**TESTOSTERONE RESTORES BODY COMPOSITION, BONE MASS AND BONE STRENGTH FOLLOWING EARLY PUBERTY SUPPRESSION IN A MOUSE MODEL MIMICKING THE CLINICAL STRATEGY IN TRANS BOYS**

**Vanessa Dubois[1,2*], Silvia Ciancia[3], Stefanie Doms[1], Sarah El Kharraz[4,5], Vera Sommers[4], Na Ri Kim[1], Karel David[1,6], Jolien Van Dijck[7], Roger Valle Tenney[7], Christa Maes[7], Leen Antonio[1,6], Brigitte Decallonne[1,6], Geert Carmeliet[1], Frank Claessens[4], Martine Cools[3,8,$], Dirk Vanderschueren[1,6,$]**

[1] Clinical and Experimental Endocrinology, Department of Chronic Diseases and Metabolism (Chrometa), KU Leuven, Leuven, Belgium

[2] Basic and Translational Endocrinology (BaTE), Department of Basic and Applied Medical Sciences, Ghent University, Ghent, Belgium

[3] Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium

[4] Molecular Endocrinology Laboratory, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium

[5] Laboratory of Cellular Metabolism and Metabolic Regulation, VIB-KU Leuven Center for Cancer Biology, VIB, Leuven, Belgium; Laboratory of Cellular Metabolism and Metabolic Regulation, Department of Oncology, KU Leuven and Leuven Cancer Institute (LKI), Leuven, Belgium

[6] Department of Endocrinology, University Hospitals Leuven, Leuven, Belgium.

[7] Laboratory of Skeletal Cell Biology and Physiology (SCEBP), Skeletal Biology and Engineering Research Center (SBE), Department of Development and Regeneration, KU Leuven, Leuven, Belgium.

[8] Pediatric Endocrinology Service, Department of Pediatrics, Ghent University Hospital, Ghent, Belgium.

$ Contributed equally

* Corresponding author: Vanessa Dubois

Basic and Translational Endocrinology (BaTE)

Department of Basic and Applied Medical Sciences

Faculty of Medicine and Health Sciences, Ghent University

Campus UZ Gent, Blok B, route 36

Corneel Heymanslaan 10

B-9000 Gent

T +32 9 332 69 44

vanessa.dubois@ugent.be

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**ABSTRACT**

Transgender youth increasingly present at pediatric gender services. Some of them receive long-term puberty suppression with gonadotropin-releasing hormone analogues (GnRHa) before starting gender-affirming hormones (GAH). The impact of GnRHa use started in early puberty on bone composition and bone mass accrual is unexplored. It is furthermore unclear whether subsequent GAH fully restore GnRHa effects, and if the timing of GAH introduction matters. To answer these questions, we developed a mouse model mimicking the clinical strategy applied in trans boys. Prepubertal 4-week-old female mice were treated with GnRHa alone, or with GnRHa supplemented with testosterone (T) from 6 weeks (early puberty) or 8 weeks (late puberty) onwards. Outcomes were analyzed at 16 weeks and compared to untreated mice of both sexes. GnRHa markedly increased total body fat mass, decreased lean body mass and had a modest negative impact on grip strength. Both early and late T administration shaped body composition to adult male levels, while grip strength was restored to female values. GnRHa-treated animals showed lower trabecular bone volume, and reduced cortical bone mass and strength. These changes were reversed by T to female levels (cortical bone mass and strength) irrespective of the time of administration, or even fully up to adult male control values (trabecular parameters) in case of earlier T start. The lower bone mass in GnRHa-treated mice was associated with increased bone marrow adiposity, also reversed by T. In conclusion, prolonged GnRHa use started in prepubertal female mice modifies body composition towards more fat and less lean mass, and impairs bone mass acquisition and strength. Subsequent T administration counteracts GnRHa impact on these parameters, shaping body composition and trabecular parameters to male values while restoring cortical bone architecture and strength up to female but not male control levels. These findings could help guide clinical strategies in transgender care.

Keywords: GnRHa, testosterone, transgender, mouse model, bone, body composition

**INTRODUCTION**

Transgender and gender diverse individuals experience a gender identity that differs from their designated sex at birth. In particular, a person designated male at birth who identifies as female is referred to as trans girl or trans woman, while a person designated female at birth who identifies as male is referred to as trans boy or trans man. The incongruence between the experienced gender and the designated sex at birth can cause psychological distress, referred to as gender dysphoria. Puberty suppression (specifically for transgender adolescents), gender-affirming hormones (GAH) and gender-affirming surgery aim to lessen the psychological burden by making physical sex characteristics more congruent with the experienced gender.[1,2]

In the last few decades, the transgender population has rapidly expanded worldwide, with a growing number of individuals seeking medical care, including children and adolescents. Whereas in adults the ratio of trans women to trans men has remained relatively stable, several studies have reported a shift among adolescent transgender individuals, with currently a trans boy:trans girl ratio of approximately 2:1.[3,4] According to international guidelines, adolescents who experience persistent gender dysphoria worsening at the onset of puberty are eligible for puberty suppression, achieved through the administration of gonadotropin-releasing hormone analogues (GnRHa).[5,6] GnRHa either block (GnRH antagonists) or repeatedly activate (GnRH agonists) the gonadotropin-releasing hormone (GnRH) receptor leading to its desensitization, with consequent suppression of gonadotrophin secretion.[7] In children presenting early at multidisciplinary transgender healthcare services and with a confirmed diagnosis, GnRHa can be prescribed from early puberty onwards (Tanner stage 2-3, corresponding to a calendar age of approximately 10-11 years for trans boys and 11-12 years for trans girls). In those willing to go through hormonal transitioning and in sufficient capacity to give fully informed consent (usually around the age of 15-16 years), GAH [testosterone (T) or estradiol (E2)] are added.[5,6]

Sex steroids play crucial roles in a variety of physiological processes, and in a sexually dimorphic way, including regulation of bone mass accrual and shaping of body composition during puberty.[8,9] As transgender adolescents starting their trajectory early will experience prolonged puberty suppression

and delayed puberty, concerns have been raised about the potential adverse effects of this treatment on the acquisition of sexually dimorphic skeletal and metabolic traits normally occurring during puberty, in particular the acquisition of peak bone mass, the gain of adult height in line with their genetic potential, and the further development of lean mass.[10]

Previous work showed that puberty suppression in transgender adolescents increased fat mass and decreased lean mass, and that subsequent GAH shaped body composition towards the experienced gender.[11] With regard to bone, several studies recently reviewed in [12] reported a decrease in areal bone mineral density (aBMD) Z-scores following GnRHa administration, which was only partially restored after GAH administration. However, long-term outcome data in transgender individuals who started medical transition from the earliest stages of puberty onwards are lacking. Furthermore, whether earlier start of GAH, when indicated, may better compensate the effects of GnRHa, is unknown. Finally, the majority of these studies used Z-scores calculated according to the designated sex at birth, making it impossible to compare the bone parameters of the transgender adolescents with those of the experienced gender. As well-controlled, long-term clinical studies on sufficiently large cohorts are challenging to perform, animal models provide a unique opportunity to shed light on these knowledge gaps, as well as to gain mechanistic insight. We recently developed a mouse model of GnRHa-mediated puberty suppression. Administration of the GnRHa degarelix (DGX) to prepubertal male mice led to a shift in body composition towards more fat and less lean mass, and was accompanied by a drastic impairment of both cortical and trabecular bone mass accrual. [13] Whether these findings also apply to female animals remained, however, to be determined, as well as the effects of GAH in this model.

Therefore, the aims of the present study were threefold: first, we developed a preclinical model to investigate the impact of long-term GnRHa started in prepuberty on body composition, bone architecture and strength, using mice. We here focused on the female-to-male transition, given the increasing prevalence of trans boys presenting at pediatric gender services. Second, we used this model to explore the potential of GAH, specifically T, to counteract the impact of GnRHa, as well as the

effects of an earlier start of GAH. We hypothesize that the negative impact of GnRHa might be enhanced by the duration of unopposed treatment and the unphysiological late addition of GAH. Third, by using untreated mice of both sexes as controls, we assessed whether the body composition and skeletal parameters at adulthood of animals subjected to GnRHa-mediated puberty suppression followed by T administration compared better to the designated sex at birth or to the experienced gender.

## MATERIALS AND METHODS

### Experimental design

Prepubertal 4-week-old female wild type C57BL/6J mice (Charles River) were treated with either the GnRHa degarelix (DGX) alone (Firmagon®, Ferring Pharmaceuticals; subcutaneous injection of 25 mg/kg every 4 weeks) to achieve complete and persistent suppression of puberty (n=12), DGX at 4 weeks supplemented with T at 6 weeks (early puberty) (n=12), or DGX at 4 weeks supplemented with T at 8 weeks (late puberty) (n=12).[13] T supplementation was achieved by inserting silastic implants (Silclear Tubing, Degania Silicone) filled with T (Sigma-Aldrich) in the dorsal region under isoflurane anesthesia, followed by meloxicam analgesia (Boehringer Ingelheim; subcutaneous injection of 5mg/kg). The daily release of T from the implants is 23 µg,[14] which is slightly supraphysiological.[15] The group treated with DGX alone received empty implants. Untreated female (n=12) and male (n=12) wild type C57BL/6J mice which were injected with vehicle and received empty silastic implants, served as controls.

Mice were group-housed (4 animals/cage) in conventional facilities at 20 °C with 12-hour light/dark cycle and had *ad libitum* access to water and standard chow, according to our institutional guidelines. Body weight was assessed weekly and body composition was determined every 2 weeks by echoMRI. One week before euthanasia, grip strength was evaluated. The day before euthanasia, animals were kept in metabolic cages for 24h to measure food intake. All animals were euthanized at adult age (16

weeks) by sodium pentobarbital overdose (Dolethal, Vétoquinol Ltd; intraperitoneal injection of 74 mg/kg) followed by cardiac puncture and bone isolation for phenotyping. Nose-to-tail length was assessed before euthanasia in anesthetized animals. Wet weights of individual fat pads and gastrocnemius muscle were measured at euthanasia. Anogenital distance and uterus wet weight were determined as readouts for sex steroid action.[16,17] Length of femur and tibia were assessed after dissection using a caliper. All animal experiments were approved by the KU Leuven ethical committee (P192/2016).

**EchoMRI**

Total body fat mass and lean body mass were measured by quantitative magnetic resonance (EchoMRI-100H Analyzer, Echo Medical Systems) according to the manufacturer's instructions.

**Grip strength**

Grip strength of the limbs was evaluated by means of a grid connected to an isometric force transducer (Chatillon DFIS-2 Digital Force Gauche, Ametek) as described before.[18] Briefly, mice were lifted by the tail and were made to hold the metal grid with all limbs. Subsequently, they were pulled backward until they could no longer hold the grid. Total-limb maximal grip strength was registered in newtons during 3 consecutive attempts, and the result was set as the average of the attempts.

**Micro-computed tomography (microCT)**

Axial (vertebral column) and appendicular (femur and tibia) murine bones were scanned using SkyScan 1272 (Bruker) with 5 µm pixel size, 0.5 mm Al filter, 60 kV, 83 µA, 180° angular rotation at 0.4° steps, and 3000 ms integration time. Images were reconstructed with the NRecon software (Bruker) and morphometric parameters were calculated using CTAn (Bruker) as previously described.[19] For trabecular bone of femora and tibiae, a 1.5 mm segment was analyzed starting at 0.75 mm from the proximal (tibiae) or distal (femora) growth plate and moving towards the diaphysis. For trabecular bone of vertebrae, we considered the whole vertebral body of lumbar 5 (L5). For cortical bone, a 0.5 mm region of interest was selected starting at 3 mm from the proximal (tibiae) or distal (femora)

growth plate and moving towards the diaphysis. 3D image rendering was performed using CTVox (Bruker).

Parameters are reported according to the American Society for Bone and Mineral Research guidelines [20] and include trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, µm), trabecular separation (Tb.Sp, µm), total cross-sectional tissue area (Tt.Ar, $mm^2$), cortical area (Ct.Ar, $mm^2$), medullary area (Ma.Ar, $mm^2$), cortical area fraction (Ct.Ar/Tt.Ar, %), cortical thickness (Ct.Th, mm), periosteal perimeter (Ps.Pm, mm), endocortical perimeter (Ec.Pm, mm), and polar moment of inertia (J, $mm^4$).

**Biomechanical testing**

At termination point, murine femora were collected and kept in PBS at -20°C. A destructive three-point bending test was performed at the midshaft region on a Bose ElectroForce testing system (TestBench LM1, EnduraTEC Systems Group, Bose Corp) as described before.[21] Span length and radius of curvature of the supports were 7 mm and 2 mm, respectively. The bones were placed with the anterior surface pointing downward and were subjected to a small stabilizing preload (1 N) and two conditioning cycles before loading until failure at a rate of 0.1 mm/second. The load-displacement curve was used to calculate ultimate bone strength.

**Bone marrow adipocyte counting**

Murine tibiae were fixed overnight in 2% paraformaldehyde and decalcified in EDTA (0.5 M; pH 7.5) for 14 days at 4 °C before being embedded in paraffin. Tissue sections of 4 µm thickness were prepared using a Microm HM 360 microtome and stained with hematoxylin and eosin (H&E) as previously described.[22] To count the adipocytes in the bone marrow, as described before,[23] bright field images were obtained using an Olympus IX83 inverted microscope with a DP73 camera. Tiled images covering the full section were taken using a 10X objective, and a region of interest (ROI) of 1 $mm^2$ was selected below the growth plate in the proximal metaphysis. Using ImageJ, the adipocytes inside the ROI were manually counted in 2-3 sections of each sample, and averaged. Growth plate thickness was assessed in ImageJ using the same images.

**Real-time quantitative PCR (RT-qPCR)**

The bone marrow fraction of murine tibiae was obtained by centrifugation of the bones for 2.5 minutes at 18000 g after removal of the proximal ends. Total RNA was subsequently extracted from the bone marrow pellet using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Copy-DNA was synthesized from 1 µg RNA using FastGene Scriptase II kit (NIPPON Genetics Europe) and random hexamer primers. The RT-qPCR reactions were performed using Fast SYBR Green Master Mix or TaqMan Fast Advanced Master Mix and the StepOnePlus Real-Time PCR system (Applied Biosystems). The relative expression levels of the target genes were calculated as a ratio to the expression levels of the *Gapdh* and *Hrprt* housekeeping genes. The primer sequences are described in Supplemental Table S1.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism v.9. To compare the five different groups, one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were performed. Statistical significance was considered to be reached when adjusted p-values were below 0.05, and the exact p-values are indicated on the graphs. Box plots are composed of a box from the 25$^{th}$ to the 75$^{th}$ percentile with the median as a line, the mean as a cross, and min to max as whiskers.

**RESULTS**

**Development and validation of a preclinical mouse model mimicking the clinical strategy as applied in trans boys**

To investigate the impact of long-term puberty suppression from early puberty onwards, followed by T administration, on body composition, bone architecture and strength, a preclinical mouse model was generated. Female mice prepubertally treated with the GnRHa degarelix (DGX) were supplemented with T at an early versus a late pubertal stage, and their outcomes were compared to those of untreated mice of both sexes (see scheme of the experimental design in Figure 1A).

Uterus weight, a common readout for sex steroid action in mice,[16] was significantly decreased by DGX and increased by T (Figure 1B,C), indicating that both treatments were effective. The increased anogenital distance and enlarged clitoris in DGX+T-treated groups compared to mice subjected to DGX alone (Figure 1D,E) confirmed effectiveness of T administration.[17]

**GnRHa administration to prepubertal female mice increases fat mass accumulation and lowers lean mass and muscle strength, effects that are counteracted by T**

Body weight was lower in females compared to males, but unaffected by either DGX or T (Supplemental Figure S1A). In contrast, DGX treatment strongly increased fat mass accumulation, with echoMRI-assessed total body fat mass being almost 2-fold higher at sacrifice in the DGX-treated group compared to control animals of either sex (Figure 2A and Supplemental Figure S1B). In particular, the effect of DGX on fat mass was restricted to white adipose tissue. Indeed, weight of subcutaneous, gonadal and perirenal fat pads was increased in DGX compared to control groups, while interscapular brown adipose tissue weight was unaltered (Figure 2B and Supplemental Figure S1C). Importantly, the stimulatory effect of DGX on fat mass accumulation was not due to an increase in food intake, as DGX-treated animals ate less compared to both control groups (Supplemental Figure S1D). DGX concomitantly lowered lean body mass, although this effect was less pronounced (Figure 2C,D and Supplemental Figure S1E). In line with the lean mass observations, grip strength was lower in females compared to males, and tended to be further reduced in the DGX-treated animals (Figure 2E).

The effects of DGX on body composition and muscle strength were counteracted by T, irrespective of the time of administration. At adult age, fat and lean masses of T-treated animals were similar to male controls, while grip strength was restored to female levels (Figure 2A,C,E). Of note, T effects on body composition occurred fast, as the decrease in fat mass and the increase in lean mass were already apparent 2 weeks after treatment initiation in both early and late groups (Figure 2A,C, and Supplemental Figure S1B,E).

Altogether, early and prolonged GnRHa therapy in female mice resulted in an increase in fat mass and a decrease in lean mass and muscle strength. These changes could all be counteracted by T, which restored grip strength to female control values while shaping body composition to male levels.

**GnRHa administration to prepubertal female mice reduces bone mass acquisition and bone strength, effects that are counteracted by T**

Trabecular bone morphology was sexually dimorphic, as evidenced by the lower BV/TV assessed by microCT at the femoral metaphysis in female compared to male control animals. DGX treatment significantly reduced female femoral BV/TV, which was restored up to the levels of female controls by late T administration. Early T administration further increased BV/TV, reaching the level of male controls (Figure 3A,B). Comparable observations were made at the tibial metaphysis (Figure 3C). The increase in BV/TV in the T groups was accompanied by a reduction in growth plate thickness (Supplemental Figure S2A), suggestive of an enhanced mineralization of the growth plate. However, the latter did not affect adult body length. Indeed, axial and appendicular bone length were lower in females compared to males, but unaffected by either DGX or T (Supplemental Figure S2B-D). In the L5 vertebral body, DGX similarly decreased BV/TV, and both early and late T treatments were able to increase BV/TV similar to female controls, but not to reach male control levels (Figure 3D). In all three bones, BV/TV was higher in the early T group compared to the late T group (Figure 3B-D). The increase in BV/TV upon T administration was mostly due to an increase in Tb.N and a concomitant decrease in Tb.Sp, as effects on Tb.Th were marginal (Supplemental Figure S3A-I). In line with the differential impact on BV/TV, the changes in Tb.N and Tb.Sp were more pronounced in the early compared to the late T group (Supplemental Figure S3A-I).

At the femoral diaphysis, DGX decreased Tt.Ar and Ct.Ar. Both parameters were restored to female control values by early and late T administration, without reaching male control levels (Figure 4A,B). Changes in Ma.Ar, Ps.Pm and Ec.Pm showed the same pattern (Figure 4C and Supplemental Figure S4A-D). These findings of reduction in cortical bone mass acquisition in the DGX group and restoration by T up to female levels in both early and late groups, were confirmed at the tibial diaphysis

(Supplemental Figure S5A-H). In line with the changes in radial bone expansion, the femoral polar moment of inertia, a proxy for bone strength, was lower in DGX-treated mice compared to female controls, and increased by both early and late T administration (Figure 4D), with a similar pattern in tibia (Supplemental Figure S5E). A three-point bending test on femur confirmed the treatment effects on bone strength (Figure 4E).

Altogether, GnRHa therapy initiated at the prepubertal stage in female mice decreased trabecular bone volume, and this effect was counteracted more efficiently by earlier start of T. In addition, reduced radial bone expansion and cortical bone strength were observed in DGX-treated animals. T administration restored those values up to female control levels without reaching male values, regardless of the time of administration.

**GnRHa administration to prepubertal female mice increases bone marrow adiposity, an effect that is counteracted by T**

Histological analysis of the murine bones revealed a higher bone marrow adiposity in female compared to male control animals, confirmed by adipocyte counting in the tibial metaphysis (Figure 5A,B). Bone marrow adipose tissue (BMAT) accumulation was markedly increased by DGX treatment, an effect that was totally reversed by T in both early and late groups (Figure 5A,B). In line with these findings, bone marrow expression levels of a panel of adipocyte markers, including the adipokines adiponectin (encoded by *Adipoq*) and leptin (*Lep*), the lipid droplet-associated protein perilipin (*Plin1*), and fatty acid binding protein 4 (*Fabp4*), were induced by DGX and strongly suppressed by T (Figure 5C-F). The mRNA levels of the key adipogenic transcription regulator peroxisome proliferator-activated receptor (PPAR)-γ (*Pparg*) remained stable (Figure 5G).

Thus, the lower bone volume in DGX-treated animals was associated with accumulation of adipocytes within the bone marrow, an effect that was completely reversed by T.

**DISCUSSION**

Puberty suppression through GnRHa is often initiated in transgender youth in order to halt the further development of secondary sexual characteristics, in most cases followed by the addition of GAH several years later.[5,6] As puberty is a period in which many sexually dimorphic traits develop under the influence of sex hormones, including body composition and peak bone mass,[8,9] the long duration of puberty suppression in transgender youth may have potential deleterious effects, which might not be fully reversed by the late exposure to sex steroids. We addressed these questions in the controlled, preclinical setting of an animal model. To our knowledge, this work, summarized in Figure 6, is the first to demonstrate the effects on bone and body composition of GnRHa initiated at the start of puberty in female mice, and to investigate the impact of timing of subsequent T initiation with regard to reversibility of GnRHa-induced effects.

In recent years, a plethora of rodent models have been developed to study the impact of gender-affirming therapy on a variety of outcomes (examples include [24-28]). The vast majority of these studies, however, mimicked the clinical approach in transgender adults. As such, those models were restricted to GAH administration and did not involve puberty suppression to avoid or halt appearance incongruent development in transgender youth. We have previously developed a model of GnRHa administration to prepubertal male mice and demonstrated a shift in body composition towards more fat and less lean mass, and a drastic impairment of both cortical and trabecular bone mass accrual.[13] Whether these observations also apply to female animals was unknown. Recently, two studies reported the use of GnRHa in female mice to suppress puberty and model the clinical trajectory proposed to trans boys. However, no data are available on the effects on bone and body composition development, as the characterization of these models was restricted to behavioral and neurobiological effects [29] or the consequences for reproduction.[30]

In our model, puberty suppression in female mice increased fat mass and decreased lean mass. These findings are in line with the observations in trans boys on GnRHa.[11,31] Of note, the increased fat accumulation in DGX-treated animals occurred in spite of a reduced food intake. The latter is in

accordance with the well-described central actions of estrogens in regulating feeding behavior.[32] Whether this diminished food intake holds true in GnRHa-treated trans boys remains to be investigated. T therapy reversed DGX-induced changes in fat and lean mass, irrespective of the timing of administration, and even shaped body composition to male control values. Again, this mirrors clinical observations. Indeed, T use for a mean duration of 2.9 years in trans boys after GnRHa-mediated puberty suppression changed body composition towards the experienced gender.[11] Regarding muscle strength, DGX treatment tended to diminish grip strength, with T restoring this parameter to female values but without reaching male control levels. A recent multicenter study including 278 adult trans men showed increased grip strength after 1 year of T,[33] but no data are available in transgender youth.

DGX-treated animals displayed reduced cortical bone mass and radial bone expansion. Importantly, these parameters were fully restored by T to control female levels, irrespective of the time of administration. It is thus plausible that the partial restoration observed in trans boys treated with GAH for at least 2 years after GnRHa-mediated puberty suppression [34-36] might be due to peak bone mass not yet being attained (age at the time of BMD assessment varied from 18 to 22 years depending on the study), while it is the case at 16 weeks in rodents.[37,38] In line with the periosteal perimeter being the most important determinant for bone strength, the latter increased upon T therapy in our model, as determined by a destructive *ex vivo* bending test, considered as the gold standard for estimating long bone fracture risk in rodents.[39] Although this finding has yet to be confirmed in humans, it is reasonable to hypothesize that fracture risk will not be increased in GAH-treated trans boys following early puberty suppression, provided that adequate T supplementation is given. Of note, a study in 18 adult trans men using T revealed an even lower fracture risk compared with age-matched cis men.[40]

Similar to our findings on muscle strength, cortical bone size and strength were increased by T up to female control levels, but did not reach male control values. This observation is in agreement with our previous work showing that skeletal sexual dimorphism is established during early puberty (i.e. before the addition of T in this model) and depends primarily on growth hormone (GH) and insulin-like growth

factor-1 (IGF-1) action. Indeed, deletion of the GH receptor in rodents, resulting in very low circulating IGF-1 levels, completely abolished sex differences in cortical bone size and strength.[41]

In our model, puberty suppression not only decreased cortical bone mass but also trabecular bone volume. Both were fully restored by T treatment. Noteworthy, trabecular bone volume was the only parameter increased to a greater extent in the early T compared to the late T group. This finding is in line with this compartment being the most sensitive to sex steroids, especially in early puberty.[13] The longer total duration of T treatment in the early group is unlikely to explain the difference, as peak bone mass was already reached at the time of bone phenotyping.[37,38] The increase in trabecular bone volume in the T groups was mostly due to an increase in trabecular number and accompanied by a reduction in growth plate thickness, suggestive of an enhanced mineralization of the growth plate. However, nose-to-tail length and lengths of tibia and femur were unaffected by either DGX or T. These data confirm recent work reporting a neutral effect on adult height of GnRHa and T in trans boys.[42]

Unexpectedly, histological evaluation of the tibiae revealed an additional effect of prolonged GnRHa administration on bone, being a dramatic but fully reversible increase in BMAT. Higher BMAT content is observed in various diseases including osteoporosis and is often associated with deterioration of bone mass.[43] In line with our findings, Nasomyont and colleagues observed greater increases in BMAT indices along with lower bone mass acquisition in transgender youth after 12 months of GnRHa compared with control participants.[44] Of note, in our model BMAT accumulation had fully disappeared following T treatment, both in the early and late T groups. Speculatively, the impact of T on BMAT could be attributable to estrogen signaling after aromatization of T into E2, as BMAT was shown to be regulated by the latter in both mice and humans.[45,46] Sex steroids modulate the differentiation of bone marrow mesenchymal stromal cells into the osteoblast and adipocyte lineages, causing a lineage shift toward the osteoblast.[47,48] Considering that in our mouse model, T increased bone mass accrual while inhibiting BMAT accumulation, we could speculate that at least part of the T effect on bone might result from actions on skeletal stem/progenitor cells in the bone marrow. In

support of this hypothesis, depletion of BMAT in mice resulted in increased bone mass secondary to enhanced endosteal bone formation, and protected from ovariectomy-induced bone loss.[49]

The main strength of this study is that we developed a mouse model able to shed light on important concerns regarding bone health in transgender youth which are at this point difficult to answer in a clinical context e.g. the impact of GnRHa started at the earliest pubertal stages and skeletal outcomes at an age when peak bone mass is already attained. Furthermore, it enabled us to explore the effects of an earlier start of GAH as well as to gain additional insight including uncovering marked changes in bone marrow adiposity. Third, using control groups of both sexes allowed to determine whether the outcomes compared better to the designated sex at birth or to the experienced gender. This study has also limitations. First, we focused on the female-to-male transition. Hence, whether our observations hold true in a male-to-female model remains to be investigated. Next, there are differences in sex steroid and bone physiology between mice and humans such as the absence of sex hormone-binding globulin in rodents, their continuous longitudinal bone growth and the lack of intracortical remodeling,[50, 51] which might limit the clinical translation of our findings. Finally, our study design did not allow to assess the relative contribution of androgen and estrogen signaling in the observed T effects.

GnRHa continuation is usually advised until gonadectomy, as a next step in the gender-affirming care. When gonadectomy is not desired by the transgender individual, GnRHa should be continued at least until adult T levels are reached, which are typically sufficient to suppress ovarian production of estrogens.[5,6] In our model, DGX was administered throughout the study to make the different T timing the only variable parameter. Although this choice makes our model more representative of transgender individuals who decide to proceed with gonadectomy rather than those stopping GnRHa after a full dose of T is reached, we don't anticipate the continuous DGX administration to have biased our results or data interpretation in any sense.

In summary, this work indicates that GnRHa administration to prepubertal female mice stimulates fat mass accumulation, lowers lean mass and muscle strength, reduces bone mass acquisition and bone

strength, and increases bone marrow adiposity. Importantly, our data demonstrate that all these effects are reversed by T, irrespective of the time of administration, with T-treated female-to-male mice achieving a body composition similar to that of adult male mice, a cortical bone mass and bone strength equal to female control animals, and trabecular bone parameters equal to male controls in case of earlier T start. Translation to equivalent clinical data in trans boys is needed, but our data suggest that trans boys may be capable of reaching a BMD and bone strength similar to cis men, at least for the trabecular compartment, with sufficiently long duration of T, provided that other measures fostering bone health (physical activity, vitamin D status, T doses,…) are in place. Thus, when confirmed in humans, our data support the safety of long-term GnRHa and subsequent GAH use in trans boys with regard to bone health.

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**FIGURE LEGENDS**

**Figure 1. Experimental design and validation of the mouse model. (A)** Four week-old female C57BL/6J mice were treated with degarelix (DGX) alone, DGX at 4 weeks supplemented with testosterone (T) at 6 weeks, or DGX at 4 weeks supplemented with T at 8 weeks. Animals were sacrificed at 16 weeks and compared to untreated mice of both sexes. **(B,C)** Representative picture (B) and wet weight (C) of the uterus from 16 week-old mice subjected to the protocol depicted in panel A (n=12/group). **(D,E)** Anogenital distance (D) and representative picture of the perineal region with the clitoris indicated by a yellow arrow (E) from the same animals (n=12/group).

**Figure 2. Body composition and muscle strength in mice prepubertally treated with GnRHa and supplemented with GAH in early or late puberty. (A)** Total body fat mass assessed by echoMRI and expressed as percentage of body weight in female C57BL/6J mice treated with degarelix (DGX) alone at 4 weeks, DGX at 4 weeks supplemented with testosterone (T) at 6 weeks, or DGX at 4 weeks supplemented with T at 8 weeks, and in untreated mice of both sexes (n=12/group). *Left panel* depicts fat mass in mice from 4 to 16 weeks of age, *right panel* shows fat mass in 16 week-old animals. **(B)** Weight of subcutaneous (*left panel*), gonadal (*middle panel*) and perirenal (*right panel*) white adipose tissue (WAT) fat pads from 16 week-old animals, expressed in mg per 100g body weight (BW) (n=12/group). **(C)** Lean body mass assessed by echoMRI and expressed as percentage of body weight in mice from 4 to 16 weeks of age (*left panel*) and in 16 week-old animals (*right panel*) (n=12/group). **(D)** Weight of gastrocnemius muscle from 16 week-old animals, expressed in mg per 100g body weight (BW) (n=12/group). **(E)** Grip strength of 16 week-old animals (n=12/group).

**Figure 3. Trabecular bone parameters in mice prepubertally treated with GnRHa and supplemented with GAH in early or late puberty. (A)** 3D reconstruction of femoral trabecular bone from 16 week-old mice subjected to the protocol depicted in Figure 1A. A representative image from each group is depicted. **(B-D)** Trabecular bone volume fraction (BV/TV) assessed by microCT in femora (B), tibiae (C) and L5 vertebral bodies (D) from the same animals (n=12/group).

**Figure 4. Cortical bone parameters in mice prepubertally treated with GnRHa and supplemented with GAH in early or late puberty. (A-D)** Total cross-sectional tissue area (Tt.Ar) (A), cortical area (Ct.Ar) (B), medullary area (Ma.Ar) (C) and polar moment of inertia (J) (D) assessed by microCT in femora from 16 week-old mice subjected to the protocol depicted in Figure 1A (n=12/group). **(E)** Ultimate bone strength assessed by a three-point bending test in femora from the same animals (n=12/group).

**Figure 5. Bone marrow adiposity in mice prepubertally treated with GnRHa and supplemented with GAH in early or late puberty. (A)** Representative H&E staining on tibiae from 16-week-old mice subjected to the protocol depicted in Figure 1A. The scale bar is 500 µm in the upper panels and 100 µm in the lower panels. **(B)** Microscopic quantification of bone marrow adipocytes in the metaphysis of tibiae from the same animals (n=12/group). **(C-G)** Relative expression levels of *Adipoq* (C), *Plin1* (D), *Fabp4* (E), *Lep* (F) and *Pparg* (G) assessed by RT-qPCR analysis on the bone marrow fraction of tibiae from the same animals (n=12/group). Data are normalized to control male levels.

**Figure 6. Summary of the mouse model developed to mimic the clinical strategy applied in trans boys, delineating the effects of GnRHa and subsequent GAH administration.** Prolonged puberty suppression impacts body composition as well as bone mass acquisition and strength in the mouse model. Several parameters are fully reversed to the levels of control adult male mice by subsequent testosterone addition, whereas some GnRHa effects are corrected partially, reaching the values seen in control adult females. Enhanced effect of an earlier start of GAH was observed on trabecular bone parameters, while the other GnRHa-induced changes were restored to the same extent irrespective of the time of GAH administration.

Figure 1

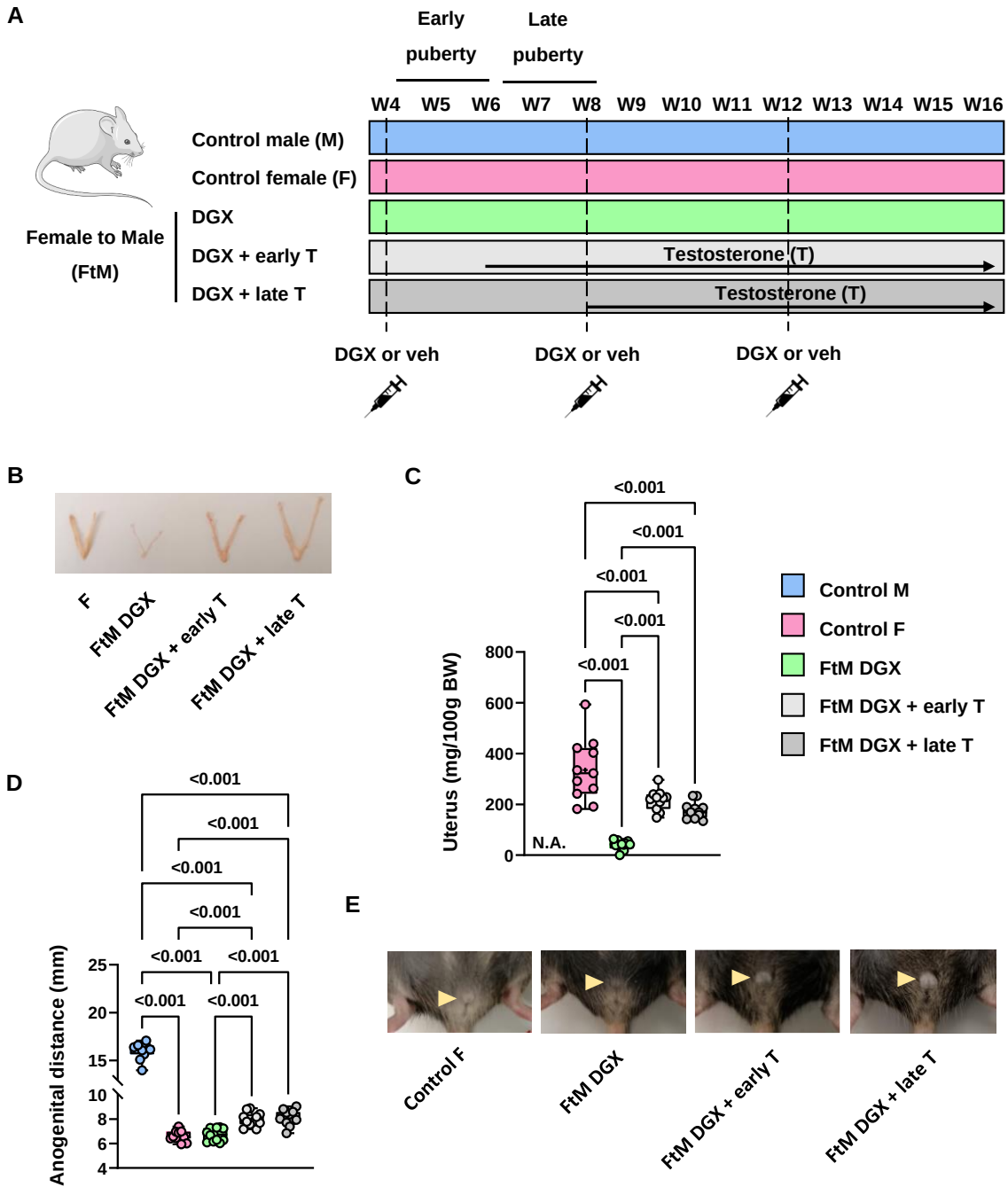

A
Early puberty
Late puberty
W4 W5 W6 W7 W8 W9 W10 W11 W12 W13 W14 W15 W16
Female to Male (FtM)
Control male (M)
Control female (F)
DGX
DGX + early T
DGX + late T
Testosterone (T)
Testosterone (T)
DGX or veh
DGX or veh
DGX or veh
B
F
FtM DGX
FtM DGX + early T
FtM DGX + late T
C
Uterus (mg/100g BW)
800
600
400
200
0
N.A.
<0.001
Control M
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T
D
Anogenital distance (mm)
25
20
15
10
8
6
4
E
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T

Figure 2

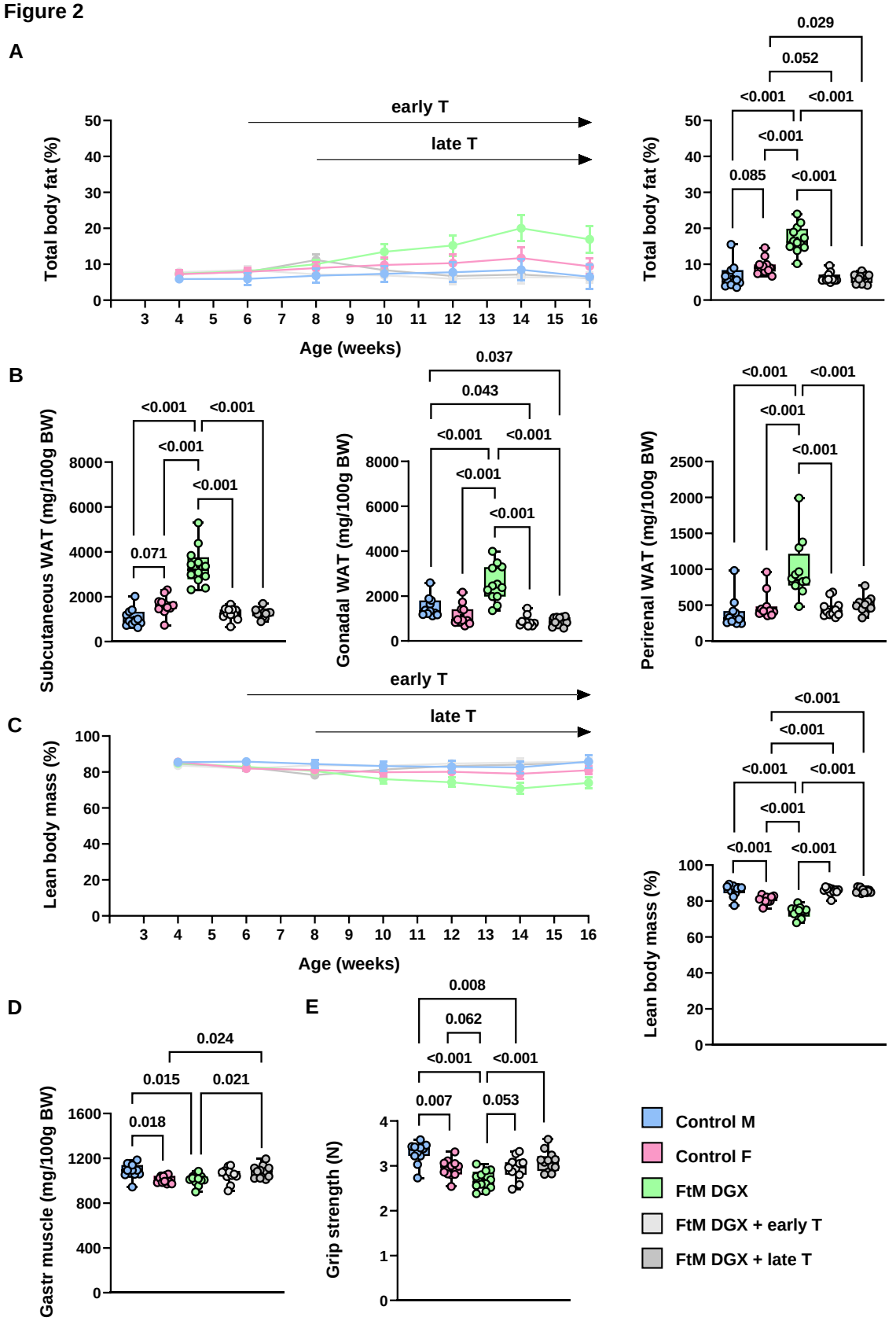

A
Total body fat (%)
early T
late T
Age (weeks)
0.029
0.052
<0.001
<0.001
<0.001
0.085
<0.001
B
Subcutaneous WAT (mg/100g BW)
Gonadal WAT (mg/100g BW)
Perirenal WAT (mg/100g BW)
0.037
0.043
0.071
C
Lean body mass (%)
D
Gastr muscle (mg/100g BW)
0.024
0.015
0.021
0.018
E
Grip strength (N)
0.008
0.062
0.007
0.053
Control M
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T

**Figure 3**

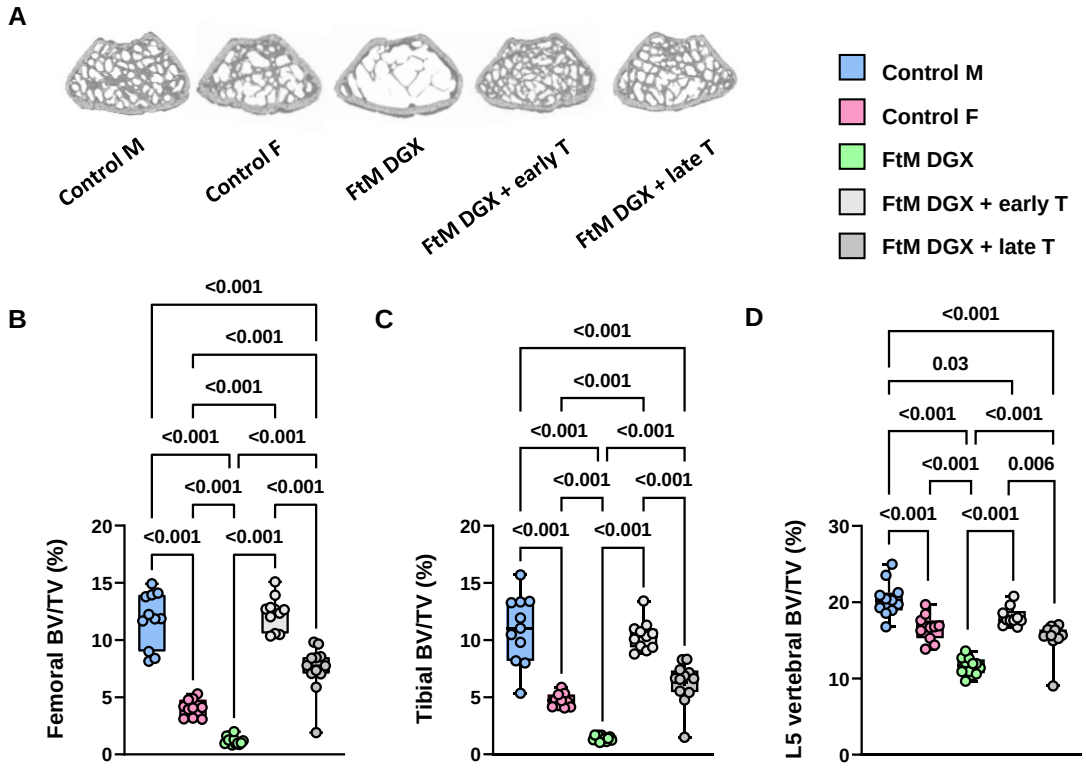

A
Control M
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T
Control M
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T
B
Femoral BV/TV (%)
C
Tibial BV/TV (%)
D
L5 vertebral BV/TV (%)
<0.001
0.03
0.006

**Figure 4**

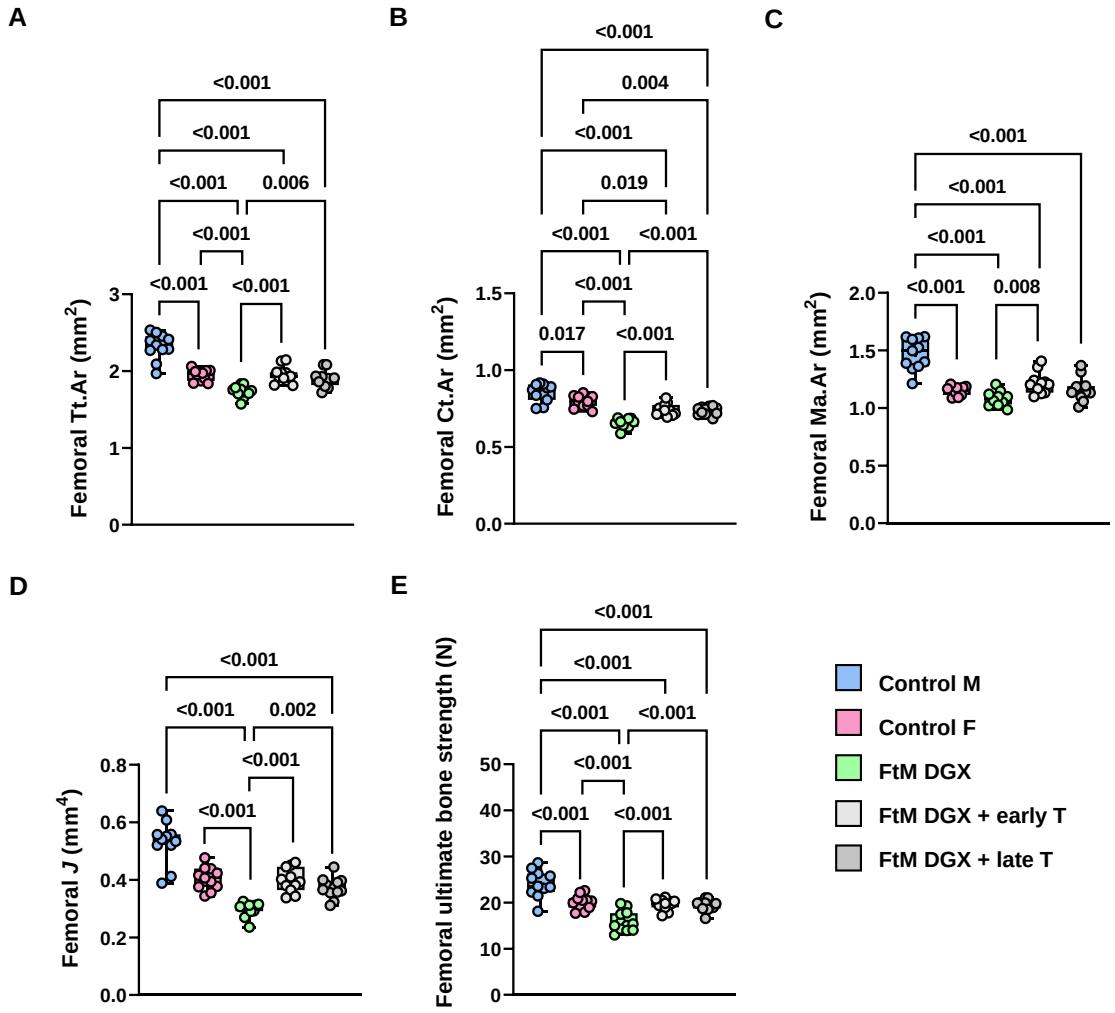

A
B
C
D
E
Femoral Tt.Ar (mm²)
Femoral Ct.Ar (mm²)
Femoral Ma.Ar (mm²)
Femoral J (mm⁴)
Femoral ultimate bone strength (N)
<0.001
0.004
0.019
0.017
0.006
0.008
0.002
Control M
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T

**Figure 5**

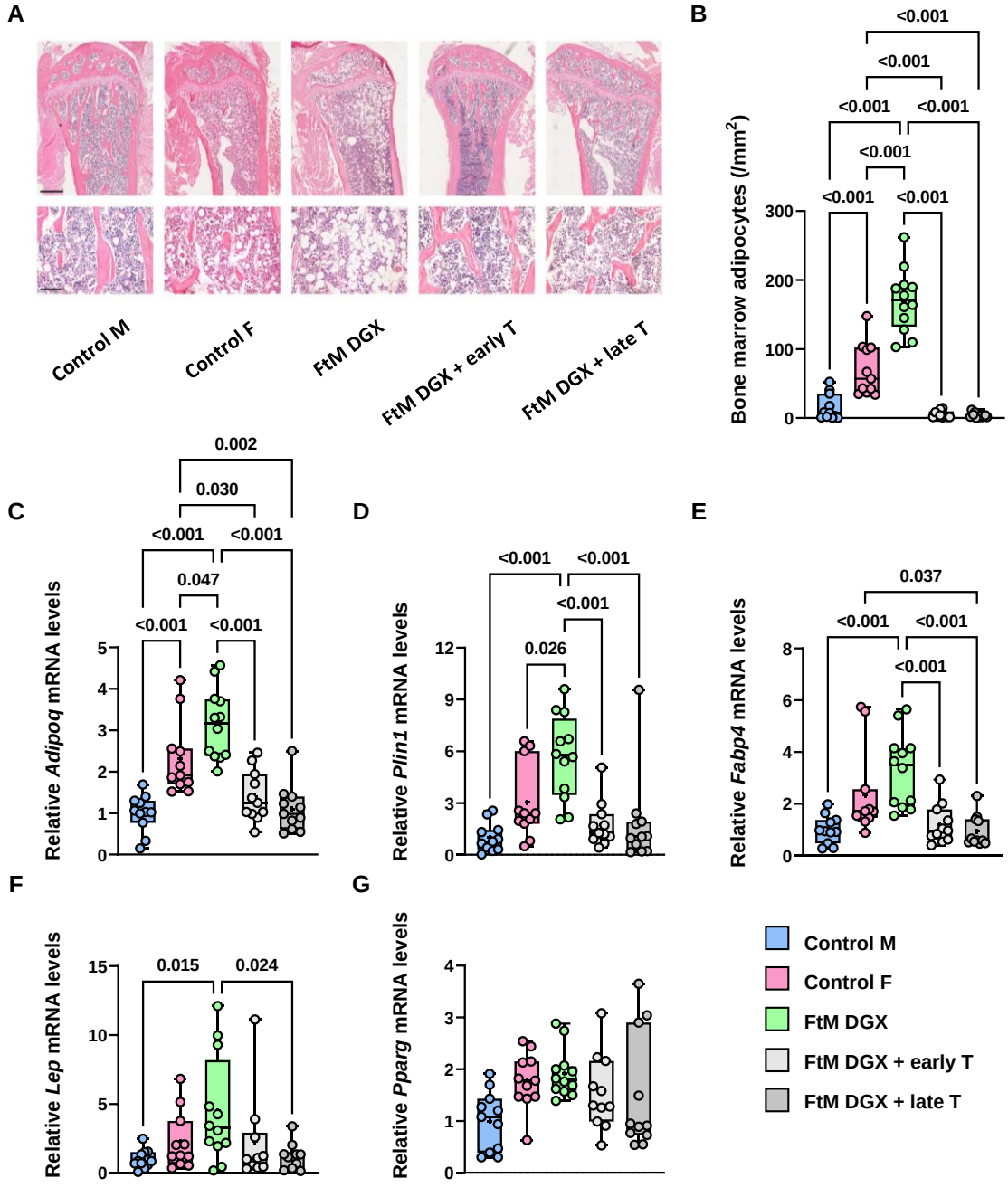
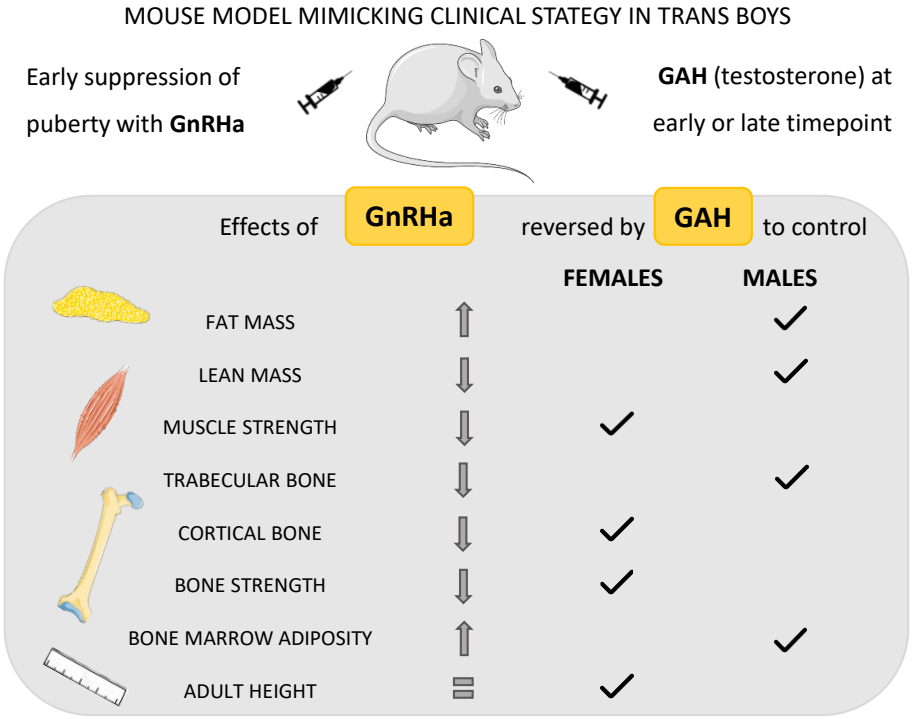

A
Control M
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T
B
Bone marrow adipocytes (/mm²)
<0.001
<0.001
<0.001
<0.001
<0.001
<0.001
<0.001
300
200
100
0
C
Relative Adipoq mRNA levels
0.002
0.030
<0.001
<0.001
0.047
<0.001
<0.001
5
4
3
2
1
0
D
Relative Plin1 mRNA levels
<0.001
<0.001
<0.001
0.026
12
9
6
3
0
E
Relative Fabp4 mRNA levels
0.037
<0.001
<0.001
<0.001
8
6
4
2
0
F
Relative Lep mRNA levels
0.015
0.024
15
10
5
0
G
Relative Pparg mRNA levels
4
3
2
1
0
Control M
Control F
FtM DGX
FtM DGX + early T
FtM DGX + late T

**Figure 6**


MOUSE MODEL MIMICKING CLINICAL STATEGY IN TRANS BOYS
Early suppression of puberty with **GnRHa**
**GAH** (testosterone) at early or late timepoint
Effects of GnRHa reversed by GAH to control
FEMALES
MALES
FAT MASS
LEAN MASS
MUSCLE STRENGTH
TRABECULAR BONE
CORTICAL BONE
BONE STRENGTH
BONE MARROW ADIPOSITY
ADULT HEIGHT