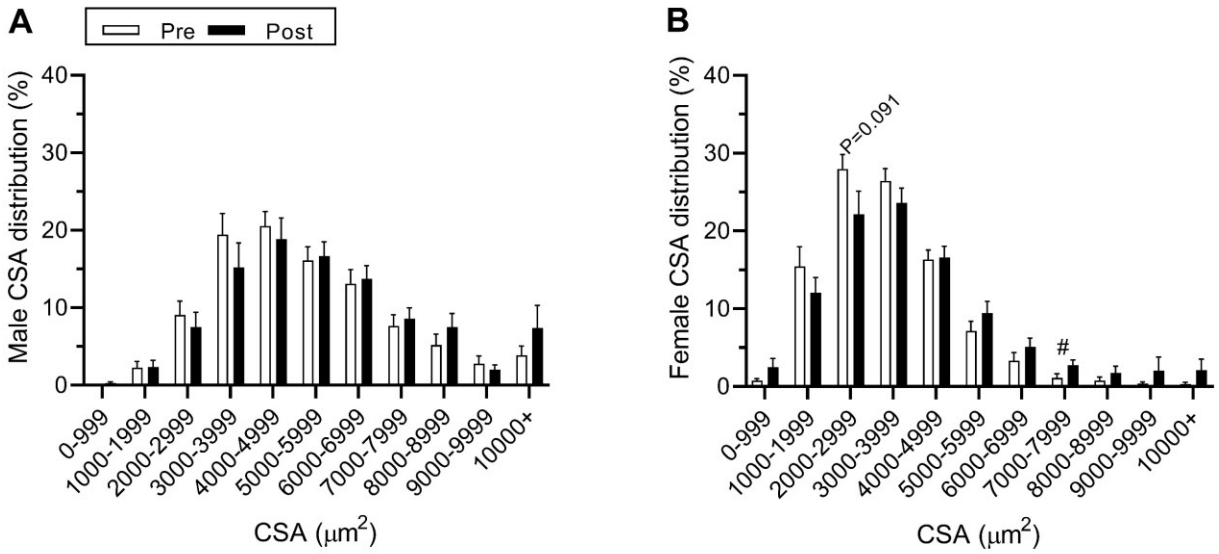
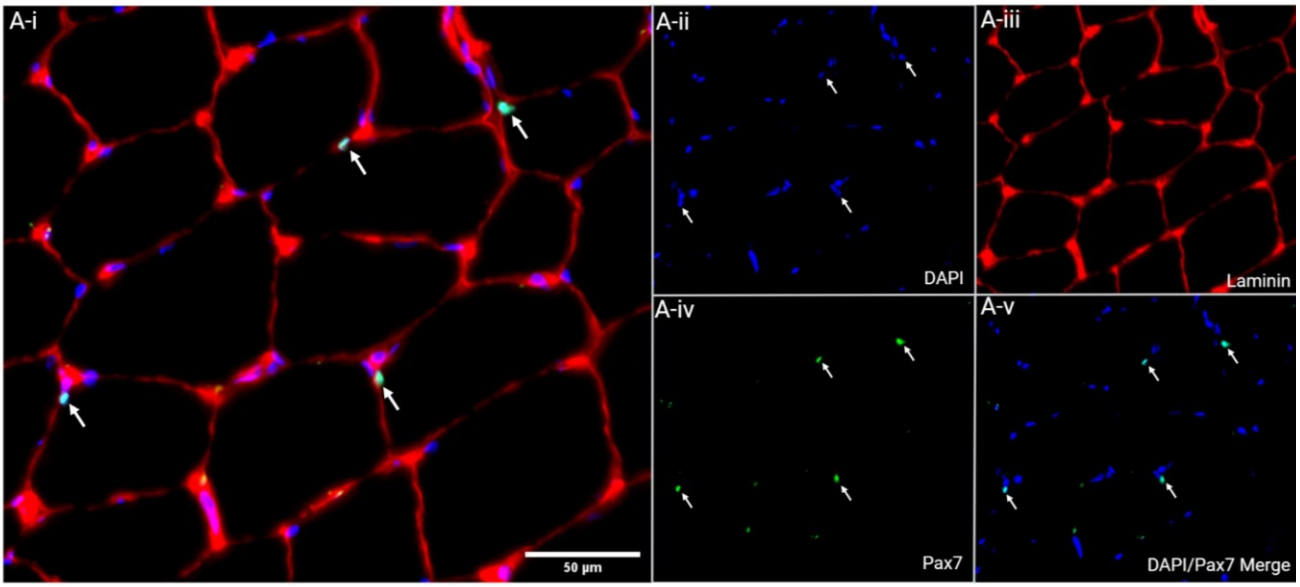
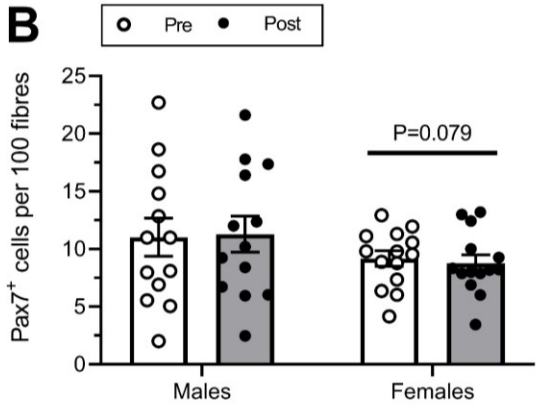


Figure 3. Influence of sex on fibre size distribution. (A) Pre- and **(B)** post-fibre size distribution (%). Data were analysed using a paired, two-tailed Wilcoxon signed-rank test with Pratt's correction. Data are expressed as mean ± SEM of N = 27 (13 males, 14 females), with bars corresponding to group means. Time effect: # P = 0.022. Trend for time effect: P = 0.091.

Both sexes had no changes in muscle stem cell content but altered fibro-adipogenic progenitor cell content. Representative MuSC images are presented in Figure 4A. There was a trend for males having a higher $Pax7^+$ MuSC content compared to females (Fig. 4B) (P = 0.079) with no time effect.





Representative FAP images are presented in Figure 5A. There was a reduction in PDGFRα$^+$ FAPs (Fig. 5B) (time effect, P = 0.044) and an increase in CD09$^+$ FAPs with time (Fig. 5C) (time effect, P < 0.0001), independent of sex. There were no sex or time effects present for PDGFRα$^+$/CD09$^+$ FAPs (Fig. 5D).

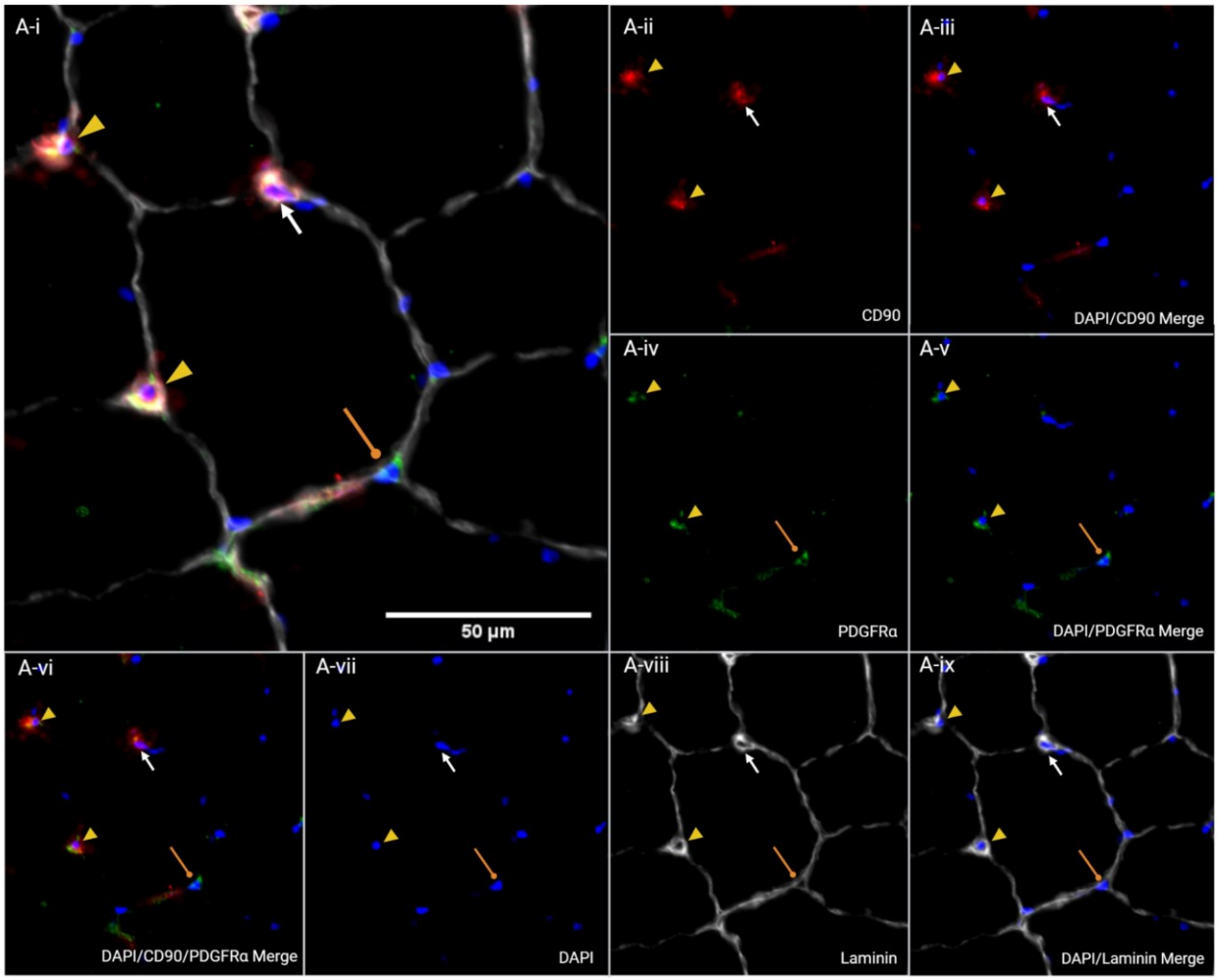
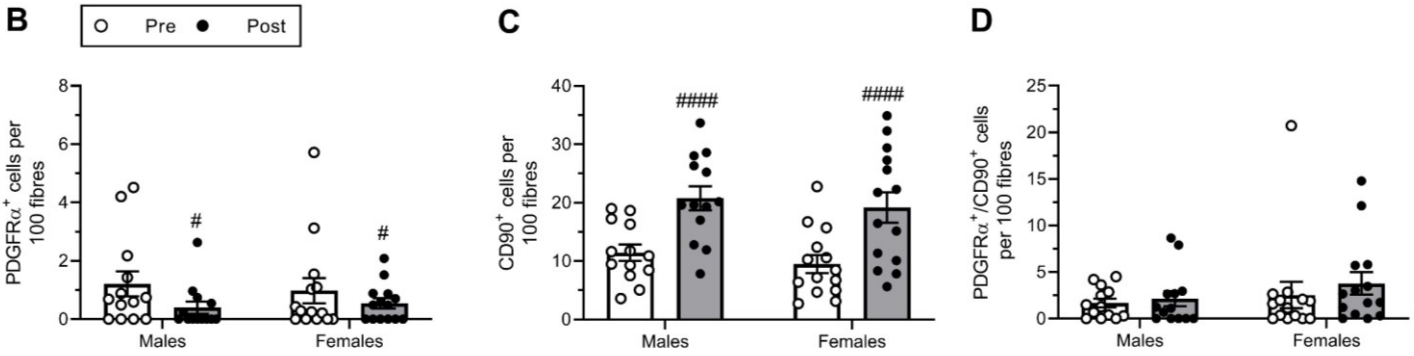


Figure 5. Influence of sex on fibro-adipogenic progenitor cell content. (A) Representative images of fibro-adipogenic progenitor immunofluorescence captured at 20×, 0.80 NA. Created with BioRender.com. **(A-i)** Merged image. Scale bar represents 50 µm. **(A-ii)** CD90-red=CD90$^+$ cells. **(A-iii)** DAPI/CD90-merge= CD90$^+$ cells colocalizing with nuclei. **(A-iv)** PDGFRα-green= PDGFRα$^+$ cells. **(A-v)** DAPI/PDGFRα-merge=PDGFRα$^+$ cells colocalizing with nuclei. **(A-vi)** DAPI/CD90/PDGFRα-merge= CD90$^+$/PDGFRα$^+$ cells colocalizing with nuclei. **(A-vii)** DAPI-blue=nuclei. **(A-viii)** Laminin-grey=fibre margins. **(A-ix)** DAPI/Laminin-merge=positioning of interstitial nuclei and myonuclei. **(A-i, ii, iii, vi-ix)** White arrows indicate CD90$^+$ cells. **(A-i, iv, v, vi-ix)** Orange line segments indicate PDGFRα$^+$ cells. **(A-i-ix)** Yellow arrow heads indicate PDGFRα$^+$/CD90$^+$ cells. ***(Fig. 5 continued on the following page).***



(B) Total PDGFRα$^+$, **(C)** CD90$^+$, and **(D)** PDGFRα$^+$/CD90$^+$ cells per 100 fibres. **(B-D)** Data were analysed using a two-factor, repeated measures ANOVA. Data are expressed as mean ± SEM of N = 27 (13 males, 14 females), with individual data points corresponding to a participant. Time effect: # P = 0.044, #### P < 0.0001.

The change in resident muscle stem cell and fibro-adipogenic progenitor cell populations are correlated. The change (post-pre) in MuSCs and PDGFRα$^+$ FAPs were not correlated for pooled data (Fig. 6A) (r = -0.286, P = 0.148). There was an inverse correlation between the change in MuSCs and PDGFRα$^+$ FAPs in males (Fig. 6B) (r = -0.651, P = 0.016). However, the change in MuSCs and PDGFRα$^+$ FAPs were not correlated in females (Fig. 6C) (r = 0.129, P = 0.661). The change in MuSCs and CD90$^+$ FAPs were positively correlated for pooled data (Fig. 6D) (r = 0.560, P = 0.002). There was a positive correlation between the change in MuSCs and CD90$^+$ FAPs in males (Fig. 6E) (r = 0.727, P = 0.005). However, the change in MuSCs and CD90$^+$ FAPs were not correlated in females (Fig. 6F) (r = 0.264, P = 0.362). The change in MuSCs and PDGFRα$^+$/CD09$^+$ FAPs were not correlated for pooled data (Fig. 6G) (r = 0.056, P = 0.782), males (Fig. 6H) (r = -0.160, P = 0.602), or females (Fig. 6I) (r = 0.405, P = 0.151).

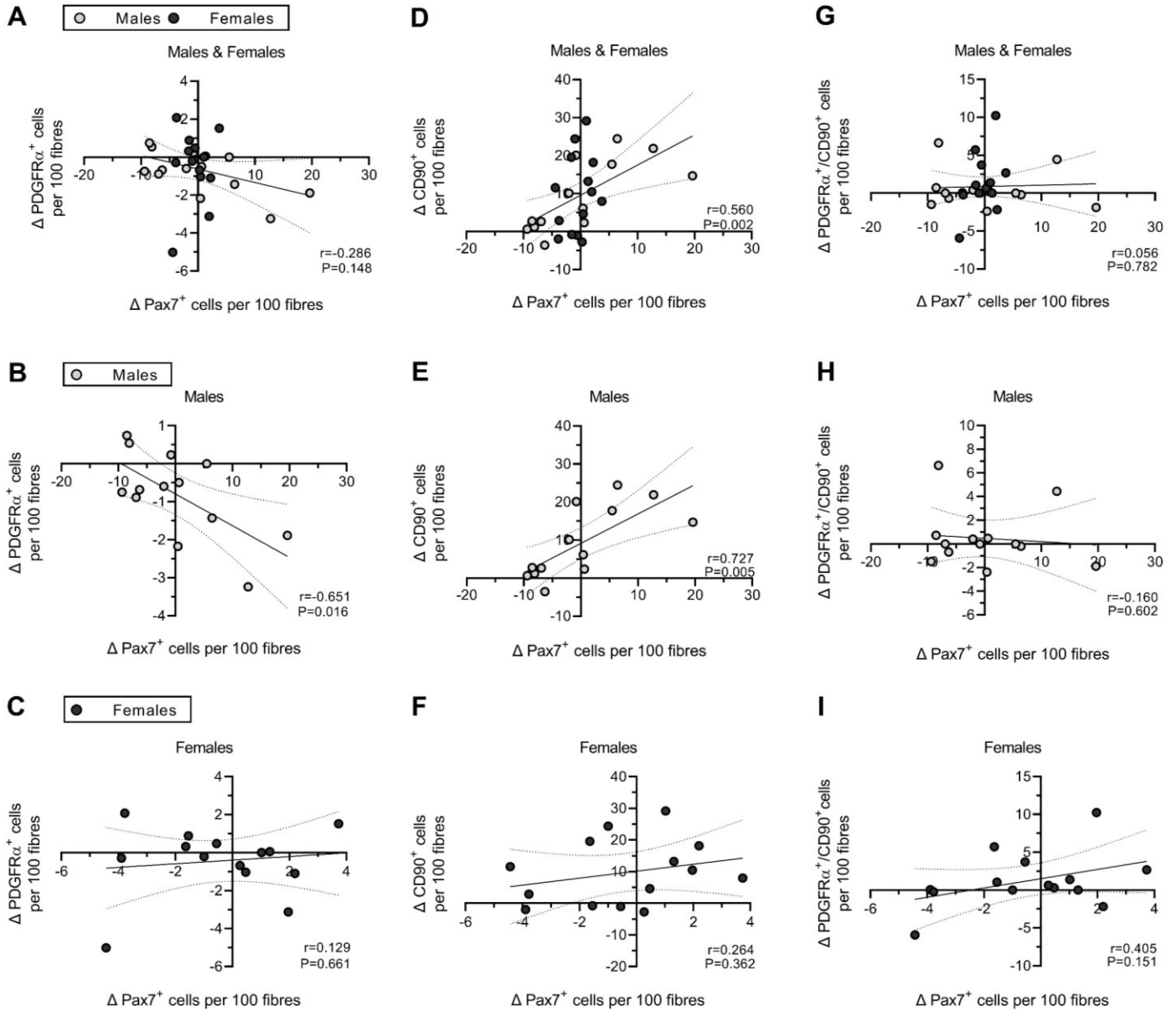


Figure 6. Correlations between the change in muscle stem cell content and fibro-adipogenic progenitor cell content. Change (post-pre) in $Pax7^+$ cells correlated against the change in $PDGFR\alpha^+$ cells in **(A)** both sexes, **(B)** males, and **(C)** females. Change in $Pax7^+$ cells correlated against the change in $CD90^+$ cells in **(D)** both sexes, **(E)** males, and **(F)** females. Change in $Pax7^+$ cells correlated against the change in $PDGFR\alpha^+/CD90^+$ cells in **(G)** both sexes, **(H)** males, and **(I)** females. **(A, D, G, H)** Data were analysed using a two-tailed, Spearman correlation test. **(B, C, E, F, I)** Data were analysed using a two-tailed, Pearson correlation test. **(A-I)** Data are expressed as a line of best fit and 95% confidence interval bands, with individual data points corresponding to a participant, N = 27 (13 males, 14 females). R and P-values are displayed directly on the graph.

2.10. Discussion

Our overall objective was to examine potential sex differences in the cellular adaptations of middle-aged adults to resistance training during a dietary controlled intervention. The results suggest that middle-aged males and females have similar cellular adaptations to 10-weeks of resistance training. Both sexes had similar increases in type II fibre CSA and changes in FAP content. Interestingly, we found that the change in MuSCs were inversely correlated to PDGFRα^{+} FAPs and positively correlated to CD90^{+} FAPs in males only. Thus, middle-aged adults appear to have similar cellular adaptations in response to 10-weeks of whole-body resistance training with some divergent sex-based responses in the relationship between MuSC and FAPs.

Our results suggest that males have greater absolute increases in leg strength than females. Notably, this result was apparent for 1-RM leg press in which males had significantly greater absolute leg strength than females pre- and post-training. In addition, males had significantly greater absolute and relative percent increases, (~95.2 kg, ~69.2%), in 1-RM leg press strength compared to females, (~47.2 kg, ~54.6%). This finding aligns with recent work conducted in young adults in which males were significantly stronger than females for absolute 1-RM leg strength before and after training; however, this effect was absent when results were expressed in relative percent terms (34). Thus, it is possible that sex-differences are more pronounced with age, as evidenced by the more distinct separation between our middle-aged adults in contrast with previous observations made in younger adults (34). The differential effects of resistance training on leg press strength gains in males and females cannot be explained by a greater hypertrophic response in males in our study. Males had significantly larger type I and II fibre CSA compared to females, but both sexes similarly increased type II fibre CSA with training. These

findings align with previous work demonstrating that type II fibres hypertrophy to a greater extent than type I fibres following resistance training in males and females of all ages (25, 34, 46–49). Furthermore, it has been shown that both young and older males and females similarly increase type II fibre CSA to a greater extent than type I fibres with training (34, 47, 48). While type I fibre hypertrophy in response to resistance training has been observed in young adults (34, 47, 48), it is absent in older adults (25, 46–50). Akin to observations reported in older adults, our participants did not experience type I fibre hypertrophy; however, the resistance training intervention induced hypertrophy in their type II fibres. Likewise, differential gains in leg press strength could not be explained by sex-based differences in fibre type responses to resistance training, as we found no significant sex differences or training effects in fibre type proportions. Similar findings have been reported by previous groups in healthy, young and older adults (25, 34, 47, 49). We speculate that the greater leg press strength gains experienced by middle-aged males in our study could be explained by differential activation of the vastus lateralis between males and females. It has been previously demonstrated that when performing leg press exercises, young females have greater muscle activation in the vastus medialis oblique compared to the vastus lateralis (51) while young males have similar activation patterns for both muscles (52). If this relationship holds in middle-aged adults, then our female participants may recruit their quadriceps muscles differently than males resulting in different strength responses.

To determine if fibre hypertrophy was mediated by myonuclear accretion differentially between males and females, we performed analyses on myonuclei and myonuclear domain. We found that males had more myonuclei in type II fibres and larger type II fibre myonuclear domain, but there were no significant training effects detected in these measures in both sexes.

Therefore, type II fibre hypertrophy occurred in absence of myonuclear addition for both males and females. These results support previous work in which young and older males had more myonuclei and greater myonuclear domains than young and older females (34, 50) and that myonuclear domain size increases in older adults with resistance training (50). In contrast, young adults increase myonuclear content in type I and II fibres without increases in myonuclear domain (34, 53). Thus, it appears that myonuclear addition is not required to support type II fibre hypertrophy in middle-aged adults. However, the lack of myonuclear addition in middle-aged adults may limit the extent to which middle-aged adults can experience fibre hypertrophy in response to resistance training.

Myonuclear addition requires MuSC activation, differentiation, and fusion (1, 9). Thus, to begin to examine the potential mechanism responsible for the lack of myonuclear addition in middle-aged adults following resistance training, we quantified MuSCs. We observed no significant sex differences for MuSCs between males and females. This finding aligns with previously reported results in MuSC content (33, 35). In response to resistance training we saw no significant changes in MuSC content in either sex which is similar to some previous observations in older adults (50); however, most studies have detected increases in MuSCs with resistance training in young and older adults (25, 28, 33–35, 49). Older adults have an attenuated MuSC response to training in which they are unable to expand their MuSC pool as effectively as young adults (25, 33). It is possible that the diminished capacity to increase MuSCs with training could be due to fibre capillarization and an aged niche. MuSCs have been shown to have a reduced proximity to fibre capillaries in aged males which impaired fibre hypertrophy (43, 54). It has been demonstrated in mice that an aged MuSC niche reduces MuSC content thus decreasing

regenerative capacity of muscle (55, 56). The exploratory nature of our work limits characterization of capillarization in middle-aged males and females; therefore, future work should be conducted to examine capillarization and the potential age impairments in MuSC expansion in middle-aged adults. However, the defective MuSC response to training in middle-aged adults may underly the lack of myonuclear accretion and may limit their hypertrophic potential.

Our study is the first to examine sex-based differences in FAPs as well as the response of FAPs to resistance training in middle-aged adults. We found that $CD90^+$ FAP content increased while $PDGFR\alpha^+$ FAP content significantly decreased with training in both sexes with no change in the $PDGFR\alpha^+/CD90^+$ population. Divergent responses between $CD90^+$ FAPs and $PDGFR\alpha^+$ FAPs is a novel finding as previous work has shown an expansion of both proliferating $CD90^+$ and $PDGFR\alpha^+$ FAPs following resistance training in humans (57). PDGFRα is a putative FAP marker in found in the skeletal muscle of both mice (58–60) and humans (61, 62). CD90 identifies a highly proliferative and progenitor-like FAP subpopulation with both adipogenic and fibrogenic potential, and CD90 is also lowly expressed in pericytes, and smooth and skeletal muscle cells in humans (7, 63)**.** Previous single cell-RNA sequencing work has identified FAP subpopulations with varying levels of *Pdgfra* and *Thy1.1* (CD90) in humans (7). Paradoxically, $CD90^+$ FAPs were previously shown to be increased in patients with Type II diabetes (7). However, in this previous work (7), bulk $CD90^+$ FAPs were evaluated without distinguishing between $CD90^+/PDGFRa^+$ and $CD90^+/PDGFRa^-$ subpopulations. Interestingly, the bulk $CD90^+$ FAPs were responsive to PDGF *in vitro* suggesting that the $PDGFRa^+/CD90^+$ subpopulation of $CD90^+$ FAPs may primarily account for the fibrotic potential of this population (7). In the present study, FAP expansion may have

occurred following resistance training to support the resistance training-induced expansion of extracellular matrix (64).

Another potential explanation for the differential responses of FAP subpopulations to resistance exercise could be their role in regulating MuSCs via paracrine factors (3, 5, 62). We observed that the change in MuSCs were inversely correlated to the change in PDGFRα$^+$ FAPs and positively correlated to the change in CD90$^+$ FAPs in males only. The positive correlation between the change in MuSCs and CD90$^+$ FAPs could indicate that they were the main stimulator of MuSC activation supporting muscular adaptations. Indeed, murine muscle-resident CD90$^+$ cells expand during muscle regeneration and gene expression analysis indicates that they express paracrine factors known to regulate satellite cells (65). Further, CD90+ FAPs are enriched for regulators of Wnt signaling (7) which is known to control MuSC differentiation (66). Future work should further evaluate the response of FAP subpopulations in response to resistance training as well as the potential for differential MuSC-FAP crosstalk between males and females.

In summary, healthy, middle-aged males and females respond similarly to 10-weeks of progressive resistance training with some differences in stem/progenitor cell responses. These findings suggests that resistance training adaptations in middle-aged adults are not different between males and females; however, the mechanisms responsible for these adaptations may be divergent. Future research should further examine sex-based differences in FAP subpopulations and their interactions with MuSCs in adults of all ages to determine if FAP signaling is impaired with age.

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Author Contributions

M.D, N.A.B, and N.A.K designed the study and provided funding. C.F.M, A.F.S, I.G.B, and R.A.A conducted the human trials and processed the tissue samples. S.A.P provided medical oversight. N.C and G.B assisted with data collection and analysis. E.R.B performed experiments, analysed data, prepared figures, and drafted the manuscript. E.R.B and M.D interpreted the results and edited the manuscript. All authors revised and approved the final version of the manuscript prior to submission.

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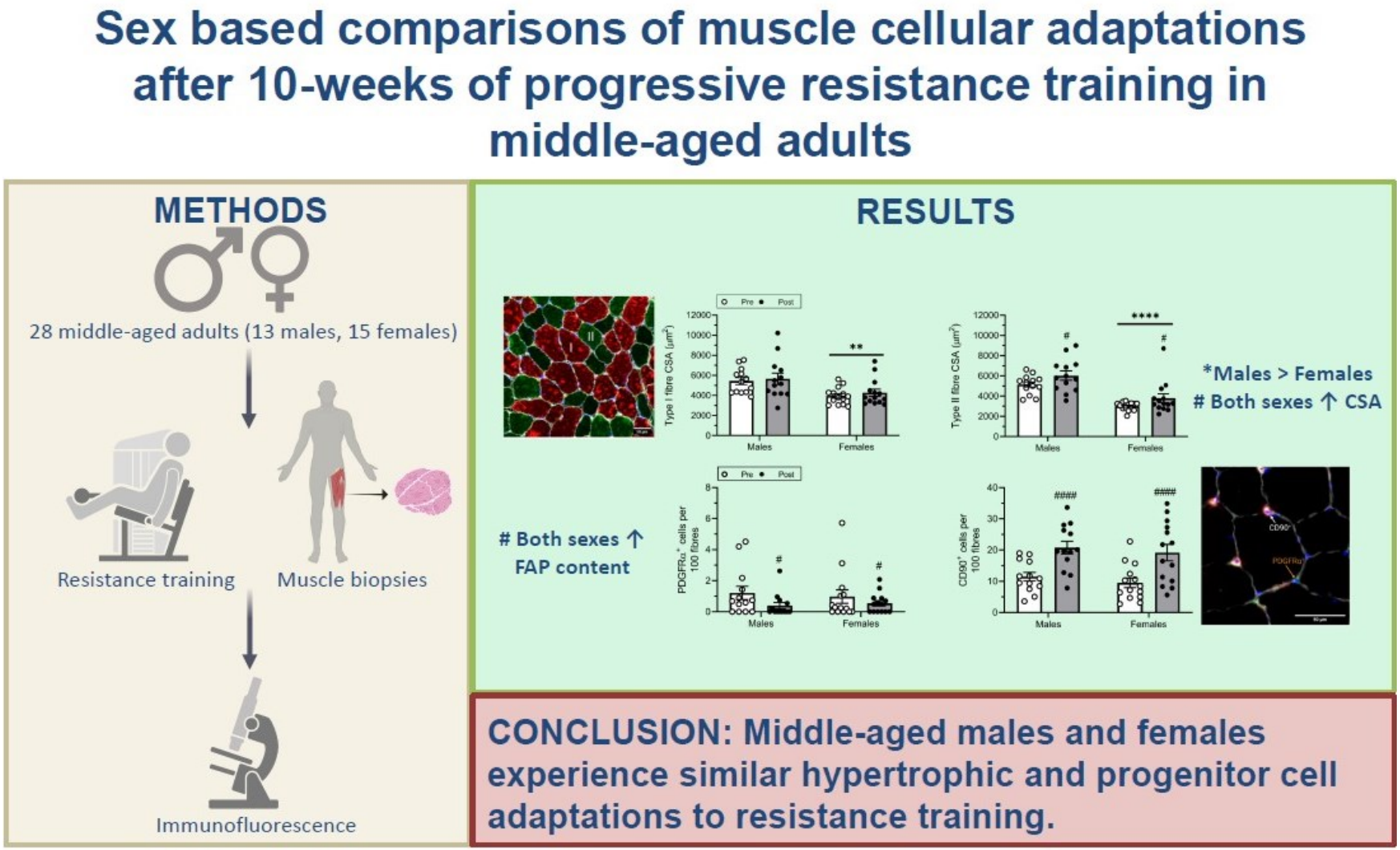
Chapter III-General Discussion

The overall purpose of this study was to investigate the potential sex-differences in resistance training-induced adaptations in middle-aged adults. No sex-differences between middle-aged males and females in the cellular adaptations to resistance training were observed. However, training increased FAP but not MuSC content. Interestingly, we found that MuSCs and FAPs were correlated in males but not females. These findings will be discussed followed by the study limitations and recommendations for future directions. A graphical abstract summarizing this study is presented in Supp. Fig. 2.

3.1. The interconnections between MuSCs and FAPs

Unlike most previous studies (1), we did not detect an increase in MuSC content in either sex in middle-aged adults. A potential explanation for this discrepancy may be that the resistance training program was insufficient to induce significant fibre hypertrophy to surpass the myonuclear domain threshold. We saw no increases in myonuclear content; thus, it is possible that the resident myonuclei were able to maintain the transcriptional capacity required for each fibre. Furthermore, it is possible that the lack of myonuclear addition could be due to a limited MuSC activation and expansion which has been previously reported in older adults (2). Although unlikely, we have to consider that our middle-aged adults had an impaired regenerative capacity that is commonly observed in older adults (3). Impaired MuSC activation with age is impacted by muscle capillarization (4) and niche thickness (5). MuSCs are closer to capillaries in young compared to older males in which activated MuSCs were found closer to capillaries while quiescent MuSCs were located further away (6). Thus, it is possible that the MuSCs in our middle-aged adults were located further away from capillaries which could have



Supplementary Figure 2. Study graphical abstract.

Illustrations created with BioRender.com.

impaired their capacity to be activated. In addition, the MuSC niche has been shown to thicken in older men which impairs MuSC activation due to limited signal transmission (5). Therefore, the niche could have been thickened in our participants; thus, limiting MuSC activation.

We next examined alterations in FAP populations in our participants. We observed that $CD90^+$ FAPs were increased; however, $PDGFR\alpha^+$ FAPs decreased following training. Fewer $PDGFR\alpha^+$ FAPs following training is opposite to what has been previously reported (7). The decline in $PDGFR\alpha^+$ FAPs may be due to factors released in the niche. FAPs are acted upon by growth factors one such being transforming growth factor beta (TGF-β) which is primarily released by macrophages (8). Specifically, TGF-β signaling has been suggested to push $PDGFR\alpha^+$ FAPs towards the fibrotic lineage over the adipocytic lineage in both mice (9) and humans (10). TGF-β signaling is thought to support muscle repair and regeneration (11, 12), and it is increased in response to resistance training (13). Therefore, it is possible that with resistance training there was increased TGF-β signaling which could have decreased $PDGFR\alpha^+$ FAPs. Furthermore, TGF-β signaling may have increased $CD90^+$ FAPs with training since they are both associated with extracellular matrix expansion (14, 15).

Moreover, there exists crosstalk between MuSCs and FAPs which is initiated by the release of paracrine factors from FAPs (8). These paracrine factors can promote the development of a pro-myogenic environment that supports MuSC expansion (8). Thus, we further investigated the relationship between these cells in response to training. In males only, we found that the change in MuSC content was inversely correlated to the change in $PDGFR\alpha^+$ FAPs and positively correlated to the change in $CD90^+$ FAPs. This positive correlation between the change in MuSCs and $CD90^+$ FAPs could indicate that they were the main stimulator of MuSC activation supporting

muscular adaptations. It is also possible that the activation of MuSCs with training caused a reduction in PDGFRα^{+} FAPs as activated MuSCs appear to inhibit FAP production as observed *in vitro* (16). It is unclear why a similar relationship was not observed in females, but it may indicate sex-based differences in MuSC-FAP communication in response to resistance training. Potential evidence in our findings to support this result, was that males had a trend towards greater MuSC content compared to females. Thus, it is possible that the greater number of MuSCs were the main driver of this correlation in males.

3.2. Study Limitations

Unfortunately, with barriers involved with human studies we were limited by the number of participants that could undergo the muscle biopsy procedure. Thus, a major limitation to this study is that there were no exercise or diet control groups; therefore, we cannot confirm that the effects observed were due to exercise or diet, and/or a combination of the two factors. It would have been ideal to have the following groups: no exercise + no diet, exercise + no diet, no exercise + diet, and exercise + diet.

Furthermore, there were several minor limitations to this study. Firstly, menstrual cycle was tracked for female participants, but not all participants had the same cycle. For instance, six-female participants were pre-menopausal, while the remainder were in menopause. If menstrual cycle were to remain consistent between participants, one would have to choose one of the following parameters: pre-menopausal, and test within the luteal or follicular phase, or post-menopausal. This limitation does not impact the interpretation of the results, since it has been shown that menstrual status does not impact skeletal muscle strength or muscle protein synthesis (17). Secondly, muscle biopsies were only taken from the lower-body. It has been

demonstrated that females have a greater capacity for relative percent increases in upper-body strength with resistance training since males typically have greater upper-body strength at baseline (18). Since our resistance training intervention included upper-body exercises, it would have beneficial to collect a biopsy from the *biceps brachii*. It would have provided additional information on if females had greater relative percent, increases in fibre CSA compared to males. Furthermore, it is possible that the male participants were performing a greater amount of work for lower-body resistance training compared to females as indicated by greater increases in lower-body strength compared to females (Table 1). This result has been previously observed in which males had a greater relative percent increase in leg-press strength compared to females(20). However, this potential limitation does not adversely impact our findings sine we did not detect any significant differences in fibre hypertrophy between the sexes. The final limitation to our study involves immunofluorescence measures. We did not examine fibre capillarization despite the fact that capillaries provide the necessary blood supply required to support MuSC mediated repair (4, 19). In addition, MuSC content was not determined for each fibre type. It is possible that MuSC content would have been altered following resistance training, specifically in type II fibres as previously reported (20–22). Ideally, we should have examined the proximity of MuSCs to capillaries to estimate regenerative capacity, as well as examined fibre-type specific MuSC content.

3.3. Research impact and future research

This study provides novel information about the resistance training-induced adaptations and lack of sex-specific responses in middle-aged adults. Our study also shows that resistance training may be used to increase CSA and strength prior to the onset of sarcopenia. Specifically,

middle-aged adults were experienced type II fibre hypertrophy with resistance training. The clinical implication of this finding is that resistance training remains an effective method to increase CSA and strength in aging adults. Therefore, whole-body resistance training may be used a method to potentially delay the onset of sarcopenia via inducing muscle hypertrophy.

Future work should examine the responses of middle-aged adults to a resistance training program along with a non-exercise control group. Specifically, the training program should implement different training intensities to determine if the lack of myonuclear addition and MuSC expansion are due to insufficient intervention difficulty or age-related impairments. In addition, measures of fibre type specific MuSC content should be conducted to determine if resistance training is inducing MuSC activation more in type I or type II fibres. MuSC proximity to capillaries should be measured to determine if MuSCs are too far away to be activated and remain in quiescence. Finally, the correlations between MuSC and FAP content should be examined further in both young and older adults to determine if they continue to be solely related in males but not females.

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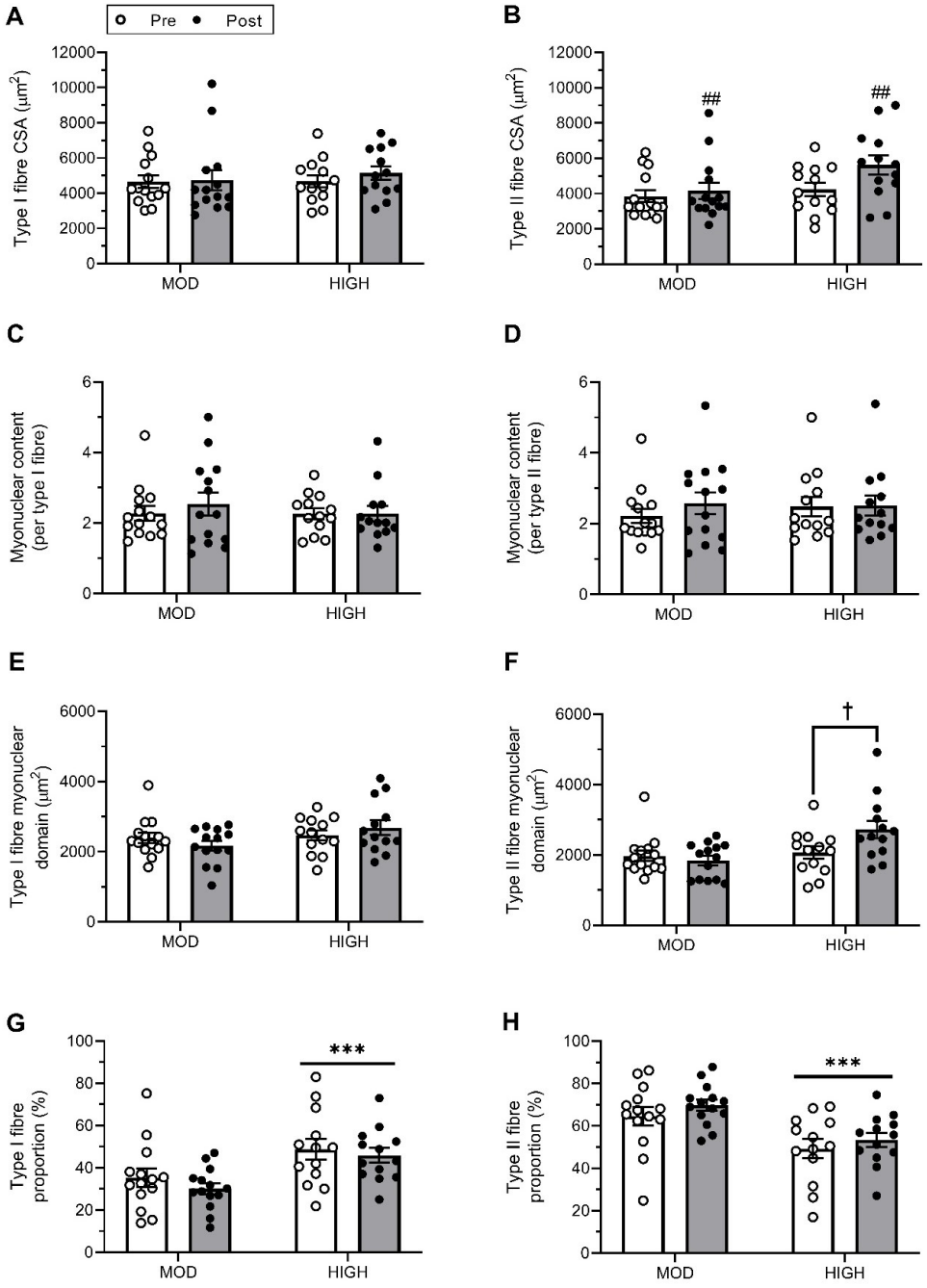
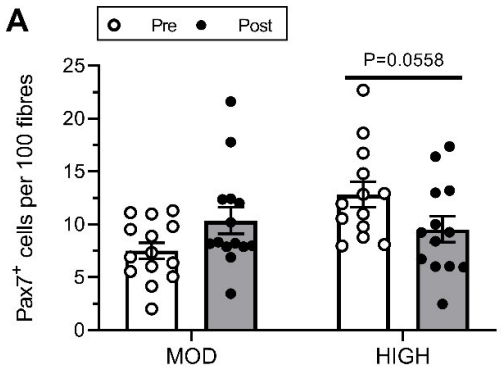
Appendix A-Supplementary Figures

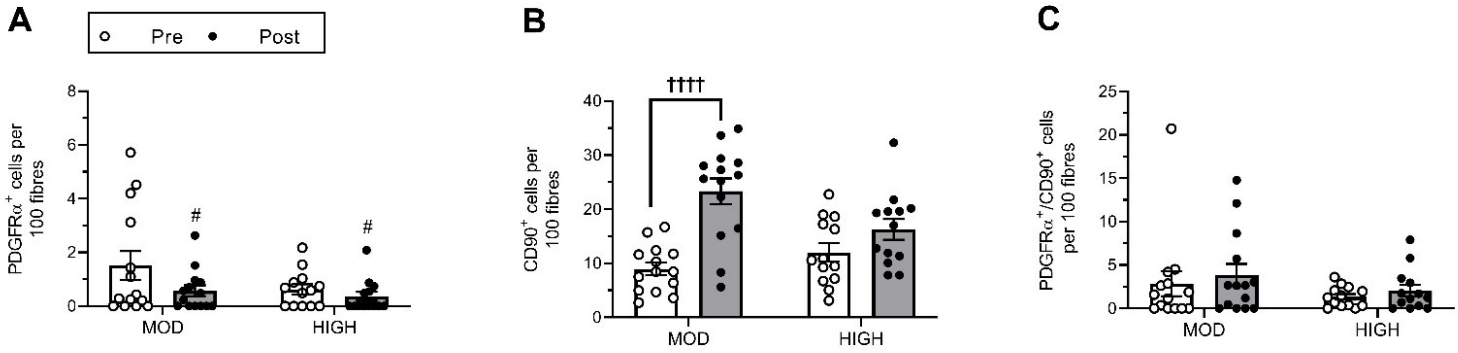
**Supplementary Figures 1 (The myogenic program pg. 13) and 2 (Study graphical abstract pg. 63) are presented within the text.*



Supplementary Figure 3. Influence of protein on fibre morphology and fibre type proportion. (A) Type I and **(B)** II fibre cross-sectional area (μm^2). **(C)** Type I and **(D)** II fibre myonuclear content. **(E)** Type I and **(F)** II fibre myonuclear domain (μm^2). **(G)** Type I and **(G)** II fibre proportions (%). **(A-H)** Data were analysed using a two-factor, repeated measures ANOVA. Data are expressed as mean ± SEM of N = 27 (14 MOD, 13 HIGH), with individual data points corresponding to a participant. Protein effect: *** P < 0.001. Time effect: ## P < 0.01. Interaction effect (protein × time): P = 0.7313 (MOD), P = 0.0360 (HIGH).



Supplementary Figure 4. Influence of protein on muscle stem cell content. (A) Total Pax7$^+$ cells per 100 fibres. Data were analysed using a two-factor, repeated measures ANOVA. Data are expressed as mean ± SEM of N = 27 (14 MOD, 13 HIGH), with individual data points corresponding to a participant. Trend for an effect of protein: P = 0.0558.



Supplementary Figure 5. Influence of protein on fibro-adipogenic progenitor cell content. (A) Total PDGFRα$^+$, **(B)** CD90$^+$, and **(C)** PDGFRα$^+$/CD90$^+$ cells per 100 fibres. **(A-C)** Data were analysed using a two-factor, repeated measures ANOVA. Data are expressed as mean ± SEM of N = 27 (14 MOD, 13 HIGH), with individual data points corresponding to a participant. Interaction effect (protein × time): P < 0.0001 (MOD), P = 0.1493 (HIGH).

Appendix B-Protocols

*Please note that each protocol has its own separate appendices and figures, so do not confuse them with the contents in the main sections of this thesis.

Cryostat Sectioning

Model: HM525 MX (Rm. 2210, 3531)
Cryostat labelled diagrams (appendices A-C).
Sectioning: -18 to -20°*C* *(no warmer or colder),* **sectioning angle=10°*, 7-10*µm** (mouse=10 µm, human=7-8 µm→smaller since we have less human samples than mouse). **Wear gloves!**
Equipment

- Forceps (min. 2 different sizes)
- Paint brushes (assorted sizes-min. 1 small (5mm), 1 large (1inch))
- OCT (optimal cutting temperature medium)
- Frosted slides (+ charged slides are best→have small + located in the corners)
- Stanley Razor blade
- New Feather S35 microtome blade→Cryostat blade
- Pencil→label slides, **do not use pen since the ink bleeds when staining**
- Gloves→nitrile preferred
- Chucks to hold sample
- Small slide box

Please see the Cryostat-Helpful Tips and Links Word Doc. for more helpful information.

Instructions for use

Setup

1. Remove all pieces and clean with 100% ethanol since you don't know if it was cleaned before you.
    a. Ensure it's 100% ethanol since if there's water it'll freeze.
2. Replace all pieces and using the menu button #1, turn the temperature down to -20°*C.*
    a. *Need to confirm selection otherwise it won't acknowledge the change. It can take between 15-30min to attain the selected temperature.*
3. *Retrieve your samples from the -80°C freezer (rm. 0510) and keep them in a dry ice box.*
4. *Select your two main section widths using buttons 2 & 3.*
    a. *Typically, 20* µm to slice OCT, and 8-10 µm for muscle.
        i. Setting two widths allows you flip between them, so that you don't need to continuously re-select in the menu.
5. Re-adjust the neck length using button #4. Try to keep between 25-28mm when starting since if it's too long the vibrations from cutting can damage your sample.
    a. *Note: as you section the neck height will decrease from 28mm since the head is moving closer to the slicing plate.*
6. Finally, you can adjust the chamber light by selecting the menu button #5 and choosing the light you prefer.
    a. *Note: if the light is too bright it could start to melt your sample and warm up the chambre.*
7. You can continuously adjust the temperature to warmer or cooler depending on how your sample is reacting to the cryostat.
    a. *Note: sticking=too warm, rolling up/cracking=too cold*

8. Adjust your sectioning angles and equipment. Place your brushes forceps in tray #12, to acclimate to temperature→if you don't it could melt your sample. Place slides and slide box on tray, #14. (Refer to appendices B and C for descriptions).
    a. Cover holes, #14 with tin foil to prevent samples from falling down holes. Add chucks, #15, to remaining holes. Add foil behind the sectioning hub since there is a large opening at the back. Add foil to the small hole beside adjustment knobs #4 & 5.
    b. Adjust sectioning angle, **10°** by toggling #9, and lining marker up to 7.
    c. Adjust platform distance and blade position using #6 (moves platform up and down), and #5 (moves blade housing left and right), respectively.
    d. Loosen #4, and slide cryostat blade into the holder. Immediately tighten and place gold cover, #7, over blade to protect yourself. **KEEP BLADE COVER ON AT ALL TIMES WHEN YOU AREN'T SECTIONING. Add Feather S35 microtome blade last.**
    e. You are ready to prep the sample.

<u>Sample Preparation in OCT</u>

Purpose: When preparing a sample to be sectioned it needs to be covered in OCT (optimal cutting temperature) medium.

A) Making the box

1. Grab the small blue, foam template square from the casserole, cryostat dish. Located on the bottom shelf, to the left of the fumehood.
2. Select a foam heart from the drawer where the lab keys are stored.
3. Use the foam template to cut a small square out of the foam.
4. Use the *Duramax tape*, found beside the foam hearts to create a border around the square. This makes a form for you to add OCT to your sample.

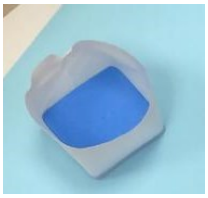


Fig. 1: Sample box

B) Preparing the sample

Although the tissue is fixed in frozen OCT, need to cover it with more OCT to ensure it's secure prior to sectioning.

Do not wait for sample to acclimatize before performing these steps. You want to ensure that the section is still frozen since it's only covered by a thin layer of OCT

1. Remove your muscle sample from the Eppendorf/original storage tube.
2. Pour OCT into the bottom of the form.
3. Place your entire sample parallel to the box, so it is laying down in the OCT in the foam box form you constructed. ***Remember the orientation of the sample, so you know how to place it on the chuck.***
    - *<u>Ex:</u> Draw a directional arrow on the tape and transfer this arrow to the OCT when you remove the tape.*
4. Cover the remainder of the sample with OCT.
5. Wait until the OCT is frozen prior to sectioning. Appx. 20-30min is sufficient to freeze the OCT.

Bring another sample to section while waiting for the prepared section to freeze, to maximize your time on the equipment.

Sample Preparation Following OCT

1. Remove sample from dry ice in foam box, and place inside cryostat. Unwrap your samples, and a*llow your samples to rest in the cryostat at -20°C for 10min prior to mounting to the chuck.*
    a. *This step is critical since it allows the samples time to acclimatize to the temperature. If you cut your samples when they're too cold it can shatter them.*
2. Take bottle of OCT at room temperature and place a small drop on chuck, #15, and grab prepared muscle sample with forceps and position on OCT in the centre of chuck. Wait appx. 5-10min or until OCT is fully frozen before mounting.
    a. If the sample is not prepared/wrapped in tin foil and it's found in an Eppendorf tube refer to the instructions below-pg. 3).
3. Take chuck with sample and mount onto hole, #3. Ensure that #2 and #1 are tight and adjusted prior to sectioning.
    a. *Note: if not secured, the sample could fall off and be damaged.*
4. You are now ready to section.

Sectioning

1. Remove gold cover off of blade, #7. Select **20µm**, button # 2 (appendix A), to cut through OCT. Ensure that the cutting angle is optimal by ensuring that the blade is cutting in the centre and flat. Continuously adjust until cutting efficiently.
    a. Ensure to adjust anti-roll, 8, during OCT slicing since you don't want to waste your sample adjusting.
    b. Throughout sectioning brush off OCT from sample and platform using brushes.
2. Once you start to see the muscle (pink) switch to **8µm**, button #2, take your practice slide and mount sample. Warm up the slide in your hand first by placing your palm on the opposite side (don't do it on the surface that you will be putting the sample) You need to warm up the slide; otherwise, the sample won't stick. Once sample is stuck to the slide, you can visualize under the microscope (start at 4X, and focus using the knobs near the back of the microscope (large=coarse and small=fine focus) Continue sectioning until you get a slice with only the cross section of muscle fibres→no longitudinal fibres.
    a. When sectioning you can either use a paintbrush brush or the anti-roll. **(I prefer the paint brush for larger samples=GA, and the anti-roll for smaller samples=TA).**
3. Once you're satisfied with your sectioning angle and sample orientation, cut a section and place it on your final slide. **DO NOT REMOVE THIS SLIDE FROM THE CRYOSTAT TO VISUALIZE.**
4. Continue steps 1-4 until you're done sectioning.

Cleanup

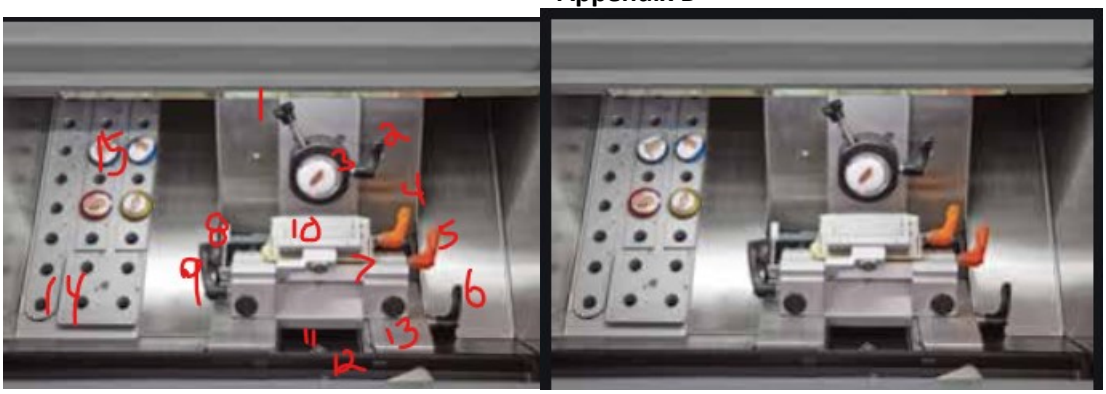
1. Once completed remove chuck from mount, #3, by loosening handles #1, and #2.
2. Replace chuck in tray, #14, and place a drop of OCT overtop of muscle to protect it. Let sit for 5-10min, or until OCT is frozen.
3. Once sample is frozen, remove from chuck using a small razor blade. Once removed replace in tin foil and put back in dry ice.
4. Place practice slides in biohazard sharps and final slides into slide box and place in dry ice. This is the only time you should remove this slide to transfer. **Do not visualize.** Remove your sectioning blade, #4, and all equipment to clean.
5. Remove all pieces of equipment and clean with 100% ethanol. Also remove all the OCT shavings from the cryostat and clean with 100% enthanol.
6. **Return cryostat neck, button #4 to original position (28mm), temperature: button #1 to -12°*C, and light to 0%.***
7. **Return your samples to -80°*C freezer, and place slides in slide box.***

Additional Notes:

- *Use the sectioning blade from left to right to get even wear, and to maximize use.*
- *If you need to section a sample the following day mark the orientation with a sharpie, so you can continue with the same orientation the following day.*
- *If using the anti-roll, ensure it is slightly over the blade. Ensure the glass is flat on the left and right sides, so it can capture the sample.* ***Do not slam the anti-roll plate over since it will chip the glass and limit the tool's effectiveness. The chips in the glass can also tear your section.***

Appendix A

Appendix B



Appendix C

General Cryostat Setup

Section preparation for staining

1. Allow sections to thaw and air-dry (15-30min).
2. Box off sections with the Vector immunopen (H-4000) and let dry (15-30min).
    a. *Be gentle when pressing the liquid out of the pen to prevent leakage of the solution onto the sample.*
3. Choose 1'AB and 2'AB sections.
4. Prepare a humid box (Fig. 1) for your samples.
    a. Line the bottom of a slide box with paper towel dampened with ddH_2O, to keep a moist environment to prevent slide dehydration. *Make sure the slides are placed across the top of the box, and not in the slide holders. They must be placed in this orientation to ensure that the liquid doesn't leak off of the slide.*
5. Proceed to fixation and staining.

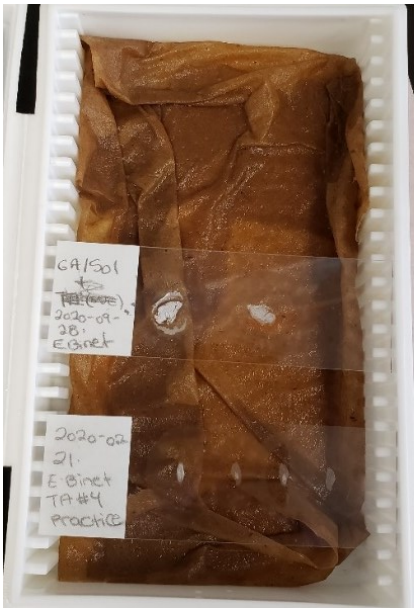


Figure 1. Humid box for staining.

Section preservation

1. Add a thin line of fluoromount (F4680) along the bottom of the slide and place a coverslip.
    a. *Be careful adding the coverslip to non-fixed sections since they are extremely fragile. Make sure the bottom of the coverslip is in contact with the entire line of the paramount to create a seal and prevent bubble formation.*
    b. *If bubbles are formed during the mounting process do not press them out or move the coverslip since it will damage the section. If step 1a is done correctly there should be limited bubble formation, so it will not cover the sections.*
    c. *Don't let the sections dry during the mounting process. Keep hydrated. Best to dump off the slide and dry any excess liquid around the sections using a kimwipe.*
2. Wait for the fluoromount to dry (15-30min) before adding nail polish around the edge of the slide and coverslip to create a seal.
    a. *Keep the slides in a cool, dry place during the setting process. The fluoromount will not create a seal in an extremely humid environment. It is best to move the slides to a dry slide box after adding the fluoromount.*
    b. *Optional step: Circle the sections on the back of the slide with sharpie to make it easier to locate the sections for imaging. Mark the + and – sections.*
3. Store the slides in the dark at (≈4°C) until imaging-best to let the slides sit O/N. Let slides sit for at least 1-2hr. prior to imaging. Leaving O/N is ideal to allow for the fluorophores to reach peak emission.

Store the slides long-term at 4°C in the dark.

Human fibre type (MHC/Laminin) immunofluorescence protocol

Block Type	Block Mixture
Initial	5% BSA, 1xPBS
Laminin	5% G/S, 1xPBS
BA-D5, SC-71, AF594	1% BSA, 1xPBS
AF488, AF647	0.5% BSA, 5% G/S in 1xPBS

1'AB	Dilution
BA-D5 (IgG-2b) Mouse anti-myosin Heavy Chain (MHC) Type 1, DSHB **[18ug/mL]**[a]	[1:100] / [0.18ug/mL] in 1% BSA, 1xPBS
SC-71 (IgG1) Mouse anti-myosin Heavy Chain Type IIa, DSHB **[20ug/uL]**[b]	[1:100] / [0.2ug/mL] in 1% BSA, 1xPBS
Laminin β-2/ γ-1 Monoclonal (A5), rat anti-laminin **(MA1-06100)** (IgG 2a), ThermoFisher Scientific **[1mg/mL]**	[1:200] /[5ug/mL] in 5% G/S, 1xPBS

[a] BA-D5 initial [36ug/mL] and [b] SC-71 initial [40ug/mL], aliquoted in 1:1 sterile glycerol.

2'AB	Dilution
Alexa Fluor 594-goat, anti-rat, IgG **(A-11007)**, Invitrogen **[2mg/mL]** →Laminin	[1:300] / [6.67ug/mL] 1% BSA, 1xPBS
Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG, Fcγ subclass 1 specific **(115-545-205)**, Jackson Immunoresearch lab **[0.8mg/mL]** → SC-71 (MHC IIa)	[1:100] / [8ug/mL] 0.5% BSA, 5% G/S, 1xPBS
Alexa Fluor 647 AffiniPure Goat Anti-Mouse IgG, Fcγ subclass 2b specific **(115-605-207)**, Jackson Immunoresearch lab **[0.85mg/mL]** → BA-D5 (MHC I)	[1:100] / [8.5ug/mL] 0.5% BSA, 5% G/S, 1xPBS

[c] AF488 initial [1.6mg/mL] and [d] AF647 initial [1.7mg/mL], aliquoted in 1:1 sterile glycerol.

Procedure	Directions
Day #1	
Section preparation	- Thaw sections. No fixation required for this stain. - **See "Section Preparation" protocol for further instructions.**
Block	- Add 5% BSA in 1xPBS and incubate 1hr. at RT (≈21°C).
Primary antibody #1	- Add laminin (MA1-06100) [1:200] in 5% G/S in 1xPBS to 1'AB sections. • *Add 5% G/S only to the 2'AB sections.* - Incubate O/N at 4°C (16-20hrs=ideal, 24hrs. max.) in the cold rm. 2115b.
Day #2	
Wash	- Remove from the cold room and wash in 1 x PBS, 3 x 5 min.
Secondary antibody #1 **In Dark**	- Add AF594 anti-rat (A-11007) [1:300] in 1% BSA, 1 x PBS - Incubate for 1hr. at RT (≈21°C). • *Add mixture to both the 1'AB and 2'AB sections.* • *Crucial from this step onwards to be in the dark since the 2'AB are light sensitive.*
Wash **In Dark**	- Wash in 1 x PBS, 3 x 5 min.
Primary antibodies #2 & 3 **In Dark**	- Add BA-D5 [1:100] and SC-71 [1:100] in 1% BSA in 1xPBS. - Incubate in the non-sterile incubator in the Burelle lab for 45min at 37°C. • *Add 1% BSA in 1xPBS only to the 2'AB sections.*
Wash **In Dark**	- Wash in 1 x PBS, 3 x 5 min.
Secondary antibodies #2 & 3 **In Dark**	- Add AF488-IgG1 (115-545-205) [1:100] and AF647-IgG-2b (115-605-207) [1:100] in 0.5% BSA, 5% G/S in 1xPBS. - Incubate for 1hr. at RT (≈21°C). • *Add mixture to both sections.*
Wash **In Dark**	- Wash in 1 x PBS, 3 x 5 min.
DAPI **In Dark**	- Add DAPI [1:20,000], 1x5min.
Wash **In Dark**	- Wash in 1 x PBS, 3 x 2 min.
Slide preservation	- Add a coverslip and seal with nail polish. - *See the "Slide Preservation," protocol for further coverslipping details.*

Human MuSC (Pax7/Laminin) immunofluorescence protocol

Block Type	Block Mixture
Initial block before Pax7	5% FBS, 5% G/S, 0.2% Triton x-100, 0.1%-NaAzide in 1xPBS
1'AB-Pax7	Dilute in 1xPBS
1'AB-LAM	5% GS, 0.2% triton x-100 in 1xPBS
2'AB-AF488	5% GS, in 95% 1xPBS

Fixation Type	Fixation Mixture
Initial	4% PFA

1'AB	Dilution
Pax7-DSHB-IgG1 **[25ug/mL]**[a]	[2ug/mL] or appx. [1:12.5]→If initial [25ug/mL] in 1xPBS.
Laminin β-2/ γ-1 Monoclonal (A5), rat anti-laminin **(MA1-06100)** (IgG 2a), ThermoFisher Scientific **[1mg/mL]**	[1:200] /[5ug/mL] in 5% G/S, 1xPBS

[a]The Pax7 supernatant concentration can differ depending on the batch. The recommended dilution is based on an initial concentration of [25ug/mL].

2'AB	Dilution
AF488-goat, anti-mouse, IgG1, **(CAT#: A-21121)** Invitrogen **[2mg/mL]**→Pax7	[1:500] / [4.0ug/mL] in 5% G/S in 1xPBS
AF594-goat, anti-rat, IgG **(CAT#: A-11007),** Invitrogen **[2mg/mL]** →Laminin	[1:300] / [6.67ug/mL] in 5% G/S in 1xPBS

Washing solution: 1xTBST [0.2% Tween 20]

**PFA is made in-house.*

Procedure	Directions
Day #1	
Section preparation	- Thaw sections and add immunopen. - **See "Section Preparation" protocol for further instructions.**
Fixation	- Add drops of 4% PFA to the sections and fix for 5min at RT.
Wash & Immunopen	- Wash 1xTBST-1x2min. • *Do an initial wash immediately after fixation to stop the action of PFA.* - Box off sections with Vector immunopen (H-4000) and let dry (10-15min). - Wash 1xTBST-2x2min.
Permeabilization	- Add 0.2% triton x-100 in 1xPBS for 10min at RT.
Wash	- Wash in 1xTBST – 3 x 2 min
Block	- Add the Pax7 block for 1hr. at RT (≈21°C) • *Pax7 block=5% FBS, 5% G/S, 0.2% Triton x-100, 0.1 NaAzide in 1 x PBS*
Wash	Wash in 1xTBST – 1 x 5 min
Primary antibody #1	- Add Pax7 [2ug/mL] in 1xPBS and incubate O/N at at 4°C (16-20hrs=ideal, 24hrs. max) in the cold room 2115b. ***18hrs. incubation** • *Only add Pax7 to the 1'AB slides. Add 1xPBS to the 2'AB slide.*
Day #2	
Wash	- Remove from the cold room and wash in 1 x TBST-3 x 5 min
Secondary antibody #1 **In Dark**	- Add AF488 goat, anti-mouse, IgG1 (A-21121) [1:500] in 5% GS, in 95% 1 x PBS. - Incubate for 2hr. at RT in the dark. • *Add the mixture to both the 1'AB and 2'AB sections.* • *Crucial from this step onwards to be in the dark since the 2'AB are light sensitive.*
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
Primary antibody #2**In Dark**	- Add laminin-rat (MA1-06100) [1:200] in 5% G/S, 0.2% triton x-100 in 1xPBS. - Incubate for 2hr. at RT in the dark. • *Only add laminin to the 1'AB slides. Add block to the 2'AB sections.*
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
Secondary antibody #2 **In Dark**	- Add AF594 goat, anti-rat, IgG (A-11007) [1:300] in 5% GS, in 95% 1 x PBS. - Incubate for 1hr. at RT in the dark. • *Add the mixture to both the 1'AB and 2'AB sections.*
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
DAPI **In Dark**	- Add DAPI [1:20,000]– 1 x 5 min
Wash **In Dark**	- Wash in 1xTBST 1 x 5 min
Slide preservation	- Add a coverslip and seal with nail polish. - *See the "Slide Preservation," protocol for further coverslipping details.*

Human FAP (PDGFRa/CD90/Laminin) immunofluorescence protocol

Block Type	**Block Mixture**
Initial block before PDGFRa	DAKO [NEAT] (X0909)
1'AB-PDGFRa, CD90 & LAM	5% GS, 0.2% triton x-100 in 1xPBS
2'AB-AF488, AF594 & AF647	5% GS, in 95% 1xPBS

Fixation Type	**Fixation Mixture**
Initial	4% PFA
After PDGFRa/AF488	2% PFA

1'AB	**Dilution**
Rabbit, PDGFRa-Anti-human/mouse **(3174S)**, IgG, **[23ug/mL]**	[1:00]/[0.23ug/mL] in 5% G/S, 0.2% triton x-100 in 1xPBS
Mouse, CD90 (Thy-1) Monoclonal (eBio5E10) **(14-0909-80)**, IgG1, **[500ug/mL]**	[1:100]/[5ug/mL] in 5% G/S, 0.2% triton x-100 in 1xPBS
Laminin β-2/ γ-1 Monoclonal (A5), rat anti-laminin **(MA1-06100)** (IgG 2a), ThermoFisher Scientific **[1mg/mL]**	[1:200] /[5ug/mL] in 5% G/S, 1xPBS

2'AB	**Dilution**
AF488-goat, anti-rabbit, IgG, **(CAT#: A-11034)** Invitrogen **[2mg/mL]**→PDGFRa	[1:100] / [20ug/mL] in 5% G/S in 1xPBS
AF594-goat, anti-rat, IgG **(CAT#: A-11007),** Invitrogen **[2mg/mL]** →Laminin	[1:300] / [6.67ug/mL] 5% G/S, 1xPBS
AF647-goat, anti-mouse, IgG1 **(CAT#: A-21240),** Invitrogen **[2mg/mL]** →CD90	[1:100] / [20ug/mL] in 5% G/S in 1xPBS

Washing solution: 1xTBST [0.2% Tween 20]

**PFA is made in-house.*

Procedure	Directions
Day #1	
Section preparation	- Thaw sections. **See "Section Preparation" protocol for further instructions.**
Fixation #1	- Add drops of 4% PFA to the sections and fix for 5min at RT.
Wash & Immunopen	- Wash 1xTBST-1x2min. *Do an initial wash immediately after fixation to stop the action of PFA.* - Box off sections with Vector immunopen (H-4000) and let dry (10-15min). - Wash 1xTBST-2x2min.
Permeabilization	- Add 0.2% triton x-100 in 1xPBS for 10min at RT.
Wash	- Wash in 1xTBST – 3 x 2 min
Block	- Add the DAKO block (X0909) for 1hr. at RT (≈21°C)
Wash	- Wash in 1xTBST – 1 x 5 min
Primary antibody #1	- Add PDGFRa, rabbit (3174S), IgG [1:100] in 5% G/S, 0.2% triton x-100 in 1xPBS and incubate O/N at at 4°C (16-20hrs=ideal, 24hrs. max) in the cold room 2115b. ***16hrs. 30min incubation** • *Only add PDGFRa o the 1'AB slides. Add the block to the 2'AB slide.*
Day #2	
Wash	- Remove from the cold room and wash in 1 x TBST-3 x 5 min
Secondary antibody #1 **In Dark**	- Add AF488 goat, anti-rabbit, IgG (A-11034) [1:100] in 5% GS, in 95% 1 x PBS. - Incubate for 2hr. at RT in the dark. • *Add the mixture to both the 1'AB and 2'AB sections.* • *Crucial from this step onwards to be in the dark since the 2'AB are light sensitive.*
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
Fixation #2 **In Dark**	- Add drops of 2% PFA to the sections and fix for 5min at RT.
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
Block	- Add 5% G/S, 5% FBS, 0.2% triton x-100, 0.1% NaAzide in 1xPBS for 1hr. at RT (≈21°C)
Wash **In Dark**	- Wash in 1xTBST-1 x 5 min
Primary antibody #2	- Add CD90 (14-0909-80), [1:100] in 5% G/S, 0.2% triton x-100 in 1xPBS and incubate O/N at at 4°C (16-20hrs=ideal, 24hrs. max) in the cold room 2115b. ***17hrs. incubation** - *Only add CD90 to the 1'AB slides. Add the block to the 2'AB slide.*
Day #3	
Wash **In Dark**	- Remove from the cold room and wash in 1 x TBST-3 x 5 min
Secondary antibody #2 **In Dark**	- Add AF647 goat, anti-mouse, IgG1 (A-21240) [1:100] in 5% GS, in 95% 1 x PBS. - Incubate for 2hr. at RT in the dark. • *Add the mixture to both the 1'AB and 2'AB sections.*
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
Primary antibody #3 **In Dark**	- Add laminin-rat (MA1-06100) [1:200] in 5% G/S, 0.2% triton x-100 in 1xPBS. - Incubate for 2hr. at RT in the dark. - *Only add laminin to the 1'AB slides. Add block to the 2'AB sections.*
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
Secondary antibody #3 **In Dark**	- Add AF594 goat, anti-rat, IgG (A-11007) [1:300] in 5% GS, in 95% 1 x PBS. - Incubate for 1hr. at RT in the dark. - *Add the mixture to both the 1'AB and 2'AB sections.*
Wash **In Dark**	- Wash in 1xTBST-3 x 5 min
DAPI **In Dark**	- Add DAPI [1:20,000]– 1 x 5 min
Wash **In Dark**	- Wash in 1xTBST 1 x 5 min
Slide preservation	- Add a coverslip and seal with nail polish. *See the "Slide Preservation," protocol for further coverslipping details.*

Image Acquisition

A) <u>Imaging on the Cell Discoverer 7 Microscope</u>

1. Turn on the computer and wait until fully on before turning on the microscope using the small button below the touch screen.
    - *If the microscope turns on before the computer, it will not function correctly, and you will need to re-start both systems.*
    - *Make sure that all commands originate from the computer software and not the touchpad on the microscope. If you flip between the two methods, the software gets confused, and the microscope stops responding.*
2. Wait until the microscope if fully on before opening the imaging application **(ZEN Blue v.2.3).**
3. Once the app is open, you can eject the sample tray and select the sample carrier: 3x slides.
4. Place the slides face down into the carrier and place them back into the microscope.
5. Close the tray and allow the microscope to automatically detect the number of slides in the sample carrier.
6. Select the sample carrier tab to view the location of the samples. To switch between slides, select the navigation pane.
7. Before imaging the light source and filters must be defined.
    - *Select the widefield tab and select the fluorophores used for imaging. In this case I am using: DAPI, AF488, AF594, and AF647. The microscope will automatically select the filters that match these fluorophores.*
    - *Select the fluorescence light source.*
8. Once the area of interest is selected, select the live tab to view your sample.
9. Select control + scroll the mouse to focus the sample. Ensure to progress from a small to large objective.
    - *In this case I am using 5x, so I will select 5x, 0.35 apochromat with a 1x magnification changer.*
10. Once the sample is in focus, you need to set the exposure times for each fluorophore of interest using the acquisition tab.
    - *You can select automatic exposure, but it sometimes over-exposes the image. To avoid this problem, you need to look at the histogram displayed below the image for fine adjustments.*
11. Once the exposures are set, you can take the image. The two methods are a snapshot, which takes an image of the field of view (FOV), or a tiled image→takes multiple images over a specific area and combines these tiles to make a full image.
    - *If the entire sample fits within the FOV you can take a snapshot.*
    - *If the sample is larger than the FOV, you must specify the area that needs to be tiled.*
12. Select the tiles section of the acquisition tab to define the tile size and area.
13. Once these parameters are set you can start the experiment to take the picture.
14. If a tiled image is taken, you need to stitch the image and export in the desired format (CZI or TIFF are most common). The images can be exported under the processing tab.

**For a more depth explanation please view the following handbook written by Jessica Rowley, HTSCA facility, Imperial College, London.* https://www.imperial.ac.uk/media/imperial-college/medicine/facilities/film/Cell-Discoverer_Handbook.pdf

B) <u>Imaging on the Zeiss M2 Axioimager Microscope</u>

This microscope belongs to the CBIA Core so treat it with care.

This microscope was chosen for the remaining analyses: MuSCs, FAPs, and capillaries since it had a specific green filter that did not capture the autofluorescence in each sample. Although the camera resolution was poor, it provided a more accurate representation of the fluorescence staining compared to the CellDiscoverer.

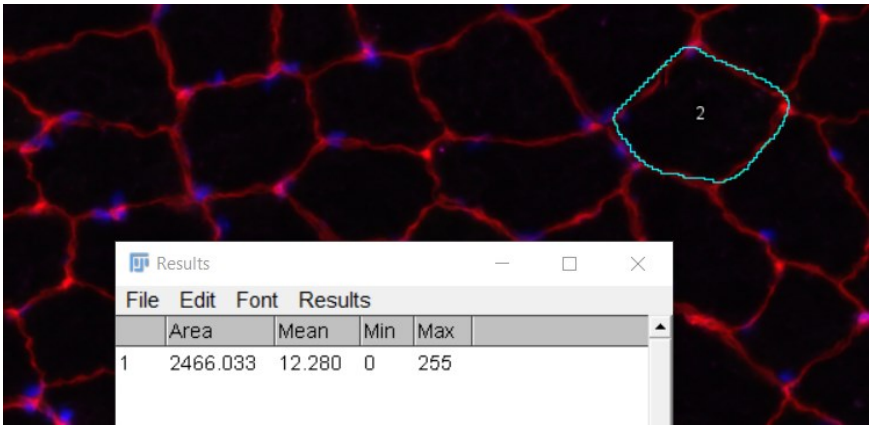
1. Turn on the following equipment in the same order as listed: lamp, battery source, microscope, and computer.
2. Wait until the microscope is fully on before turning on the computer.
3. Wait until the microscope if fully on before opening the imaging application **(ZEN Blue v.2.3).**
    - *This is an upright microscope with automatic capabilities.*
    - *I preferred to use manual commands for the gross focus, and automatic controls for the fine focus.*
4. This microscope allows only 1 slide to be observed at a time and it is placed face down.
5. The following steps to acquire the image are the same as the CellDiscoverer protocol since it uses the same software.
6. Place the slides face down into the carrier and place them back into the microscope.
7. Before imaging the light source and filters must be defined.
    - *Select the widefield tab and select the fluorophores used for imaging. In this case I am using: DAPI, AF488, AF594, and AF647. The microscope will automatically select the filters that match these fluorophores.*
    - *Select the fluorescence light source.*
    - *This microscope uses two cameras: for fluorescence imaging we require the monochrome camera.*
8. Once the area of interest is selected, select the live tab to view your sample.
9. Select control + scroll the mouse to focus the sample.
10. Once the sample is in focus, you need to set the exposure times for each fluorophore of interest using the acquisition tab.
    - *You can select automatic exposure, but it sometimes over-exposes the image. To avoid this problem, you need to look at the histogram displayed below the image for fine adjustments.*
11. Once the exposures are set, you can take the image. The two methods are a snapshot, which takes an image of the field of view (FOV), or a tiled image→takes multiple images over a specific area and combines these tiles to make a full image.
    - *If the entire sample fits within the FOV you can take a snapshot.*
    - *If the sample is larger than the FOV, you must specify the area that needs to be tiled.*
12. Select the tiles section of the acquisition tab to define the tile size and area.
13. Once these parameters are set you can start the experiment to take the picture.
14. If a tiled image is taken, you need to stitch the image and export in the desired format (CZI or TIFF are most common). The images can be exported under the processing tab.

<u>Note:</u> This is an excellent teaching microscope since it allows understanding the light paths required for visualization.

Image Analyses

A) Measuring CSA

1. Open up TIF file. Can open through Image J or drag the image into the program to open it.
2. Select which channel you want to be looking at. Files may not always be merged. Can split or merge channels. You need to know which colour represents what.
    a. *Image →Colour →Merge or split (Select which channels you want based on the colour); or **shift + Z to open channels.***
    b. Adjusting merged image:
        i. *Image →colour →channels tool*
        ii. *Image →adjust →brightness/contrast →drop down, pick colour, and adjust each channel individually.*
        iii. **You need to adjust the colour and brightness/contrast, since sometimes the image appears black even though there's stains present. Select *colour and channel →cannot be composite since won't individually change colours.***
        iv. Make sure you know which channel corresponds to which staining colour.
3. Determine the scale based on microscope imaging parameters. The image should say the distance per pixel in the image.
    a. Click analyze →set scale →Click global if you want the same measurements for other pictures.
4. Measure CSA based on the laminin. Once selected save ROI and measure CSA.
    *a. Analyze →Measure or **Click-m. To measure everything at one select cntrl-A to select everything in the ROI manager and click measure.***
    b. Take values and copy into an excel doc. The units are in um, since already specified the scale.



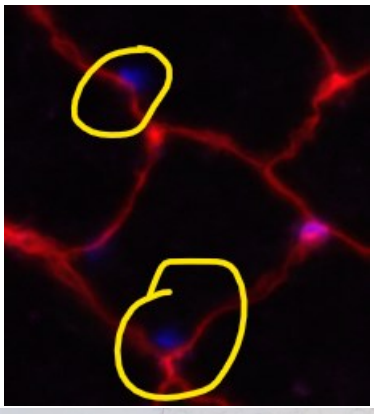
Make sure you hit measure at the beginning, so it measures the ROIs as you go, otherwise you can to go individually select each ROI and hit measure.

5. To adjust measurements, click on *analyze →set measurements.* Choose the measurements you'd like to have.

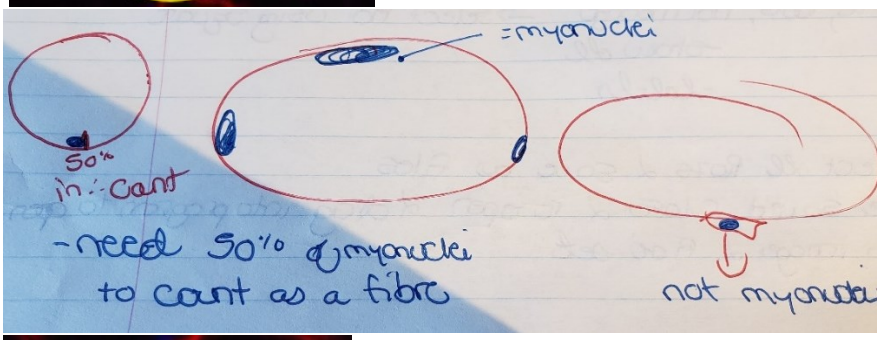
B) Measuring myonuclei

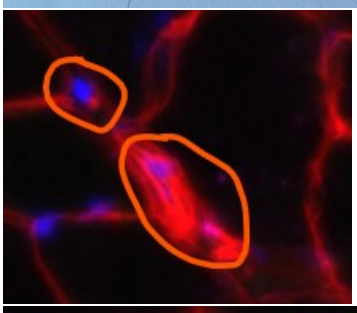
Same steps, but myonuclei in the muscle fibre appear 50% or more inside the fibre.

The myonuclei stain is not skeletal muscle fibre specific, so need to count which ones within laminin. The ones surrounded by a laminin are not skeletal muscle, thus the basal lamina cannot cover more than 50% of the myonuclei. Some fibres don't have any myonuclei.

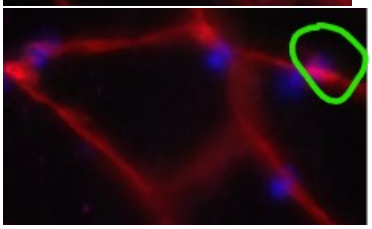


-Yellow, are considered myonuclei since not surrounded by lamina, so they're inside fibre.





-Orange are not considered myonuclei since they're surrounded by laminin.



-Green considered myonuclei since min50% inside the fibre.

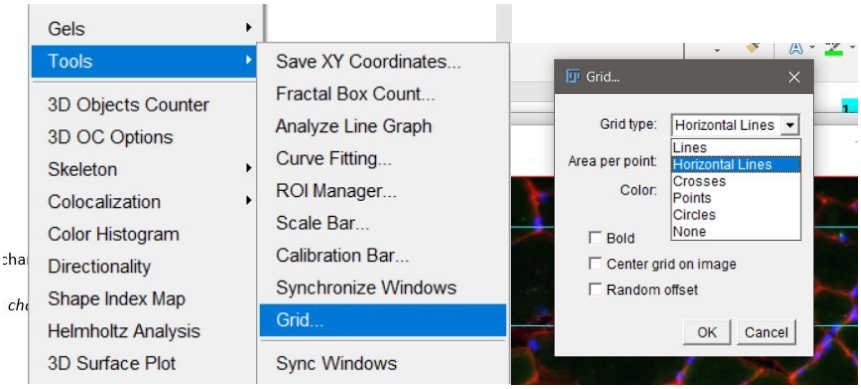
C) Measuring fibre type

1. For the fibres anaylsed for CSA determine the fibre type through the positive MHC isoform expressed.

   Ex: a positive stain for BA-D5, will have colour→green, Type I fibre.
   SC-71+ is Type IIa, therefore BA-D5- and SC-71- is a Type IIx fibre since there's no stain.

D) Measuring MuSCs and FAPs

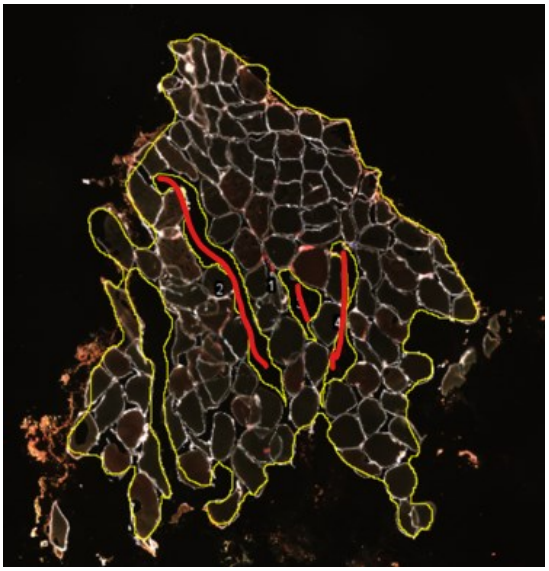
1. Open the image and select the channels tool and brightness and contrast toggle switch.
    - *You will need to use the channels tool throughout the image analysis to turn individual channels on and off.*
2. Under the *analyze* tab, select *tools →grid →horizontal lines →choose the line colour*
3. A dialogue box opens with different grid types. Select *horizontal bars* to cover the entire image.



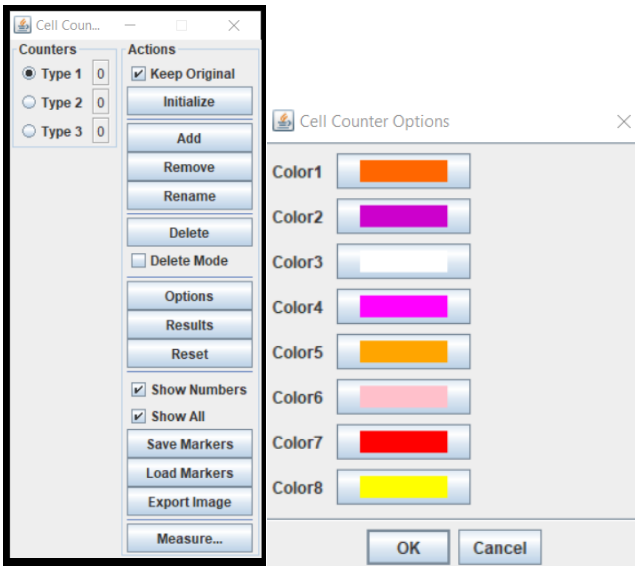
4. You can now perform your stain analyses. You count the amount of + stains per each row of the grid. Unlike myonuclei (mn)/fibre in which you must count the amount per fibre, this stain is not fibre specific, and you count it using the grid.
5. Once the image is open and the grid is added you need to open the cell counter plugin.
6. Under the *plugins* tab, select *analyze →cell counter →cell counter (listed twice, IDK why).*
7. A dialogue box opens with the cell counter plugin.
8. You need to select *initialize* to start the counter program. A separate window opens with your image
9. If you want to keep the original image open, you need to select *keep original.*
10. You can then choose a counter type and start clicking on the areas you wish to count.
11. Each counter type represents a separate thing you are counting. You can specify the counter colour by clicking *options.*
    a. Ex: Type I=fibres, type II=myonuclei, type III=satellite cells.
12. When counting move left→right and from top→bottom across the image by using the grid.
    - *Turn off the Pax7 channel when counting the fibres and myonuclei to eliminate the chances of being tempted to count fibres and myonuclei with Pax7 that probably shouldn't be counted.*
    - *If you need to delete a marker than you added a long time ago, you click on delete mode and select the marker you want to delete. If not, you can just click delete, and it will delete the most recently added point.*
13. Once you are finished counting you can save the counts by clicking *results* and save the point by clicking *save markers.* You can then save your results in a separate Excel file.
14. If you want to re-open your saved markers over an image you cannot drag the file into image J as you would do for MHC analyses. You need to repeat steps **5-9,** click *load markers*, and choose your saved marker file you wish to open over the image.

General CSA of entire area analysed

1. Circle the area you wish to quantify and add it to the ROI manager.
2. If there is an area within the centre of the muscle section you don't wish to quantify, you can circle that are, and later subtract it from the ROIs.
3. For example: The yellow areas encircle the areas of the section that were analysed, but the red areas indicate holes in the fibre that should not be quantified. Therefore, those ROIs in the ROI manager will be subtracted from the sum of the "good areas."
    a. $5000\mu m^2$-$1000\mu m^2$-$50\mu m^2$-$25\mu m^2$=$3925\mu m^2$ quantified (Arbitrary numbers chosen)



***The steps to analyse FAPs are the same as for Pax7, but instead nuclei in the interstitial space colocalize with PDGFRa, CD90, or PDGFRa/CD90.**



Cell counter options