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Functional changes to Achilles tendon and enthesis in an adolescent mouse model of testosterone hormone therapy

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Abstract

Purpose/aim: Some youth seek puberty suppression to prolong decision-making prior to starting hormone therapy to help align their physical sex characteristics with their gender identity. During peripubertal growth, connective tissues such as tendon rapidly adapt to applied mechanical loads (e.g. exercise) yet if and how tendon adaptation is influenced by sex and gender-affirming hormone therapy during growth remains unknown. The goal of this study was to understand how pubertal suppression followed by testosterone influences the structural and functional properties of the Achilles tendon using an established adolescent mouse model of testosterone hormone therapy.

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Author contributions

MLK, LH, and AS conceived and designed research; LH, MLK, TP, PC, SS, SB, NM, and CDC performed experiments; LH, SB, and SG analyzed data; LH, BH, and MLK interpreted results of experiments; LH prepared figures; LH and SG drafted the manuscript; LH, BH, SS, NM, and MLK edited the manuscript; All authors approved the final version of manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Materials and methods: C57BL/6N female mice were assigned at postnatal day 26 to the following experimental groups: control (vehicle treated), gonadotropin release hormone analogue (GnRHa) treatment alone to delay puberty, testosterone (T) alone after puberty, or delayed puberty with T treatment (i.e. GnRHa followed by T).

Results: We found that pubertal suppression using GnRHa with and without T, as well as treatment with T alone post-puberty, increased the ultimate load of tendon in female mice. Additionally, we found that GnRHa, but not T treatment resulted in a significant increase in cell density at the Achilles enthesis.

Conclusions: These findings demonstrate that delayed puberty and T have no negative influence on structural or functional properties of mouse tendon.

Keywords

Biomechanics; enthesis; estrogen; tendon; testosterone

Introduction

Sex differences have been associated with increased risk of musculoskeletal soft tissue injury, especially for patients assigned female at birth (AFAB)[1]. The risk of injury is especially elevated in AFAB athletes, who have a four- to eightfold increase in anterior cruciate ligament tears (ACL) compared to assigned male at birth athletes (AMAB)[2,3]. This increased risk is proposed to be linked to the regulation of sex hormones[1,2]. A prospective study of the rate of ACL tears in AFAB skiers showed tears were 2.4 times more frequent during the follicular and ovulatory phase of menstruation, when estrogen is peaking[2]. Additionally, sex differences in the biomechanical properties of tendon and ligament are significant in the duration where the sex hormonal expression profile is substantially different between women and men[1].

Sex hormones have been identified to regulate structure and function in many musculoskeletal tissues[4–6]. The enthesis contains a cellular gradient of tenocytes, chondrocytes as well as osteocytes which remodel their ECM[7]. While sex hormones have been shown to influence chondrocyte development and inhibit proliferation[8–10], it is currently unknown how these hormones affect the morphology and cellular density in the developing enthesis. Tendons express estrogen receptors, and estrogen plays a critical role in collagen synthesis in tendon[11]. For example, estrogen deficiency in rats following ovariectomy led to ~30% reduction in collagen content in the Achilles tendon[12]. Additionally, estrogen increases tenocyte proliferation *in vitro*[13]. Taken together, these data suggest that estrogen plays a crucial role in cellular density and healthy tendon formation. Similar effects have been observed when looking at the effects of testosterone on tendon. T testosterone (T) contributes to increased tendon stiffness in AMAB individuals by increasing collagen turnover and content[1]. Furthermore, T may indirectly reduce tendon and ligament laxity by downregulating the expression of relaxin receptors, which modulate joint elasticity[14]. While these studies demonstrate the role of sex hormones in mature tendon composition, adaptation, and injury risk, the impact of sex steroids on tendon function during pubertal growth has not yet been explored.

Transgender and gender diverse youth seek gender affirming care to align their physical sex characteristics with their gender identity, which is important and beneficial for the overall mental health and wellness of the individual[15]. One approach to gender affirming hormone therapy (GAHT) in peripubertal youth is the use of gonadotropin release hormone analogue (GnRHa) to prevent further development of the endogenous secondary sex characteristics corresponding to the individual's sex designated at birth[16]. Gonadotropin release hormone (GnRH) is released by the hypothalamus, stimulating the release of pituitary gonadotropins to activate the production of estrogen and testosterone. Inhibition of the hypothalamopituitary gonadal (HPG)-axis by GnRHa leads to hypoestrogenia in female mice and, when combined with testosterone treatment, mimics a current clinical treatment option of gender-affirming therapy in AFAB adolescents. Additionally, another approach to GAHT is the use of T alone without puberty suppression[16]. To date, the majority of GAHT research has revolved around cardiovascular, endocrine, and metabolic health[17], with a limited number of studies investigating GAHT-mediated changes in bone health[17,18]. However, connective tissues like tendon are critical for skeletal growth and mobility and are responsive to the regulation of sex hormones. Therefore, it is crucial to understand the potential effects of pubertal suppression directly followed by T treatment (peripubertal GAHT) and well as T only treatment (post-pubertal GAHT) on functional properties of connective tissues, like tendon, to inform clinical decision-making on therapeutic intervention, as well as training and injury recovery, for peripubertal transgender and gender diverse patients.

Previously, Dela Cruz et al. validated a mouse model of transmasculine gender affirming care by implanting peripubertal mice with GnRHa and T or T alone[19,20]. Mice with GnRHa implants had reduced levels of luteinizing hormone (LH) and estradiol, key drivers of female sexual development, and decreased uterine and ovarian weight[19]. Mice given T, either with or without GnRHa, had sustained elevated levels of T and suppressed LH levels compared to control and GnRHa-only groups[19]. Therefore, we utilized this mouse model to test the effects of transmasculine GAHT on musculoskeletal tissues.

We and others have recently reported the effects of testosterone on skeletal growth in which puberty suppression followed by T treatment (peripubertal GAHT) led to significantly increased trabecular bone density and skeletal muscle strength in female-born mice[21]. In this study, we aimed to identify how tendon function is influenced by GnRHa treatment. Specifically, we measured tendon structure and function in young adult female mice who were subjected to puberty suppression, peripubertal and post-pubertal GAHT by assessing changes in mechanical properties, cell density and collagen structure and alignment.

Materials and methods

Experimental design

All procedures were approved by the Institutional Animal Care and Use Committees at the University of Michigan. Mice were obtained post-euthanasia from a collaborative study by Dela Cruz et al. following tissue harvests for *in vitro* fertilization (IVF)[19]. C57BL/6N female mice ($n = 26$) and age-matched male mice ($n = 6$) were used in this study. Female mice were assigned to one of the four experimental groups to mimic GAHT in human

adolescents: Sham only (Sham + Sham, sham surgery with empty silastic tubing implants throughout the duration of the experiment; $n = 7$), GnRHa only (GnRHa + Sham, for pubertal suppression only; $n = 6$), testosterone (T) only treatment (Sham + T, post-pubertal GAHT; for masculinizing transition only; $n = 7$), and GnRHa + T treatment (peripubertal GAHT; combined pubertal suppression and masculinizing transition; $n = 6$). On postnatal day 26 (P26, 1 month (M); prior to the onset of puberty in female mice), mice were either implanted with GnRHa (GnRHa + Sham, GnRHa + T; Goserelin acetate implant, 3.6 mg, ZoladexRV, Astra Zeneca, UK) or an empty implant (Sham + Sham, Sham + T). At 3 weeks post-implantation (1.5 M), mice were either subcutaneously implanted with crystalline testosterone (T, 10 mg dissolved in ethanol; Sham + T, GnRHa + T; Sigma Aldrich, Testosterone C-IIIN, Catalog number: T1500–5 G, St. Louis, MO, USA) in silastic tubing, which was previously shown to provide sustained release for extended periods of time without replacement[22], or with empty silastic tubing (Sham + Sham, GnRHa +Sham). At 3 months (3 M), mice were euthanized by cardiac puncture exsanguination under isoflurane anesthesia, weighed, and hindlimbs were dissected for either biomechanical testing and/or histology[19] (Figure 1). An additional group of naïve C57BL/6N male ($n = 4$) and female ($n = 4$) mice were euthanized at 1 M (start of GAHT treatment, prior to the onset of puberty in female mice) and at 3 M (terminal GAHT treatment time point) for RNA isolation and quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR).

Biomechanical testing of Achilles tendons

At the time of euthanasia, one hindlimb from each mouse was immediately frozen at −20 °C. Simple randomization of samples prior to Achilles tendon dissection was performed, and tester was blinded to all experimental groups. Twenty-four hours before the start of mechanical testing, limbs were thawed at 4°C and prepared for photogrammetry and biomechanical testing. Both the plantaris tendon and muscle were carefully removed and the Achilles tendons and calcaneus were left intact. Cross-sectional areas (CSA) of the Achilles tendon were measured using photogrammetry with a custom pinch clamp holder attached to a motor controller (Arduino, Ivrea, Italy). With the muscle and Achilles hanging free of the pinch clamp, at least 50 consecutive images of the pinch clamped tendon were acquired using a 12 mm focal length lens (Basler Fujinon Lens, Ahrensburg, Germany). Using Metashape software (Agisoft, St. Petersburg, Russia), images were aligned and converted first to a sparse point then to a dense point cloud, to create an STL surface mesh of the tendon. CSA was defined as the smallest area from the STL surface mesh generated from the Achilles tendon and was measured using a slice analysis tool in Dragonfly (Comet Technologies Canada Inc., Montreal, Canada).

A custom 3D printed fixture secured the calcaneus into place (FormLabs 3B, Somerville, MA, USA), and the proximal Achilles tendon was clamped in a textured grip and screwed into place (Imada, Northbrook, IL, USA). The assembled grip with the secured Achilles tendon was placed into a phosphate-buffered solution (PBS) bath at 37°C using a temperature controller (MA160, Biomomentum Inc., Laval, Quebec, Canada) and secured with a pin to a tensile testing frame with a multi-axis load cell (±70 N; Mach-1 VS500CST,

Biomomentum, Laval, Quebec, Canada). Samples were preloaded to 0.1 N, and gauge length was measured as the distance between the secured calcaneus and the textured grip.

The Achilles tendons were then preconditioned for 10 cycles (±0.1 N at 0.1 mm/sec) followed by a load-to-failure test at 0.1 mm/sec. Off—axis load (forces in X and Y, torques in X, Y, Z) were collected to assess off-axis loading for the duration of the experiment. Using the Mach-1 Analysis and a custom R script (v4.2.2 or later, The R Project for Statistical Computing, Vienna, Austria), the mechanical properties of the Achilles were calculated from force-displacement data. Maximum stress of the Achilles tendon was calculated as the maximum force divided by the CSA. Strain was calculated as the displacement at failure divided by the original gauge length. Maximum load, maximum stress, and maximum strain were calculated using R. Stiffness and linear modulus were determined from load-displacement and stress-strain data, respectively, using piecewise linear segmentation by dynamic reprogramming recursion package (dpseg) in R[23]. Data from female mice were compared using a one-way ANOVA and corrected for multiple comparisons using Tukey's multiple comparison tests (Prism v9.0+; GraphPad, LaJolla, CA, USA).

Histology preparation and imaging

At the time of euthanasia, hindlimbs from all groups (n = 3 per group) were dissected and fixed in 4% paraformaldehyde for 24 hr then decalcified in 14% ethylenediaminetetraacetic acid (EDTA) prior to paraffin embedding. Distal hindlimbs were sectioned at 5 μm in the sagittal plane, stained with Hematoxylin and Eosin (H&E) or Picrosirius Red (PSR) (Stat Labs, Catalog numbers: HXHHEPT (hematoxylin), STE0143 (eosin), SKU #: KTPSRPT (PSR) McKinney, TX, USA) and mounted with acrylic mounting media, Shandon Mount, Catalog number: 1900331 ThermoFisher, Waltham, MA, USA). H&E stained slides were imaged on a bright-field microscope (ECLIPSE Ni-U, Nikon) at 10× and analyzed using QuPath[24]. PSR stained slides were imaged with a 10× objective on an epifluorescence microscope (dmi600b, Leica) and acquired using a Kiralux polarization camera (Thorlabs camera, Model: C505MUP1; Thorlabs, Newton, NJ, USA)[25]. One section per animal was used for H&E and PSR analyses. Hindlimbs were fixed in the same anatomical position and anatomical landmarks were used to ensure samples collected were at a consistent sagittal plane location.

QuPath analysis

Cell density was calculated from H&E images using QuPath[24]. First, the enthesis and insertional tendon areas were defined using morphological features. Next, color deconvolution was performed by QuPath to digitally separate stains within each image. Positive cell detection was then performed to differentiate cells. Hematoxylin-positive nuclei were identified to determine cell number and cell density (cells/mm^2. QuPath cell density analysis was performed by two blinded reviewers, and all cell and area counts were averaged. Enthesis and insertional area and cell density from female mice were compared using one-way ANOVAs and corrected for multiple comparisons using Tukey's multiple comparison tests (Prism v9.0+; GraphPad, LaJolla, CA, USA). Cell number and area

correlation plots were analyzed using simple linear regression and Pearson's correlation tests (Prism v9.0+; GraphPad, LaJolla, CA, USA).

Quantitative polarized light imaging (qPLI) analysis

The degree of linear polarization (DoLP) and the angle of linear polarization (AoLP) images were acquired using a polarization camera (Thorlabs, Newton, New Jersey, USA) and a circular polarizing lens (Edmund Optics, Barrington, New Jersey, USA). The mean DoLP and standard deviation of the AoLP were analyzed using Math and SciPy Stats libraries in Python (v3.12.1, Python, Wilmington, Delaware, USA). Data from female mice were compared using a one-way ANOVA and corrected for multiple comparisons using Tukey's multiple comparisons tests (Prism v9.0+; Graphpad, LaJolla, CA, USA).

RNA isolation and qRT- PCR

We quantified gene expression of enthesis and tendon-specific genes: *Tnc*, *Tnmd*, *Scx*, and *Sox9* (Integrated DNA Technologies) using qRT-PCR. Achilles tendons from aged-matched female and male mice at 1 M and 3 M (n = 4/group) were immediately dissected under ribonuclease (RNase)—free conditions and snap-frozen in liquid nitrogen. Tissues were mechanically pulverized in TRIzol, and total RNA was isolated using spin-columns (PureLink RNA mini kit, Catalog number: 12183018A,Thermo Fisher Scientific) with on-column genomic DNA digestion (RNase-free DNase, Catalog number : 79254, QIAGEN). The quality and quantity of RNA were checked with a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA with a 260/280 ratio greater than 2.0 were reverse transcribed to cDNA (SuperScript IV VILO Master Mix, Catalog number: 11756050, Thermo Fisher Scientific). After cDNA conversion, 10 ng of cDNA was used per reaction. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad) with Power SYBR Green PCR Master Mix (Catalog number: 4367659, Thermo Fisher Scientific). *Rplp0* was used as the reference gene. Relative expression was calculated by $2^{-\Delta Cq}$, where ΔCq was calculated by Cq (gene of interest)—Cq (reference gene: *Rplp0*). Reported relative expression was calculated by $2^{-\Delta\Delta Cq}$ where experimental groups were compared to 1 M males. Primer information is provided in Table 1. Data ($2^{-\Delta\Delta Cq}$) were compared using two-way ANOVAs and corrected for multiple comparisons with Tukey's multiple comparison tests (Prism v9.0+; Graphpad, LaJolla, CA, USA).

Results

Achilles tendons in female mice treated with GAHT were mechanically stronger

The dosing scheme was validated as GnRHa-only and GnRHa+T groups remained in diestrus 21 days after implantation and GnRHa +T and T only treated mice had elevated T levels compared to controls with no significant change to estradiol levels (data published in Dela Cruz et al.[19]. Additionally, treatment with T only led to significantly increased body weight of female mice, Figure 2(A)) (data also published in[19]. All treatments (GnRH + Sham, Sham + T, and GnRHa+T) resulted in an increase in Achilles tendon load at failure (maximum load) compared to Sham + Sham controls (Figure 2(B)). Additionally, treatment with T (Sham + T, GnRHa +T) increased variation in stiffness (Figure 2(C)). We also found

that only treatments with GnRHa, with and without T (i.e., GnRHa + Sham, GnRHa + T) had significantly increased tendon stress at failure (maximum stress, Figure 2(E)). Finally, we did not identify any significant differences between groups related to CSA (Figure 2(D)) or linear modulus (Figure 2(F)).

Female mice treated with GnRHa had increased cellular density in the enthesis but no change to collagen organization

GnRHa treatment alone (GnRHa + Sham) led to significantly increased enthesis cell number and density compared to all other groups (Figure 3(A,B)). We found no significant differences between enthesis area between all groups (Figure 3(C)). We also found that there was not a strong correlation between area of the enthesis and cell number when comparing all groups ($r^2 = 0.329$; $p = 0.0510$ Figure 3(D)). Furthermore, we also saw no change in cell number or density in any treatment group in the insertional tendon and there was not a strong correlation between area of the insertional tendon and cell number when comparing all groups ($r^2 = 0.1402$; $p = 0.2305$) (Figure 3(I-L)). We found that collagen organization of the enthesis and insertional tendon was also not affected by any GAHT treatment strategy (Supplemental Fig. S1).

Tendon markers are age dependent in both pre- and post-pubertal mice and Tnmd was sex dependent in pre-pubertal mice

To identify if tendon and enthesis markers differ between sexes (without GAHT treatment) at key time points used in this study, we measured relative levels of tendon markers *Scx, Tnc, Tnmd*, and cartilage marker *Sox9* at 1 M (start point of GAHT) and 3 M (end point of GAHT). We found that gene expression of tendon and enthesis markers, *Scx*, *Tnc* and *Tnmd*, were age dependent (Figure 4(A,C,D)). Relative gene expression at 3 M in the male-born mice was reduced for *Tnc* and *Tnmd* compared to 1 M in male mice (Figure 4(C,D)). Additionally, we saw no change in the relative expression of *Sox9* regardless of sex or age (Figure 4(B)). Finally, *Tnmd* was the only marker we selected that was significantly different between male and female mice, and female mice had a reduced expression of *Tnmd* at 1 M compared to male mice (Figure 4(D)).

Discussion

The potential effects of pubertal suppression, as well as peripubertal, and post-pubertal GAHT, on the structural and functional properties of musculoskeletal tissues is an important and understudied topic. In tendon, the effects of estrogen and T have recently been reported to have similar effects despite differing mechanistic targets during healing[26]. RNA sequencing performed on the supraspinatus tendon 2 weeks post laceration suggested these hormones can mediate gene expression during the healing process[26]. From this, we can infer that sex hormones may have differing mechanistic targets during development and homeostasis as well. Therefore, understanding the differences between sex hormones and their impact on tendon health are important for youth who may use GnRHa to delay or inhibit secondary sex characteristics.

The role of sex hormones on tendon structure and function has not been explored extensively in pre-pubescent populations, and data from adult populations is limited in scope and yields conflicting results. Previous studies have addressed how treatment with hormones following menopause, disease or injury have varying effects on tendon morphology and function[27–29]. For example, treatment with estradiol can lead to increased tensile strength, stiffness, and maximum load in Achilles tendons in an adult rat model of tendinitis[27]. Oral estradiol replacement therapy (ERT) has been shown to increase collagen synthesis and reduce fibril size in tendons of postmenopausal AFAB patients compared to patients without ERT (i.e., reduced estrogen levels)[28]. However, ERT therapy can also reduce tendon stiffness in postmenopausal patients, and this has been associated with immature crosslinking from increased synthesis[28]. Taken together, this data suggests the absence of estrogen via menopause impacted the structure and function of tendon, which was restored with estrogen supplementation. Interestingly, in the current study, we found that puberty suppression with GnRHa improved tendon function with or without T in female mice. Treatment with T alone also led to increased maximum load of the tendon, however when we accounted for changes in tendon size, the maximum stress remained the same as female controls. If and how GAHT treatment influenced other tendons, such as co-contracting tendons like the plantaris, was not investigated in this study. However, we expect that the effects of GAHT on all force-transmitting tendons would be comparable, given that T plays a known role in skeletal muscle hypertrophy[30–32]. This has been supported in recent literature investigating the role of GAHT in a post-pubertal mouse model by Dubois et al., who showed delayed treatment with T led to increased gastrocnemius muscle mass[21]. We also did not find organizational changes associated with GnRHa or T treatment, suggesting that during peripubertal growth, the tendon is not negatively affected by these treatments. These data taken together showcase the importance of estrogen and testosterone throughout various states (i.e., disease, injury, menopause) on factors that influence overall collagen deposition and remodeling that could lead to improved tendon function. Additionally, we show that peripubertal and post-pubertal GAHT have no negative impact on tendon structure. Future studies should continue to explore how hormonal changes regulate and improve tendon structure and function in the context of tendon health. Furthermore, as sex differences are prevalent in injury risk and repair future studies should expand on how GAHT may alter the healing properties of tendon.

Although the effects of sex hormones on the proliferation and growth of cartilage and bone has been recently studied[8–10], little is known about the role of sex hormones in tendon and enthesis[26]. For example, ovariectomized mice exhibit increased chondrocyte proliferation compared with sham controls, and these results were negated when inhibiting estrogen receptor b (ERb) using transgenic knockout mice[8]. These findings and others have suggested that estrogen is a negative regulator for chondrocyte proliferation[9]. These studies are supported by our findings that hormone suppression with GnRHa had a significant impact on cell distribution at the tendon-bone enthesis, which is a fibrocartilaginous tissue. However, we also found that puberty suppression with GnRHa followed by T treatment rescued these changes in enthesis cell density compared to controls. In tendon-derived cells from male rats, treatment with estrogen and estrogen-like analogues significantly increased cell proliferation *in vitro* while estrogen agonists only decreased proliferation when given

in the presence of estrogen[13]. Our findings that both peripubertal and post-pubertal GAHT models had no effect on the cellular density of the insertional tendon suggest that hormone regulation is not a major regulator of proliferation in tendon (Figure 4). The differences in findings between ours and previous studies could be explained by lower levels of circulating estrogen in the Achilles tendon than the doses given *in vitro*.

We were surprised to observe the emergence of possible sex-dependent genes in tendon, specifically *Tnmd*, a regulator of collagen fiber growth and maturation[33]. It has been shown that loss of *Tnmd* in mice does not produce a severe developmental phenotype[34]. However, *Tnmd* deficient mice do exhibit reduced tendon cell density, reduced tenocyte proliferation, and elevated collagen fibril size[34]. Additionally, *Tnmd* may also play a similar role in the enthesis. In mouse enthesis mesenchymal cells, single-cell RNA sequencing defined a subpopulation termed as “enthesoblasts,” which had the most enriched profiles of matrix deposition and tissue development[35]. These cells had high expression of tendon and chondrogenic markers which included *Tnmd*[35]. Carroll et al. showed that *Tnmd* may also be negatively regulated by estrogen-like compounds, as *Tnmd* expression was increased in tendons from ovariectomized female rats, and treatment with genistein, an estrogen analogue, restored *Tnmd* expression to control levels[36]. Taken together, we believe an increase in *Tnmd* could be a possible mechanistic target responsible for the increase in tendon function in response to hormone suppression with GnRHa with and without testosterone. While there was a significant decrease of *Tnc* over time in male mice, we also noticed a trending decrease over time in female mice ($p = 0.0964$) with no significant difference between male and female mice at 3 M for *Tnc*. Furthermore, for *Tnmd*, males and females were significantly different at 1 M and the overall two-way ANOVA showed a significant interaction of sex and time indicating the change over time for *Tnmd* is sex-dependent. However, more data is needed to conclude if the change over time of *Tnc* is also sex-dependent. A limitation of our current study was the inability to examine gene expression results from just the enthesis, our results are representative of the whole tendon. Future studies should explore changes in gene expression in the enthesis as well as how changes in *Tnmd* are associated with changes to testosterone and pubertal suppression in the context of tendon health.

In summary, this work demonstrates that pubertal suppression with GnRHa and T-containing GAHT can influence enthesis and tendon function in female mice. While puberty suppression through GnRHa does change enthesis cellular behavior, post-pubertal therapies (e.g., GnRHa + T) do not have any significant effect on the histological properties of the enthesis or tendon. Additionally, all forms of GAHT significantly improved the overall tendon function of female-born mice. Taken together, these findings begin to answer important questions of the potential effects of pubertal suppression of peripubertal GAHT and post-pubertal GAHT on the functional properties of the tendon and enthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hansen M, Kjaer M. Sex hormones and tendon. In: Ackermann P, Hart D, editors. Metab Influ Risk Tendon Disord [Internet]. Vol. 920. Cham: Springer International Publishing; 2016 [accessed 2024 Jan 18]. p. 139–149. doi:10.1007/978-3-319-33943-6_13.
2. Lefevre N, Bohu Y, Klouche S, Lecocq J, Herman S. Anterior cruciate ligament tear during the menstrual cycle in female recreational skiers. Orthop Traumatol Surg Res. 2013;99(5):571–575. doi:10.1016/j.otsr.2013.02.005. [PubMed: 23764504]
3. Hewett TE, Zazulak BT, Myer GD. Effects of the menstrual cycle on anterior cruciate ligament injury risk: a systematic review. Am J Sports Med. 2007;35(4):659–668. doi:10.1177/0363546506295699. [PubMed: 17293469]
4. Cauley JA. Estrogen and bone health in men and women. Steroids. 2015;99:11–15. doi:10.1016/j.steroids.2014.12.010. [PubMed: 25555470]
5. Bay-Jensen AC, Slagboom E, Chen-An P, Alexandersen P, Qvist P, Christiansen C, Meulenbelt I, Karsdal MA. Role of hormones in cartilage and joint metabolism: understanding an unhealthy metabolic phenotype in osteoarthritis. Menopause. 2013;20(5):578–586. doi:10.1097/gme.0b013e3182745993. [PubMed: 23615651]
6. Velders M, Diel P. How sex hormones promote skeletal muscle regeneration. Sports Med. 2013;43(11):1089–1100. doi:10.1007/s40279-013-0081-6. [PubMed: 23888432]
7. Steltzer SS, Abraham AC, Killian ML. Interfacial tissue regeneration with bone. Curr Osteoporos Rep. 2024;22(2):290–298. doi:10.1007/s11914-024-00859-1. [PubMed: 38358401]
8. Chen J, Kamiya Y, Polur I, Xu M, Choi T, Kalajzic Z, Drissi H, Wadhwa S. Estrogen via estrogen receptor beta partially inhibits mandibular condylar cartilage growth. Osteoarthr Cartil. 2014;22(11):1861–1868. doi:10.1016/j.joca.2014.07.003.
9. Talwar RM, Wong BS, Svoboda K, Harper RP. Effects of estrogen on chondrocyte proliferation and collagen synthesis in skeletally mature articular cartilage. J Oral Maxillofac Surg. 2006;64(4):600–609. doi:10.1016/j.joms.2005.12.006. [PubMed: 16546639]
10. Claassen H, Schicht M, Paulsen F. Impact of sex hormones, insulin, growth factors and peptides on cartilage health and disease. Prog Histochem Cytochem. 2011;45(4):239–293. doi:10.1016/j.proghi.2010.11.002. [PubMed: 21251699]
11. Leblanc DR, Schneider M, Angele P, Vollmer G, Docheva D. The effect of estrogen on tendon and ligament metabolism and function. J Steroid Biochem Mol Biol. 2017;172:106–116. doi:10.1016/j.jsbmb.2017.06.008. [PubMed: 28629994]
12. Ramos JE, Al-Nakkash L, Peterson A, Gump BS, Janjulia T, Moore MS, Broderick TL, Carroll CC. The soy isoflavone genistein inhibits the reduction in a chilles tendon collagen content induced by ovariectomy in rats. Scand J Med Sci Sports [Internet]. [2012 [accessed 2024 Jan 18];22(5). doi:10.1111/j.1600-0838.2012.01516.x.
13. Maman E, Somjen D, Maman E, Katzburg S, Sharfman ZT, Stern N, Dolkart O. The response of cells derived from the supraspinatus tendon to estrogen and calciotropic hormone stimulations: in vitro study. Connect Tissue Res. 2016;57(2):124–130. doi:10.3109/03008207.2015.1114615. [PubMed: 26646255]
14. Dehghan F, Muniandy S, Yusof A, Salleh N. Testosterone reduces knee passive range of motion and expression of relaxin receptor isoforms via 5α-dihydrotestosterone and androgen receptor binding. Int J Mol Sci. 2014;15(3):4619–4634. doi:10.3390/ijms15034619. [PubMed: 24642882]

15. Lee JY, Rosenthal SM. Gender-affirming care of transgender and gender-diverse youth: current concepts. Annu Rev Med. 2023;74(1):107–116. doi:10.1146/annurev-med-043021-032007. [PubMed: 36260812]

16. Coleman E, Radix AE, Bouman WP, Brown GR, De Vries ALC, Deutsch MB, Ettner R, Fraser L, Goodman M, Green J, et al. Standards of care for the health of transgender and gender diverse people, version 8. Int J Transgender Health. 2022;23(sup1):S1–S259. doi:10.1080/26895269.2022.2100644.

17. Dubon ME, Abbott K, Carl RL. Care of the transgender Athlete. Curr Sports Med Rep. 2018;17(12):410–418. doi:10.1249/JSR.0000000000000545. [PubMed: 30531457]

18. Van Caenegem E, Taes Y, Wierckx K, Vandewalle S, Toye K, Kaufman J-M, Schreiner T, Haraldsen I, T'Sjoen G. Low bone mass is prevalent in male-to-female transsexual persons before the start of cross-sex hormonal therapy and gonadectomy. Bone. 2013;54(1):92–97. doi:10.1016/j.bone.2013.01.039. [PubMed: 23369987]

19. Dela Cruz C, Wandoff A, Brunette M, Padmanabhan V, Shikanov A, Moravek MB. In vitro fertilization outcomes in a mouse model of gender-affirming hormone therapy in transmasculine youth. FS Sci. 2023;4(4):302–310. doi:10.1016/j.xfss.2023.08.001.

20. Dela Cruz C, Kinnear HM, Hashim PH, Wandoff A, Nimmagadda L, Chang FL, Padmanabhan V, Shikanov A, Moravek MB. A mouse model mimicking gender-affirming treatment with pubertal suppression followed by testosterone in transmasculine youth. Hum Reprod. 2023;38(2):256–265. doi:10.1093/humrep/deac257. [PubMed: 36484619]

21. Dubois V, Ciancia S, Doms S, El Kharraz S, Sommers V, Kim NR, David K, Van Dijck J, Valle-Tenney R, Maes C, et al. Testosterone restores body composition, bone Mass, and bone strength following early puberty suppression in a mouse model mimicking the clinical strategy in trans boys. J Bone Min Res. 2023;38(10):1497–1508. doi:10.1002/jbmr.4832.

22. Hashim PH, Kinnear HM, Cruz CD, Padmanabhan V, Moravek MB, Shikanov A. Pharmacokinetic comparison of three delivery systems for subcutaneous testosterone administration in female mice. Gen Comp Endocrinol. 2022;327:114090. doi:10.1016/j.ygcen.2022.114090.

23. Machne R, Stadler PF. Dpseg: piecewise linear segmentation by dynamic programming [Internet]. 2020 [accessed 2024 Jul 11]; doi:10.32614/CRAN.package.dpseg.

24. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, McQuaid S, Gray RT, Murray LJ, Coleman HG, et al. QuPath: open source software for digital pathology image analysis. Sci Rep. 2017;7(1):16878. doi:10.1038/s41598-017-17204-5. [PubMed: 29203879]

25. Iannucci L, Riak MB, Lake SP. The effect of extracellular matrix properties on polarized light-based analysis of collagen fiber alignment in soft tissues. In: Ramella-Roman J, Ma H, Vitkin I, Elson D, Novikova T, editors. Polarized light opt angular momentum biomed diagn 2022 [Internet]. San Francisco, United States: SPIE; 2022. p. 4. [accessed 2024 May 31] doi:10.1117/12.2614438.

26. Tashjian RZ, Zitnay J, Kazmers NH, Veerabhadraiah SR, Zelada AC, Honeggar M, Chalmers PN, Henninger HB, Jurynec MJ. Estrogen and testosterone supplementation improves tendon healing and functional recovery after rotator cuff repair. J Orthop Res. 2024;42(2):259–266. doi:10.1002/jor.25695. [PubMed: 37756152]

27. Wang F, Shan H, Song G, Chen S, Zhang C, Liu Y, Wu T. 17β-estradiol attenuates inflammation and tendon degeneration in a rat model of achilles tendinitis. Immunopharmacol Immunotoxicol. 2022;44(4):556–564. doi:10.1080/08923973.2022.2065639. [PubMed: 35404181]

28. Hansen M, Kongsgaard M, Holm L, Skovgaard D, Magnusson SP, Qvortrup K, Larsen JO, Aagaard P, Dahl M, Serup A, et al. Effect of estrogen on tendon collagen synthesis, tendon structural characteristics, and biomechanical properties in postmenopausal women. J Appl Physiol. 2009;106(4):1385–1393. doi:10.1152/japplphysiol.90935.2008. [PubMed: 18927264]

29. Marqueti RC, Prestes J, Paschoal M, Ramos OHP, Perez SEA, Carvalho HF, Selistre-de-Araujo HS. Matrix metallopeptidase 2 activity in tendon regions: effects of mechanical loading exercise associated to anabolic-androgenic steroids. Eur J Appl Physiol. 2008;104(6):1087–1093. doi:10.1007/s00421-008-0867-7. [PubMed: 18810485]

30. Horwath O, Apró W, Moberg M, Godhe M, Helge T, Ekblom M, Hirschberg AL, Ekblom B. Fiber type-specific hypertrophy and increased capillarization in skeletal muscle

following testosterone administration in young women. J Appl Physiol. 2020;128(5):1240–1250. doi:10.1152/japplphysiol.00893.2019. [PubMed: 32191598]

31. Sinha-Hikim I, Artaza J, Woodhouse L, Gonzalez-Cadavid N, Singh AB, Lee MI, Storer TW, Casaburi R, Shen R, Bhasin S. Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. Am J Physiol-Endocrinol Metab. 2002;283(1):E154–E164. doi:10.1152/ajpendo.00502.2001. [PubMed: 12067856]
32. Basualto-Alarcón C, Jorquera G, Altamirano F, Jaimovich E, Estrada M. Testosterone signals through mTOR and androgen receptor to induce muscle hypertrophy. Med Sci Sports Exerc. 2013;45(9):1712–1720. doi:10.1249/MSS.0b013e31828cf5f3. [PubMed: 23470307]
33. Shukunami C, Yoshimoto Y, Takimoto A, Yamashita H, Hiraki Y. Molecular characterization and function of tenomodulin, a marker of tendons and ligaments that integrate musculoskeletal components. Jpn Dent Sci Rev. 2016;52(4):84–92. doi:10.1016/j.jdsr.2016.04.003. [PubMed: 28408960]
34. Docheva D, Hunziker EB, Fässler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol. 2005;25(2):699–705. doi:10.1128/MCB.25.2.699-705.2005. [PubMed: 15632070]
35. Fang F, Xiao Y, Zelzer E, Leong KW, Thomopoulos S. A mineralizing pool of Gli1-expressing progenitors builds the tendon enthesis and demonstrates therapeutic potential. Cell STEM Cell. 2022;29(12):1669–1684. e6. doi:10.1016/j.stem.2022.11.007. [PubMed: 36459968]
36. Carroll CC, Patel SH, Simmons J, BDh G, Olson JF, Chemelewski K, Saw S, Hale TM, Howden R, Sabbaghi A. The impact of genistein supplementation on tendon functional properties and gene expression in estrogen-deficient rats. J Med Food. 2020;23(12):1266–1274. doi:10.1089/jmf.2019.0293. [PubMed: 32345111]

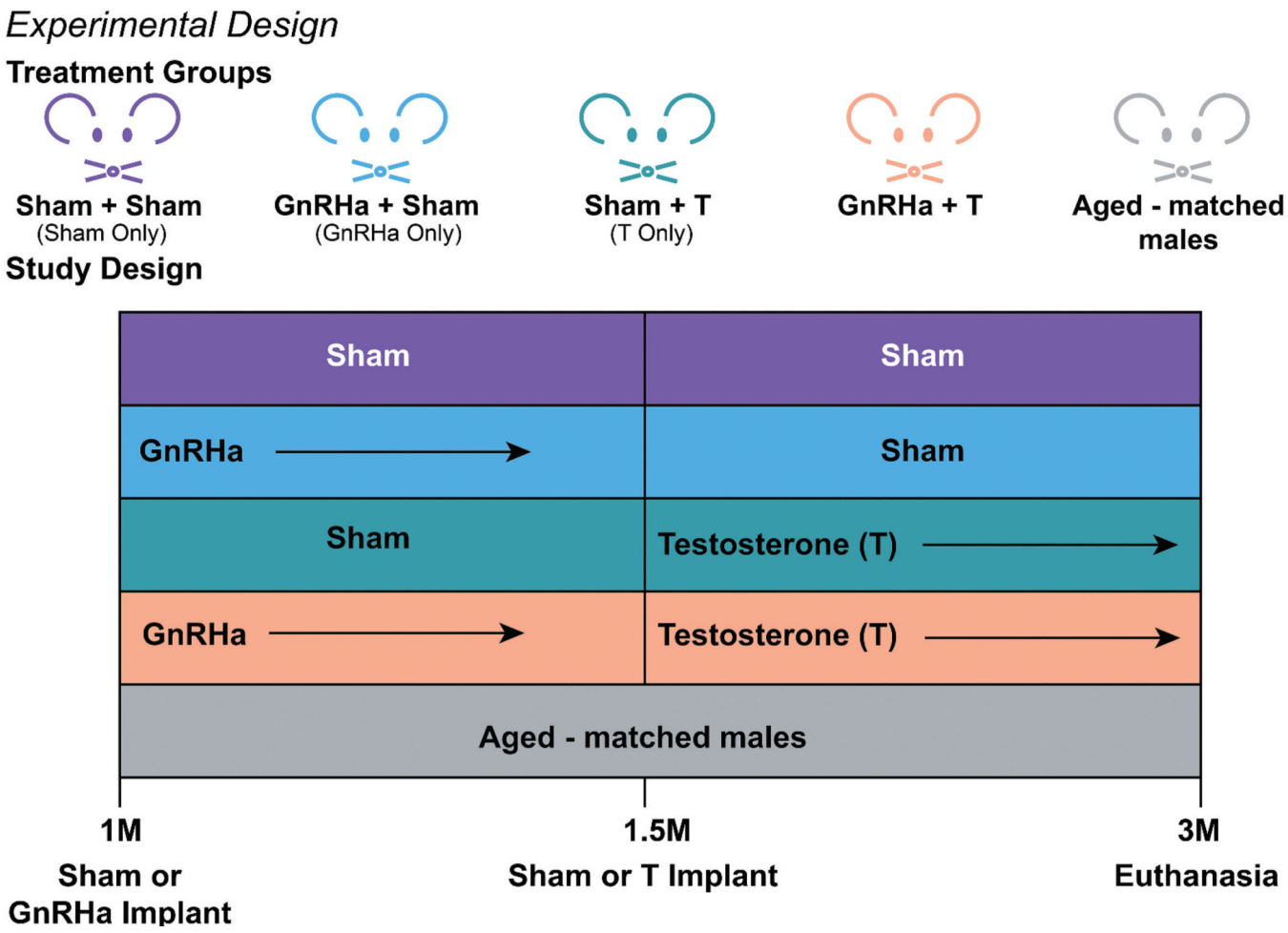

Figure 1.
Summary of experimental design. *M* = month.

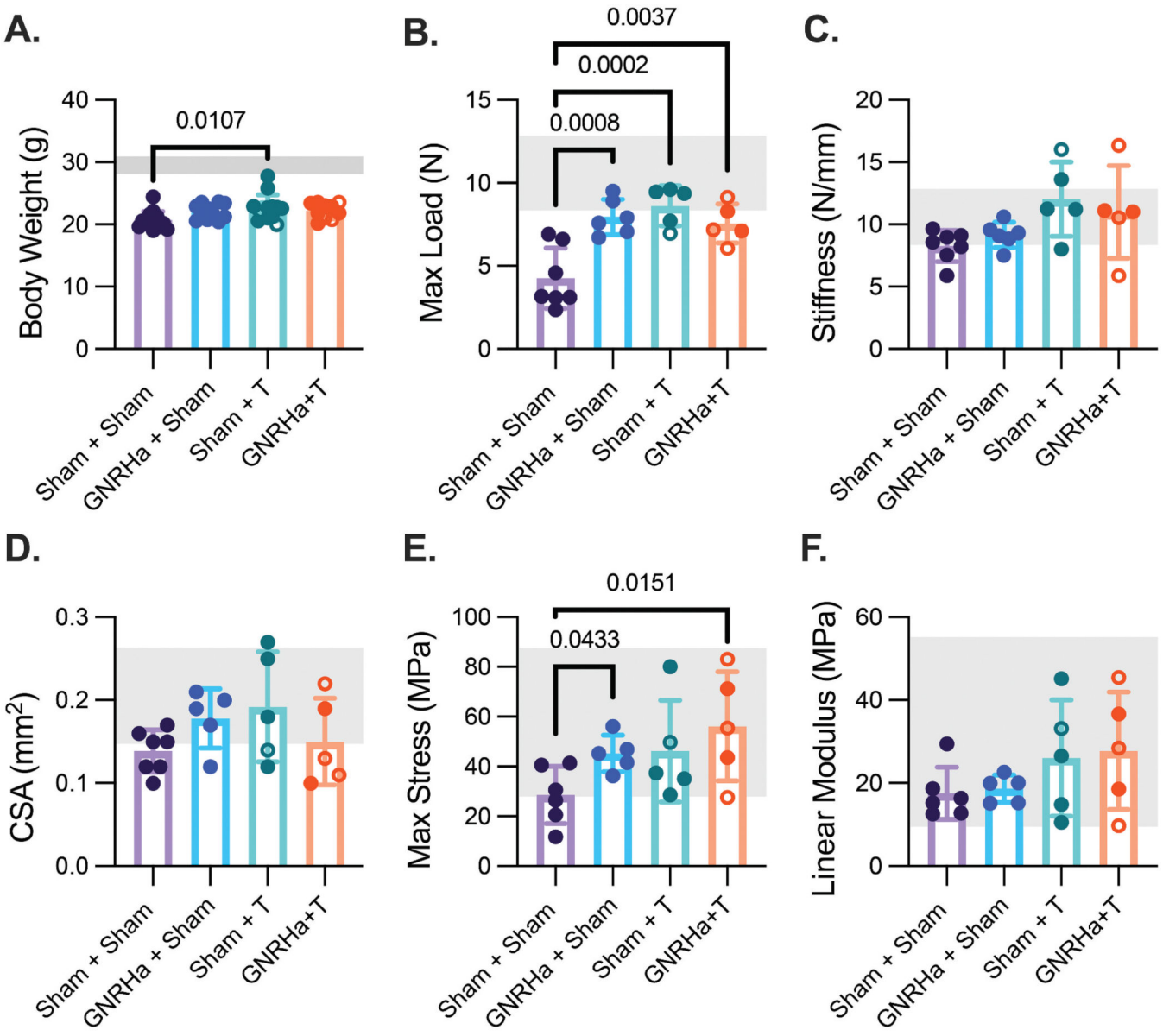

Figure 2.

Maximum load of Achilles tendons was significantly increased in all treatment groups compared to sham controls while GnRHa treatment with and without T resulted in increased maximum stress for Achilles tendons. treatment with T significantly increased the body weight (g) of female mice after treatment (some mice included in this study were previously reported in Dela Cruz et al.[19] (A). Maximum load (N) of Achilles tendons was significantly increased in all groups compared to controls (sham + sham) (B). Achilles tendon stiffness remained unchanged between all groups (C). Cross sectional area (CSA) (mm) of Achilles tendons remained unchanged between all groups (D). Maximum stress (MPa) of Achilles tendons was significantly increased in GnRHa treated mice with or without testosterone (GnRha + sham, GnRHa + T) compared to controls (sham + sham) (E). Linear modulus (MPa) of achilles tendons remained unchanged between all groups (F). Data are presented as

biological replicates (individual dots) and mean ± standard deviation. Mice euthanized at 3 M are represented using filled circles; mice euthanized after a two-week T cessation (3.5 M) are represented as open circles. Gray horizontal bars represent range for age-matched male control mice, $n = 3$. Data from female mice were compared using a one-way ANOVA and corrected for multiple comparisons using Tukey's multiple comparisons tests (prism v9.0+; Graphpad, LaJolla, CA, USA).

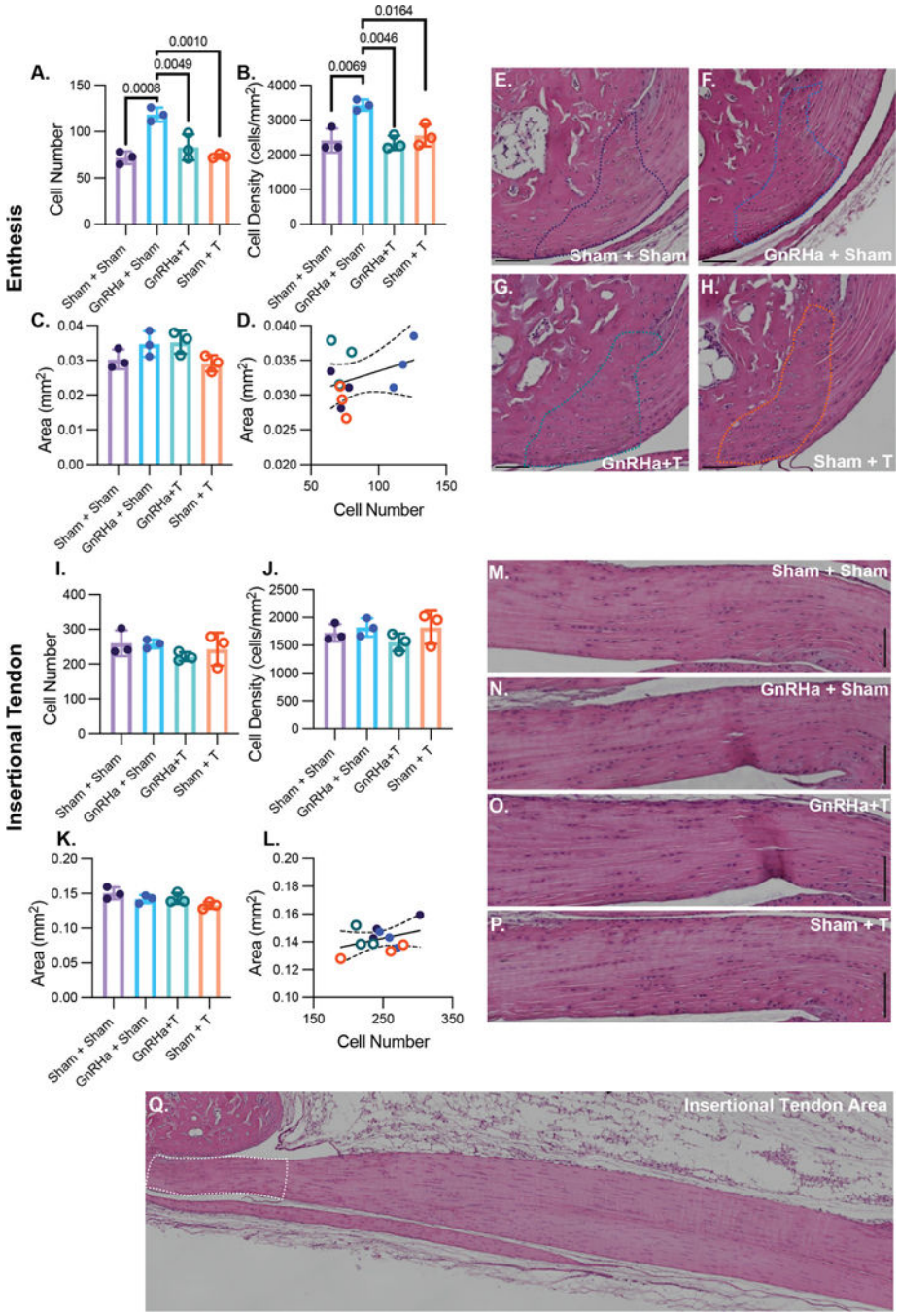

Figure 3.

GnRHa significantly increased cellular number and density in the Achilles enthesis.Treatment with GnRHa (GnRHa + Sham) significantly increased Achilles enthesis cell number compared to all groups (A). Treatment with GnRHa (GnRHa + Sham) significantly increased Achilles enthesis cell density (cells/mm^2) compared to controls (Sham + Sham) and GnRHa + T treated female mice (B). Achilles enthesis area (mm^2) remained unchanged between all groups (C). Linear regression of area and cell number of the Achilles enthesis between all groups, $r^2 = 0.329$ with Pearson's correlation test

(D). Representative images of Achilles enthesis areas used for cell number and density calculations (E-H). Achilles insertional tendon cell number remained unchanged between all groups (I). Achilles insertional tendon cell density (cells/mm^2) remained unchanged between all groups (J). Achilles insertional tendon area (mm^2) remained unchanged between all groups (K). Linear regression of cell number and area of the Achilles insertional tendon between all groups, $r^2 = 0.1402$ with Pearson's correlation test (L). Representative images of Achilles insertional tendons used for cell number and density calculations (M-P). (Q) Representative image of insertional tendon area compared to tendon body. Data are presented as biological replicates (individual dots) and mean +/− standard deviation. n = 3 mice per group. Data were compared using a one-way ANOVAs and corrected for multiple comparisons using Tukey's multiple comparisons tests.

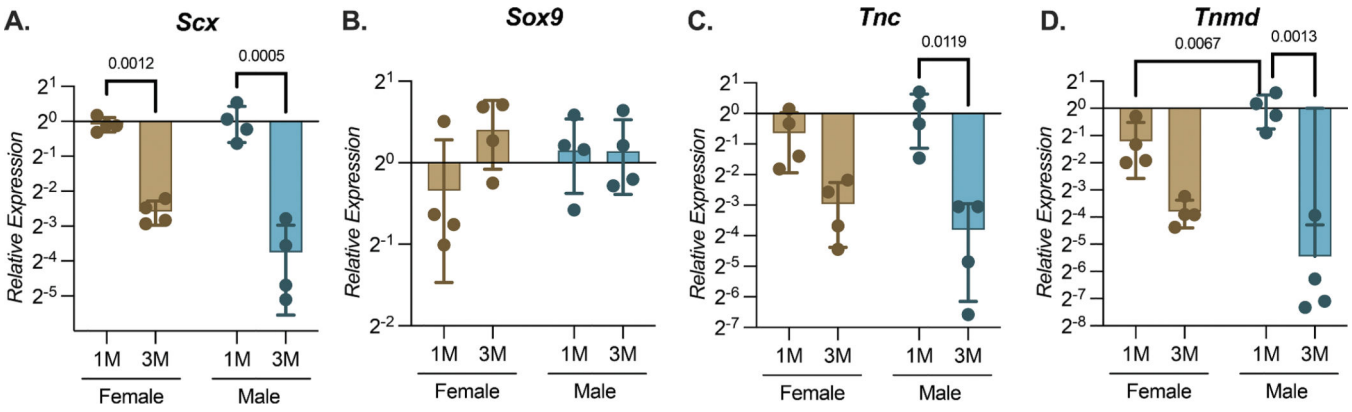

Figure 4.

Scx and *Tnc* gene expression were age dependent in mouse Achilles tendon while Tnmd is age- and sex-dependent in prepubertal mouse Achilles tendon. *Scx* expression was significantly reduced in both female and male Achilles tendons at 3M compared to 1M (A). *Sox9* expression was not different between female and male mice at either age (B). *Tnc* expression was significantly reduced in male tendons at 3M compared to 1M and trended down, although not significant, for females at 3M compared to 1M (C). *Tnmd* expression was significantly higher in male born mice compared to females at 1M and was significantly reduced at 3M compared to 1M for males (D). Data are presented as individual mice (biological replicates; individual dots) and mean +/− standard deviation. n = 4 per sex per age. Experimental groups compared to 1M males for relative expression.

Table 1.

qPCR primer information. IDT predesigned qPCR assays used with intercalating dyes, primers only.

Assay ID	Gene Query	NCBI Gene Symbol	Ref Seq #	Detects All Variants	Exon Locations
Mm.PT.58.43894205	Rplp0	Rplp0	NM_007475(1)	Yes	5–6
Mm.PT.58.8090418	Tnc	Tnc	NM_011607(1)	Yes	18–19
Mm.PT.58.13530921	Tnmd	Tnmd	NM_022322(1)	Yes	5–6
Mm.PT.58.31750069	Scx	Scx	NM_198885(1)	Yes	1–2
Mm.PT.58.42739087	Sox9	Sox9	NM_011448(1)	Yes	1–2