DOSE-RESPONSE RELATIONSHIPS BETWEEN GONADAL STEROIDS AND BONE TURNOVER IN MEN

Joel S. Finkelstein, M.D.

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Version 1.20

Summary of changes for V.20

The following changes have been made in the protocol to shorten and simplify it and to make it Specific for Specific Aim 1b in men age 60 and higher.

The original protocol consisted of 3 Specific Aims. Aim 1 had 2 parts:

- Specific Aim 1a. Dose-response studies in men age 20- 50 treated Goserelin acetate and various doses of a testosterone gel. This aim is finished.
- Specific Aim 1b. Dose-response in men age 60 and over treated Goserelin acetate and various doses of a testosterone gel. This aim is the subject of the manuscript under consideration by the NEJM
- Specific Aim 2: Dose-response studies in men age 20- 50 treated Goserelin acetate and various doses of a testosterone gel plus anastrozole to suppress the conversion of T to E. This aim is finished.
- Specific Aim 3: Dose-response studies in men and 20-50 treated Goserelin acetate, 5 gm of testosterone gel daily and various doses of estradiol. This Aim was never funded and it was therefore dropped

A. Deleted sections 1.2. 1.6, and 1.8 from Background as they are not directly relevant to the ongoing research.
B. Deleted old section 1.10 (very old “preliminary” data) and replaced with summary of published results from Specific Aims 1a and 2.
C. Deleted Specific Aim 2 from Section 2 and Protocol for Specific Aim 2 (Section 5.2)
D. Revised Section 5.1 so that it only reflects the Protocol for Aim 1b, particularly the fact that the double placebo group (Group 6) was randomized along with the other five groups in the protocol for Aim 1b.
E. Updated outcome measures
    1. Deleted serum osteocalcin, urine NTX, and urine DPD as outcome measures
    2. Added serum CTX as the primary measure of bone resorption.
F. Edited section 5.1 so that it would reflect only the protocol in men age 60 and over.
G. Removed PTH and urine studies from the list of measurements performed at each visit (section 5.1)
H. Updated the methodologies used to measure testosterone and estradiol (section 5.3.3.7
I. Deleted section 5.3.3.1 to 5.3.3.5 and 5.3.3.8
J. Reorganized outcome measures by groups e.g. Bone measures, Body Composition Measures etc. (section 6.1)
K. Revised Power Calculations (section 6.1.1) using data from the 20-50-year-old men who underwent the same protocol. Power calculations are now available for serum CTX and total body fat mass.
L Revised Data Analysis (section 6.1.1) to reflect the plan designed by the Principal Investigator and approved by our biostatistician (see NEJM 369:1011-22)
M Deleted sections 6.2.1 and 6.2.2 since Specific Aim 2 is complete and has been published
N Deleted section 6.3.2 since Specific Aim 3 was removed from the protocol previously (see V.17 below)
O. Deleted section 10 since the Amendment 43 sub study has been completed.

Summary of changes for V.19

a. If a subject's testosterone level rises above 1500ng/dL and either the subject's liver function tests are two times higher than the upper limit of the reference range or the hematocrit is >50%, the subject will be discontinued from the study.
b. If a subject's testosterone level rises above 1500 ng/dL but the subject's liver function tests are less than two times the upper limit and the hematocrit is less than or equal to 50%, the subject's dose will be lowered to the next highest dose and continued to be monitored at the lower dose. If the testosterone level continues to be >1500 ng/dL at the subject's next visit after switching to the lower dose, he will be withdrawn from the study.

Summary of changes for V.18

a. Added "minimal risk" procedures (DXA, HR-pQCT, cognitive testing, and physical functioning to screening consent to facilitate scheduling at the baseline visit to reduce participant burden.

Summary of changes for V.17

b. Removed Aim 3 from the protocol.
c. Removed PSA testing as an eligibility criterion and as a safety measure in the 60-75-year-old men

Summary of changes for V.16

a. Change the remuneration for participation from $1000 to $500

Summary of changes for V.15

a. Allow recruitment of subjects who failed to qualify for another research protocol in men in our unit.

Summary of changes for V.14

a. Solvay Pharmaceuticals changed to Abbott Laboratories as the supplier of AndroGel.

Summary of changes for Version 1.13.

a. Add 24-hour urine collection prior to the baseline and final visits to assess the effects of androgens on kidney stone risk profiles.
b. Measure urinary flow rates at the baseline and final visits to assess the effects of androgens on symptoms of prostate obstruction

Summary of changes for Version 1.12:

a. The entrance criteria for men over age 60 will be modified such that subjects will be excluded if:
   - They are > 75 years old
   - They report difficulty walking 2 blocks
   - Their Framingham risk score is $\geq$ 20
   - Their hematocrit is > 50
   - Their systolic BP is >160 or diastolic BP is >95.
b. Maximal dose of testosterone gel will be reduced from 10 g/day to 7.5 g/day
c. If systolic BP is >160 or diastolic BP is >95 at any follow-up visit, subjects will be asked to return to have his blood pressure re-checked within 1 week. If the SBP is still > 160 or the DBP is still > 95 the subject will be withdrawn from the study.
d. If hematocrit is >50 at any follow-up visit, subjects will be asked to return to have his hematocrit re-checked within 1 week. If the hematocrit is still > 50 the subject will be withdrawn from the study.
e. Language has been added to the consent form to inform subjects of possible cardiovascular disease risk.
f. A DSMB will be formed consisting of 3 individuals. One member will be a cardiologist, one will be an endocrinologist, and one will be an oncologist. The DSMB will review the safety of the protocol at least every 6 months.

1. BACKGROUND AND SIGNIFICANCE

1.1 Introduction and importance of the proposed research

The role of androgen deficiency in many of the physiologic changes of aging remains unclear. Many of the changes that accompany aging including bone loss, muscle wasting, fat accumulation, decreased strength, and decreased sexual function may be related to the gradual decline in serum testosterone levels with age (1-4). Although we know that bone loss, muscle wasting, fat accumulation, decreased strength, and decreased sexual function occur when men become severely hypogonadal and improve when testosterone is replaced to mid-normal young adult levels, the level of androgen or estrogen deficiency at which adverse physiological changes begin is unknown. Moreover, although we know that there is a linear dose-response relationship between serum testosterone levels and muscle volume, strength, and fat mass when serum testosterone varies from low-normal and very high levels (5), the dose-response relationships between testosterone levels and important physiological processes (e.g. bone turnover, body composition, strength, lipids, and hypogonadal symptoms) has never been explored at the lower range. Studies of the lower portion of these dose-response curves are particularly important because they will mimic the testosterone levels that occur with normal aging. The data derived from the proposed studies will help to clarify the degree of androgen and estrogen deficiency at which various adverse changes begin, and the dose-response relationships between various end-points and gonadal steroids as levels vary from normal to severely hypogonadal. This information should help clinicians make rational decisions on the use of androgen replacement in men. Moreover, these studies will help determine the proportion of testosterone action that is explained by conversion to estrogens. Thus, the proposed studies will provide unique information on the physiological effects of androgens and estrogens on bone metabolism and other steroid-responsive processes in men.

1.3 Differential effects of androgens and estrogens on bone

In women, estrogen deficiency is known to be the major cause of postmenopausal bone loss. The relative roles of estrogens and androgens in the pathogenesis of male osteoporosis are much less clear though gonadal steroids clearly play a key role in the regulation of bone metabolism in men. For example, the dramatic increase in bone mineral content and density that occurs during puberty in boys is induced by the pubertal increase in serum testosterone (and/or estrogen) production (8-12). As in women, men who become severely hypogonadal lose bone rapidly (31, 32). Despite these well-known observations, the physiological effects of androgens and estrogens on bone metabolism in men have not been well studied.

For many years it was assumed that androgens were the primary regulators of bone metabolism in men. This assumption was based on several clinical observations including finding that men have higher cortical BMD and lower fracture rates than women (33). Several observations support the hypothesis that androgens themselves play an important role in bone metabolism. First, human osteoblast and osteoclast-like cells contain androgen receptors (34-37) suggesting that androgens might affect bone formation and resorption directly. Second, aromatizable and nonnegotiable androgens stimulate osteoblast proliferation and differentiation in vitro (38, 39) and decrease the resorption activity of osteoclasts in cell culture, an effect that is blocked by co-treatment with the specific androgen antagonist hydroxyflutamide (34). Third, both aromatizable and non-aromatizable androgens prevent the increase in osteoclastogenesis and trabecular bone loss that occurs after orchidectomy in rodents (40-43). Fourth, femoral cortical thickness of androgen-resistant rats and spine and hip BMD of androgen-resistant humans are lower than those in male controls (44-47). Finally, castration increases bone turnover and causes bone loss in both estrogen receptor (ER) alpha and ER alpha and beta knockout mice and this high turnover bone loss can be prevented by testosterone administration, but not estradiol administration (48, 49). Moreover, selective ER blockade does not impair the bone-sparing effect of testosterone in orchidectomized rats (50) whereas androgen receptor blockade does block the bone sparing effect of testosterone in ER alpha knockout mice (48). These findings demonstrate that the estrogen receptor is not required for androgen-mediated skeletal maintenance in mice and strongly suggest an independent role for androgens themselves in skeletal homeostasis. Taken together, these observations suggest a direct role for androgens in the regulation of bone metabolism that does not require aromatization to estrogens, though each of the models leading to this conclusion has limitations.

Several lines of evidence indicate that estrogens also play an important, if not pivotal role, in male bone metabolism. First, most epidemiological studies have found that serum estrogen levels in men are associated more strongly with BMD than are serum testosterone levels (21, 51, 52). Moreover, lower estradiol levels, but not lower androgen levels, are associated with vertebral fractures in men (53). These epidemiological data are difficult to interpret, however, both because of some discordant results and because of the limited physiological relevance of associations between single hormone levels and BMD (a variable that represents the cumulative effects of a lifetime of bone accrual and resorption).

Other approaches also suggest that estrogens help regulate bone metabolism in men. As with androgens, estrogen receptors are expressed on both osteoblast and osteoclast-like cells suggesting that estrogens might affect bone formation and resorption directly (54-59). Femur length, trabecular thickness, and serum osteocalcin levels are reduced in male mice with severe estrogen deficiency due to targeted gene inactivation of the aromatase gene (ArKO) (60). In addition, ArKO mice are osteopenic (60). Cortical bone mineral content and bone size are decreased in male mice with estrogen resistance due to targeted inactivation of the estrogen receptor alpha gene (ERKO) or both the estrogen receptor alpha and beta genes (DERKO) (61, 62). There is no apparent bone phenotype in male mice with inactivation of the estrogen receptor-β-gene alone (62). These knockout experiments clearly show a role for estrogen in the male skeleton but cannot differentiate whether the effect of estrogens is on skeletal development or on skeletal maintenance and whether the findings results from direct hormonal effects on bone.

During the last 10 years, a small number of adult men who have the human equivalent of the mouse knockouts of the estrogen receptor-alpha and aromatase genes have been identified. Smith et al. described a 28-year old man with a mutation in the estrogen receptor-α (ER-α) gene resulting in estrogen resistance (63). This man had markedly elevated serum estrone and estradiol levels (145 and 119 pg./mL, respectively), normal serum concentrations of total and free testosterone, tall stature (204 cm), and continued linear growth. His skeletal phenotype was further marked by incomplete closure of the epiphyses, severe osteopenia (lumbar spine T score of – 3.1), and elevations in biochemical markers of both bone formation and resorption. Pharmacological estrogen administration had no measurable effect on any of these parameters. Three men with mutations in the gene encoding the aromatase enzyme have been described (64-66). As expected, their serum estradiol levels are extremely low and their serum testosterone levels are high. Like the man with estrogen resistance, these men exhibit tall stature, incomplete closure of the epiphyses, and osteopenia. When treated with estrogen, their epiphyses closed and BMD increased dramatically (65, 66). Taken together, these findings strongly suggest that estrogen is needed for normal skeletal development and to achieve a normal peak BMD. The role of estrogens in the maintenance of adult male bone metabolism cannot be determined from these men, however.

We, and others, have studied the roles of androgens and estrogen in the maintenance of normal bone turnover in men. Administration of anastrozole, a potent aromatase inhibitor, to 8 young men for 10 weeks did not alter markers of bone formation even though estradiol levels decreased by nearly 50% (67). We recently administered anastrozole or placebo for 12 weeks to 37 elderly men with mild hypogonadism. As in the prior study, serum E_2 levels decreased significantly in men treated with anastrozole but there were no changes in biochemical markers of bone formation or resorption (see section C.3). Interpretation of both of these studies is confounded, however, by concomitant increases in serum T levels in these men. Direct administration of estradiol to men with prostate cancer with GnRH analog-induced hypogonadism reduces bone resorption markers modestly but does not affect bone formation markers (68). In men undergoing male-to-female gender reassignment, estradiol administration increased spinal bone density and reduced bone turnover, even in the setting of markedly low testosterone levels (69, 70). These latter two observations demonstrate that estrogens (often administered in <u>pharmacological</u> doses) have anti-resorptive effects in men, as they do in women.

Two recent studies have provided important information related to the <u>physiological</u> effects androgens and estrogens in the maintenance of bone metabolism in men. Falahati-Nini et al. administered a GnRH analog, an aromatase inhibitor, transdermal testosterone, and transdermal estradiol to 59 elderly men for three weeks and then selectively removed various agents to induce states of selective testosterone deficiency, selective estradiol deficiency, or combined testosterone and estrogen deficiency for an additional 3 weeks (71). They

then examined a series of bone formation and resorption markers. Using this model, they concluded that estradiol plays the major role in the maintenance of bone resorption though they could not rule out a role for testosterone, probably because their study period (3 weeks) may have been too short to assess bone turnover adequately. Effects of androgens and estrogens on bone formation markers were less clear, particularly because formation markers decreased during short term hypogonadism in contrast to the well-known increases that occur during long-term hypogonadism. We recently treated 70 young men with a GnRH analog alone (to create severe androgen and estrogen deficiency), a GnRH analog plus topical testosterone (to maintain normal testosterone and estradiol levels), or a GnRH analog plus topical testosterone plus an aromatase inhibitor (to create selective estrogen deficiency) for 12 weeks (72) (see C.1). Using this model, we found that both androgens and estrogens play physiologically important roles in the maintenance of bone turnover in adult men. Studies such as these and such as those that we propose here are needed to clarify the physiological roles of androgens and estrogens in adult male bone metabolism.

1.4 Gonadal steroids and bone turnover in men

It is well known the BMD is lower than expected in hypogonadal men (33) and this bone loss appears to be due to an increase in bone resorption that is greater than the concomitant increase in bone formation (30-32, 72-75). Long-term hypogonadism increases both markers of bone resorption (urinary deoxypyridinoline, N-telopeptide, and hydroxyproline excretion and serum N-telopeptide), as well as markers of bone formation (serum osteocalcin, and bone-specific alkaline phosphatase) (30-32, 72-75). For reasons that are unclear, biochemical markers of bone formation (serum osteocalcin, carboxy-terminal propeptide of type I procollagen, and amino-terminal propeptide of type I procollagen) decline significantly in the first few weeks of GnRH analog-induced hypogonadism (71, 72). This change may reflect direct actions on osteoblasts before complicated osteoblastic responses to bone resorption dominate or may be due to the initial increase in testosterone levels after GnRH analog administration. The increase in bone turnover in men with long-term hypogonadism is consistent with data from animal models of hypogonadal bone loss. In mice, for example, orchidectomy increases osteoclastogenesis and bone turnover and this increase is prevented by administration of either aromatizable or non-aromatizable androgens (43). In aged male rats, castration increases trabecular and endosteal bone resorption, though testosterone also decreases periosteal bone formation (76).

Several studies have examined the effects of androgen administration on bone turnover in both normal and hypogonadal men. Supraphysiological doses of testosterone increase bone formation markers in hypogonadal men (77). In normal young men, pharmacological doses of intramuscular testosterone increase serum OC levels with no effect on urinary OHP excretion (78). We observed similar increases in bone formation markers when we administered supraphysiological doses of testosterone to normal men with GnRH analog-induced hypogonadism (see section C.2). In contrast, a modest dose of testosterone reduces bone resorption and formation markers in middle aged osteoporotic men (79). In a 3-year study of elderly men with normal or mildly low serum testosterone levels, transdermal testosterone administration did not alter markers of bone turnover (80) although intramuscular testosterone reduced bone resorption significantly in a short-term study of a similar population (81). In young men with primary or secondary hypogonadism, doses of intramuscular testosterone that produce normal “trough” testosterone levels (i.e. the level achieved just before the next testosterone dose) decrease markers of bone formation and resorption significantly (82). Similarly, administration of a topical testosterone gel (which maintains stable testosterone levels throughout the day) in doses that produce mid-normal range testosterone levels, also suppresses bone turnover back into the normal range in mildly hypogonadal men (83). Bone resorption markers also decline in normal men with GnRH analog-induced hypogonadism who receive doses of intramuscular testosterone that produce “trough” testosterone levels that are slightly below the normal range (see section 1.10.2). It seems likely that the ability of intramuscular testosterone to suppress bone resorption despite slightly low “trough” levels is due to “peak” testosterone levels that are transiently supraphysiological. Taken together, these findings suggest that physiological and supraphysiological doses of testosterone inhibit bone resorption while pharmacological doses, or doses that produce transiently elevated levels of testosterone (such as intramuscular preparations), may also stimulate bone formation. Additionally, because the testosterone administered in these studies is also converted to estrogen, it is impossible to determine whether observed effects on bone turnover are related to androgen action, estrogen action, or a combination of the two. Finally, because testosterone gels

maintain a more physiological profile of testosterone than parenterally-injected testosterone, these data suggest that administration of testosterone by a topical gel is preferred to intramuscular administration when studying the physiological effects of gonadal steroids on bone turnover.

1.5 Bone density in men with severe hypogonadism due to castration or GnRH analogs

Hypogonadism is present in approximately 15-36% of men with documented osteoporosis (84-86) and elderly men with hypogonadism are 6.5 times more likely to have a minimal trauma hip fracture than are eugonadal elderly men (87). Thus, conditions of gonadal steroid deficiency, though diverse, underscore the integral importance of normal gonadal function in male bone integrity. Unlike in women in whom severe primary hypogonadism is common (due to the menopause), severe primary hypogonadism (with pre-pubertal testosterone levels) is rare in men and is almost exclusively due to castration. BMD declines and bone turnover increases in men who are castrated (32, 88-90). In a retrospective study of 235 men with prostate cancer, BMD of the femoral neck was 13% lower in men who underwent castration versus those who did not (88). In addition, after 7 years of observation, nearly 30% of men who underwent castration had experienced an osteoporotic fracture compared with 1% of men who were not castrated. The most common cause of severe hypogonadism currently is GnRH analog therapy for prostate cancer. GnRH analogs are potent, reversible inhibitors of the hypothalamic-pituitary-gonadal axis and reliably reduce serum testosterone and estradiol to pre-pubertal levels, though there is a short initial period (1-2 weeks) during which testosterone levels increase. Thus, GnRH analog administration provides a powerful model to study the effects of severe hypogonadism on bone metabolism in men. GnRH analog therapy reduces BMD and increases bone turnover in men with prostate cancer or benign prostatic hypertrophy (31, 73-75, 90-92). Prospective studies demonstrate that GnRH analog-induced bone loss is progressive over 12 to 18 months, though there is considerable variation among individuals (31, 73-75). As with castration, long-term GnRH analog therapy increases fracture risk in men with prostate cancer (93). Because serum testosterone and estradiol levels can be reversibly suppressed to castrate levels with GnRH analogs, these agents provide an ideal model to investigate the effects of selective replacement with various doses of androgens and estrogens on bone metabolism in men (i.e. a “Leydig cell clamp”, see 5.1).

1.7 Gonadal steroids and body composition

The anabolic and nitrogen-retaining effects of androgens in animals have long been known and many recent studies have examined the effects of androgens on body composition in humans. Observational studies suggest that muscle mass is decreased and fat mass is increased in men with low circulating serum androgen levels (17, 99, 100). We recently reported that GnRH analog-induced hypogonadism increases fat mass and decreases lean mass in men with prostate cancer when assessed by DXA (101). Surprisingly, when body composition was assessed by computed tomography in these men, we found that abdominal fat increased due to increases in subcutaneous fat with no changes in visceral fat (101), a pattern of fat change that differs from that reported in most cross-sectional studies of hypogonadal men. These data demonstrate that severe androgen and estrogen deficiency cause undesirable changes in body composition in men.

The effects of T replacement in hypogonadal men have also been well studied. We treated 36 hypogonadal men (7 with primary and 29 with secondary hypogonadism) with replacement doses of testosterone enanthate (82). Testosterone therapy reduced subcutaneous fat mass and increased lean muscle mass in these men (82). Similar findings have been reported in other studies of men with hypogonadism due to pituitary or gonadal disease (77, 102-105). In fact, significant changes in lean mass, muscle size, and muscle strength were seen with standard doses of T replacement in just 7 hypogonadal men in only 10 weeks (105), suggesting that it is likely that we will see significant effects of androgen depletion within 16 weeks in a much larger group of men in this proposal (see section D). In older men with mildly reduced serum testosterone levels (i.e. functional hypogonadism or “andropausal” men), the effects of androgen administration on body composition are also generally positive but beneficial effects on strength have not been firmly established (81, 98, 106, 107).

Pharmacological doses of testosterone increase muscle size and strength in men who are eugonadal at baseline (108) and fat free mass increases progressively as the dose of exogenous testosterone increases (5). The latter study utilized a design similar to that proposed in section D of this application except that testosterone was given by intramuscular injection so that nadir T levels ranged from 253 to 2,370 ng/dL after doses of 25 to 600 mg of testosterone enanthate weekly and peak levels were likely much higher. No information is available on the degree of hypogonadism necessary to induce maladaptive changes in body composition or on the minimal amount of androgen replacement that is necessary to prevent those changes from occurring in profoundly hypogonadal men. The current proposal will examine the dose-response relationship between androgens and body composition should provide that important information.

Estrogens also play an important role in regulation of body composition. Surprisingly, estrogen seems to reduce fat mass. For example, fat mass is increased in male mice with homozygous inactivation of either the estrogen receptor-alpha gene (109) or the aromatase gene (110). In addition, estrogen administration prevents increases in fat mass due to castration in male mice (41). In postmenopausal women, estrogen replacement reduces weight gain and fat accumulation (111). We recently found that fat mass increases less in men with prostate cancer when treated with bicalutamide (an androgen receptor blocker that increases serum estradiol levels) than when similar men are treated with a GnRH agonist (Smith and Finkelstein, unpublished data). Thus, in this proposal we also plan to explore the dose-response relationships between estradiol and several measures of body composition in normal men.

1.9 Gonadal steroids and erectile function, fatigue, quality of life, and vasomotor symptoms

Loss of libido and sexual function are cardinal signs of severe hypogonadism. In addition, severely hypogonadal men often experience the same vasomotor flushing (hot flashes) that occurs in postmenopausal women. Still, the degree to which testosterone and/or estradiol levels must fall to affect sexual function or other symptoms is unknown. When normal men are treated with a GnRH agonist to suppress gonadal steroid levels and then replaced with testosterone in a dose that produces low-normal serum T levels (mean 302 ng/dL), sexual function in maintained (126). Similarly, sexual function is similar in men treated with a GnRH agonist and a variety of testosterone doses that produced nadir serum T levels ranging from 253 ng/dL to 2370 ng/dL (5) or a single dose of T that produced mean T levels above 800 ng/dL (119). In contrast, transdermal T replacement improves sexual function (libido and potency) in frankly hypogonadal men (mean baseline T <100 ng/dL) (103, 127). Testosterone also increases sense of energy and decreases irritability, tiredness, nervousness, sadness, and anger in frankly hypogonadal men (103, 128). In this proposal, we will utilize validated instruments to assess the dose-response relationships between gonadal steroids and sexual function, vasomotor symptoms, fatigue, and quality of life to establish the level of androgen and/or estrogen deficiency at which alterations in these parameters occur. This information may help provide a better understanding of the pathogenesis of some important symptoms of aging

1.10 Progress Report: Key results from Specific Aims 1 and 2.

The protocols for Specific Aims 1 and 2 have been completed and the primary results have been published. Briefly we recruited 198 health men age 20-50 and randomly assigned them to receive a GnRH agonist (goserelin acetate) plus either 0, 1.25, 2.5, 5, or 10 g of a testosterone gel daily for 16 weeks (Cohort 1) or the same doses of testosterone gel plus anastrozole to lower serum estradiol to castrate levels (Cohort 2). We found distinct dose-response relationships between testosterone dose and total body lean and fat mass, subcutaneous fat area, thigh muscle area and leg press strength and sexual function in Cohort 1 (1) as well as serum CTX levels and L4 trabecular BMD (2) but not in spine or hip BMD by DXA or intraabdominal fat area by CT. The dose response relationships were quite variable with some measures (e.g. fat mass) worsening with very mild reductions of gonadal steroids whereas others (e.g. lean mass and erectile function) were maintained until testosterone levels fell to near castrate levels (1). By repeating this experiment in a separate group of 20-50 year old men who also received a potent aromatase inhibitor, and comparing the dose-responses with those in cohort 1, we were able to determine whether changes in each outcome measure were due to androgen deficiency, estrogen deficiency, or both. We found that the accumulation of body fat, the increase in bone resorption, and the decline in trabecular BMD that occur when men become severely hypogonadal are largely, if not exclusively, due to estrogen deficiency (1, 2). Moreover, estrogen

deficiency is responsible for much of the decline in sexual function in hypogonadal men (1). Lean mass, muscle area, and muscle strength are regulated exclusively by androgens (1).

We have also published manuscripts describing the hormonal basis for vasomotor symptoms in men (3), the role of FSH in the regulation of bone turnover in men (4), and the role of gonadal steroids in the glcosylation of immunoglobulins (5), and the regulation of beta natriuretic peptide levels by gonadal steroids (Bachman et al., in press).

2. SPECIFIC AIMS

Gonadal steroids have major effects on bone metabolism in men. Data from epidemiological and experimental studies suggest that both androgens and estrogens play important roles in bone development and in adult skeletal homeostasis. Bone mineral density (BMD) is reduced in men with hypogonadism due to gonadotropin hormone-releasing hormone deficiency (GnRH) (94, 131), castration or GnRH analog administration (31, 73, 74, 88, 91), or acquired pituitary or testicular disease (82, 95, 97, 132). Despite the well-documented reductions in BMD in these various patient groups, these clinical disorders are relatively uncommon. Serum testosterone and free testosterone levels also decline, however, as normal men age and serum testosterone levels are below the lower end of the normal adult male range in approximately 20% of men over age 60 (4, 16, 133-135). Because hypogonadism leads to bone loss, many millions of men may be at risk for developing osteoporosis due to the normal decline in sex steroids that occurs with aging. Thus, it is important to determine the approximate levels of gonadal steroids at which bone loss begins to establish which men are at risk for hypogonadism-induced bone loss as they age. The overarching goal of this proposal is to determine the dose-response relationship between gonadal steroids and bone turnover in adult men and then to dissect the distinct dose-response relationships of androgens and estrogens on bone turnover in adult men. Secondary aims are to determine the dose-response relationships between gonadal steroids and body composition, strength, lipoproteins, libido, and quality of life measures. The specific aims for this proposal are:

Specific Aim 1: To determine the levels of testosterone and estradiol at which bone turnover increases in normal young and old men.
When adult men develop hypogonadism, serum testosterone (T) and estradiol (E_2) levels both decline. In adult men who become severely hypogonadal, i.e. men with serum testosterone and estradiol levels similar to those of pre-pubertal boys, bone turnover increases. The degree of hypogonadism required to increase bone turnover is unknown, however. To investigate this issue, we will treat normal adult men with a GnRH analog to reduce serum testosterone and estradiol to pre-pubertal levels for 16 weeks. Normal men age 20-50 and 60-75 will be randomly assigned to receive no testosterone replacement or 1 of several graded doses of a topical testosterone gel (1.25 gm, 2.5 gm, 5 gm or 10 gm/day) to produce mean testosterone and estradiol levels that range from pre-pubertal to high-normal. (Note the 10 gm dose was later reduced to 7.5 gm/day for men age 60-75). Because most of the circulating estradiol in men comes from peripheral aromatization, lowering serum testosterone levels will induce proportionate reductions in serum estradiol levels. Biochemical markers of bone turnover will be measured every 4 wk. Serum testosterone and estradiol levels will be related to bone turnover markers to establish the level of each gonadal steroid at which bone turnover begins to increase. Dose-response relationships will also be established for body composition, strength, lipids, and symptoms of hypogonadism.

Hypotheses: Bone resorption will begin to increase when serum testosterone levels fall to 200 to 300 ng/dL in young and old men and then increases progressively as serum testosterone (and estradiol) levels are reduced further. Dose-response relationships for changes will vary depending on the endpoint with symptoms of hypogonadism (e.g. decreased libido) requiring the greatest decreases in testosterone levels.

3. SUBJECT SELECTION

3.1 Study population

For each Specific Aim, we will recruit men who meet the following criteria:

Inclusion criteria:

1. Age 20-50 (completed) or 60 -75

Exclusion criteria:

1. History of significant cardiac, renal, pulmonary, hepatic, benign prostatic hyperplasia, or malignant disease, current alcohol or illicit drug abuse, or major psychiatric disorders.
2. Current diagnoses of disorders known to affect bone metabolism including hyperthyroidism, hyperparathyroidism, osteomalacia, or Paget's disease.
3. Current use of medications known to affect bone metabolism including estrogens, androgens, anti-estrogens, bisphosphonates, denosumab, calcitonin, fluoride, oral or inhaled glucocorticoids, suppressive doses of thyroxine, lithium, pharmacological doses of vitamin D (greater than 2000 IU/day), or anti-convulsants.
4. Cognitive or intellectual impairment that precludes complete understanding of the study protocol.
5. History of deep vein thrombosis, pulmonary embolism, or clotting disorders.
6. Serum 25-OH vitamin D < 15 ng/mL
7. Serum PTH < 10 or > 65 pg./mL
8. Serum TSH < 0.5 or > 5.0 U/L
9. Serum calcium > 10.6 mg/dL
10. Serum creatinine > 2 mg/dL
11. Serum AST or ALT > 2x the upper limit of normal
12. Serum bilirubin > 2 mg/dL
13. Serum alkaline phosphatase > 150 U/L
14. Plasma hemoglobin < 11 gm/dL.
15. Fracture within the last 6 months.
16. Serum testosterone level < 270 or > 1070 ng/dL
17. History of violent behavior.

Additional exclusion criteria for men age 60-75:

1. Systolic blood pressure > 160
2. Diastolic blood pressure > 95
3. Framingham risk score $\geq$20%
4. Hematocrit > 50%
5. Unable to walk 2 blocks without difficulty
6. IPSS score >19 (for men over age 60 to 75)

Note: If screening serum 25-OH vitamin D levels are < 15 ng/mL or if serum PTH levels are > 65 pg./mL, subjects will be treated with vitamin D (400-800 units/day) and/or calcium (500 mg BID) for 2 to 12 weeks and the test will then be repeated. If the 25-OH vitamin D level is $\leq$ 12 ng/mL the investigator will have the option of treating the subject with 50,000 units of vitamin D daily for a week or 3 times weekly for up to 1 month and then repeating the level.

Informed consent for the following tests, all of which are classified as “minimal risk” by the IRB and so that research assistants may obtain consent, will also be obtained at the screening visits so that these tests may be conducted at a time that facilitates the scheduling of the subjects’ other screening and baseline visit testing in order to minimize participant burden by decreasing the length of the visits.

1. DXA of hip, spine, and total body
2. HR-pQCT of radius and tibia

3. Tests of cognitive function (East Boston Memory Test and Ray Complex Figure Drawing Test)
4. Tests of physical function (Sit to stand, 40 foot walk, and grip strength)
5. 24-hour urine collection for kidney stone risk

3.2 Recruitment strategy

We will send letters in batches of 10,000 each to men whose age is within the study age range and who live within a 10-mile radius of Massachusetts General Hospital. The letter will describe the study and will contain a brief eligibility questionnaire and will ask about the subject's willingness to receive a call with further information about the study. Interested subjects will be asked to return a postage pre-paid return envelope that is included with the letter. The names and addresses used for these mailings will be obtained by purchasing commercially-available mailing lists that are in the public domain. We have used this strategy to recruit subjects successfully for many studies in the past. To enhance recruitment of minority subjects, a disproportionately high number of these letters will be sent to men who live in areas with a high percentage of minority subjects. Other strategies that will be used to enhance recruitment of minority subjects will include placing IRB-approved ads in local newspapers that service minority areas and posting IRB-approved notices at local community health centers.

To ensure compliance with HIPAA regulations, subjects being pre-screened by telephone will be informed of the nature and sensitivity of all questions at the beginning of the conversation. They will be asked whether this is an appropriate time for them to answer these questions and told how long the phone call is expected to take. All questionnaires or screening tools that will be used will be approved by Partners HealthCare Human Research Committee before they are used. Subjects will be offered the option of completing the pre-screening in person if they wish.

To maintain confidentiality of subjects pre-screened by telephone, we will record only the subject's first name or initials at the beginning of the screening conversation. We will explain to the subject that he will be asked a set of questions to determine eligibility and that at the end, only if he appears to be eligible and is interested in pursuing the study, will he be asked to provide contact/identifying information (e.g. last name, address, birth date, Social Security number or hospital medical record number). By following this procedure, two distinct categories of healthcare information will be created during pre-screening: 1) healthcare information on non-identifiable individuals and 2) healthcare information on identifiable individuals. For those who have not provided identifying information at the end of the screening conversation, their privacy and the confidentiality of their information are protected. Because this healthcare information is not identifiable, it is not subject to HIPAA privacy regulations. For those who have provided identifying information at the end of the screen conversation, we will obtain permission from the Partners HealthCare Human Research Committee to temporarily waive the HIPAA requirement to obtain written authorization until the subject meets with study staff to discuss the study further and sign the consent form. At that time, the subject will be asked to sign the written authorization to use and disclose his identifiable healthcare information and will be given a copy of the hospital's Privacy Notice unless he has previously received one during interactions with the hospital. For subjects who do not ultimately pursue the study, all identifiable information will be blacked out or cut off as soon as it is clear that the individual will not be enrolled or, alternatively, all screening information on those individuals will be shredded.

Subjects will be paid a total of $500 if they complete the entire study. Subjects will receive $250 after the first 8 weeks and the remaining $250 at the completion of the study (week 16). Subjects who complete both 24-hour urine collections will be paid an additional $50 after their final study visit.

4. SUBJECT ENROLLMENT

4.1 Randomization

Subjects will be randomly assigned to various treatment groups by a statistician on the MGH General Clinical Research Center (GCRC) using a standard computerized randomization program.

4.2 Informed consent

Written informed consent will be obtained using a consent form that has been approved by the Partners Institutional Review Board before subjects undergo any study procedures other than asking simple questions to determine potential eligibility and interest in the study. Separate consent forms will be administered for the screening visit and for the remainder of the protocol visits. For the screening visit, informed consent will be obtained by a licensed physician, nurse practitioner, or qualified research assistant who has been approved by the IRB. We are asking permission to allow research assistants to administer the screening consent form because the screening visit involves minimal risk (i.e. the only procedure is a single blood draw and no medications are administered). For the remainder of the study protocol, informed consent will be obtained by a licensed physician who has been approved by the IRB. All subjects will be informed regarding the purpose of the research, the details of the study protocol, risks and benefits, alternatives to participation, costs, reimbursements, their right to privacy and confidentiality, their right to refuse to participate or withdraw from the study at any time, their rights in the event of a study-induced injury, and whom to contact for questions about the study. Subjects will be given a copy of their signed consent form and an additional copy will be kept in our research files.

4.3 Treatment assignment and randomization

See sections 4.1 and 5.

5. STUDY PROCEDURES AND BIOSTATISTICAL ANALYSIS

Because each specific aim shares common elements but involve**s** distinct cohorts and has some distinct issues of data analysis, they are presented separately and sequentially.

5.1 Protocol for Specific Aim 1

Subjects will be screened on the MGH General Clinical Research Center (GCRC) or at the MGH Osteoporosis Research Center. After obtaining informed consent for the screening procedures, subjects will undergo a complete history and physical examination. If no exclusionary findings are noted during the history and physical examination, blood will be drawn to measure hemoglobin, routine chemistries (including serum calcium, liver function tests, and creatinine), a lipid panel, and serum levels of PTH, 25-OH vitamin D, TSH, and T. We will recruit a cohort of 180 men (30per group) age 60 to 75 who meet the entrance criteria specified in section 3.1.

Subjects who are successfully screened will be randomly assigned by a computer to one of 6 groups. All subjects in Groups 1 to 5 will receive a GnRH agonist goserelin acetate (Zoladex®; AstraZeneca, Wilmington, DE) 3.6 mg sc every 4 weeks for 16 weeks. Subjects in Group 1 will receive goserelin acetate and placebo Androgel®. Subjects in Group 2 will receive goserelin acetate every 4 weeks plus 1.25 gm of a topical testosterone gel (Androgel®, Abbott Laboratories, Chicago, IL) daily for 16 weeks. Subjects in Group 3 will receive goserelin acetate every 4 weeks plus 2.5 gm of Androgel® daily for 16 weeks. Subjects in Group 4 will receive goserelin acetate every 4 weeks plus 5 gm of Androgel® daily for 16 weeks. Subjects in Group 5 will receive goserelin acetate every 4 weeks plus 10 gm of Androgel® daily for 16 weeks.
Subjects in Group 6 received placebos for both goserelin acetate and for testosterone. Note that for subjects in Group 5 the testosterone dose was initially 10 g/day but was reduced to 7.5 g/day in December 2010 due to concerns based on retrospective studies suggesting that testosterone therapy might increase the risk of cardiovascular disease in men. All subjects, will be blinded with respect to group assignment. Subjects will be asked to maintain the usual level of physical activity. Dietary calcium intake will be assessed by a research dietitian and adjustments made through diet or supplements so that calcium intake is between 1000 and 1200 mg/day. Missed doses of medication will be considered acceptable for all aims it the investigator feels that there is no impact on subject safety and that the remaining data are likely to be usable.

Subjects will be seen on the GCRC at 4 week intervals for 16 weeks (0, 4, 8, 12, and 16 weeks). For each aim, there will be a window of +/- 2 weeks for each visit. If a subject misses a visit, the investigator will determine whether that will impact safety or data integrity. At each visit, compliance with Androgel® will be assessed by a structured interview and by reviewing a medication diary. A standardized series of questions will be posed to each subject to assess potential side effects of the study drugs. Subjects will be given a new 4-week supply of Androgel® or placebo gel (except at week 16). A fasting blood sample will be collected. For each aim, non-fasting blood samples will be acceptable if the investigator feels that the data are still likely to be usable. After the blood sample has been obtained, subjects will be given their goserelin or placebo injection (note that no injection is given at week 16). The blood tests listed below as well as anthropometric measures, and questionnaires will be performed at each visit. If the investigator feels that a test result needs to be repeated, that will be permitted. DXA, CT scans, physical function tests (in men 60 and older), and strength assessments will be performed at 0 and 16 weeks only. Subjects who discontinue participation at or after week 8 will be asked if they are willing to have an early discontinuation visit in which all procedures normally done at week 16 will be performed.

1. Blood studies
    a. CBC
    b. Routine chemistry panel (including calcium, liver function tests, and creatinine)
    c. Testosterone
    d. Estradiol
    e. Sex hormone-binding globulin (SHBG)
    f. Lipid panel
    g. PSA (if the participant "opts-in" to have their PSA measured at baseline and week 16)

3. Bone density and body composition by dual-energy x-ray absorptiometry (DXA)
a. Whole lumbar vertebrae (DXA in the PA projection)
b. Lumbar vertebral bodies (DXA in the lateral projection)
c. Proximal femur
d. Total body BMD
E. Fat and lean mass
c.
4. Body composition by computerized tomography (CT)
    a. Thigh muscle and fat area
    b. Abdominal subcutaneous fat (at L4 level)
    c. Intra-abdominal fat (at L4 level)
5. Quality-of life, fatigue, vasomotor symptoms,, and erectile function
6. 1 RM leg press strength
7. Anthropometric measures (weight, height, BMI, waist and hip circumference)
8. Bone microarchitecture by Xtreme CT of the distal radius and distal tibia
9. Cognitive function (Rey Complex Figure Drawing and East Boston Memory Test)
10. Physical Function Tests (sit-to-stand speed, 40 ft. walk speed, and grip strength) in men 60 and older

Subjects age 60-75 will be asked to participate in 2 additional urologic evaluations. First, subjects will be asked to collect a 24-hour urine for a kidney stone risk profile within 4 weeks of their baseline visit. The collection kits will be mailed to the subjects and will contain a pre-prepared Federal Express return mailer in which they place a small aliquot of the 24-hour urine sample and then drop it any Federal Express mailbox. Subjects will repeat the 24-hour urine collection within 2 weeks of their final study visit. Second, subjects will be asked to have measurements of urine flow rates at baseline and the final visit. This test requires simply that they urinate into a flow meter. Both of these measures are being conducted to generate preliminary data that can be used to determine the feasibility, as well as the required sample size, of a potential future study examining the effects of gonadal steroids on urologic disorders.

For each aim missing laboratory data will be considered acceptable if the investigator feels that there is no impact on subject safety and that the remaining data are likely to be usable. Subjects can skip any questionnaire items that they so choose.

5.3. Technical Methods

5.3.1 Dual-energy x-ray absorptiometry (DXA).

DXA measures spinal bone density with a reproducibility that is 3-4 times better than prior techniques such as dual photon absorptiometry (DPA) or quantitative computed tomography (QCT) (136). This advance has greatly facilitated longitudinal human studies of bone loss. Measurements of BMD and body composition by DXA are made using a Hologic QDR 4500 and software version 11.2. BMD is measured in the lumbar spine (posterior- anterior (PA) and lateral projections), proximal femur (femoral neck, trochanter, and total), and total body according to the Hologic Manual of Operations. All scans are performed in the array scan mode.

5.3.1.1 DXA spine scans. Subjects are positioned supine on the table with a foam block under their lower legs. For PA spine scans, a region of interest from L1 to L4 is scanned. Vertebrae that are obviously fractured or contain areas of focal sclerosis are manually excluded from the analyses. For lateral scans, a region of interest including L2 to L4 is scanned. In addition to excluding abnormal vertebrae identified on the PA spine scans, vertebrae with noticeable overlap from ribs or the pelvic brim are eliminated from analyses of lateral spine scans. For follow-up measurements, Hologic's "Compare" feature is used to match the region of interest (ROI) to the baseline scan. All scans are reviewed by an expert in bone densitometry (who is not involved with the study and is blinded to treatment) to assure quality. Our short-term in vivo reproducibility, based on duplicate scans performed on the same day with repositioning are 0.005 g/cm^2 and 0.014 g/cm^2, respectively, for PA and lateral spine measurements. To assure long-term stability of the instrument, an anthropomorphic hydroxyapatite spine phantom is measured daily. These values vary less than 1%.

5.3.1.2 DXA proximal femur scans. Subjects are positioned using Hologic's Hip Positioner System. Unless there are physical limitations (e.g. prior fracture, amputation, etc.), the left hip is scanned. The ROI is determined according to standard procedures in Hologic's DXA manual so that the dashed line goes down the center of the femoral neck and the solid line that separates the trochanteric from the inter-trochanteric region terminates at the inflection point of the lower curvature of the greater trochanter. The femoral neck rectangle is positioned as close as possible to the greater trochanter without actually touching it and no portion of the ischium or lesser trochanter is included in the femoral neck box. For follow-up measurements, Hologic's "Compare" feature is used to match the ROI to the baseline scan. Our short-term in vivo reproducibility are 0.007 g/cm^2 and 0.006 g/cm^2, respectively, for femoral neck and total hip measurements.

5.3.1.3 DXA total body scans. Subjects are positioned supine with their arms at their sides and at least 1-inch of space between the hands and thighs. Feet are loosely bound with at least 1 inch of separation. If the subject is too large to fit in the scan area, the left arm is extended and values for the right arm are substituted at a later time. All metal and plastic is removed if possible. Sub-regions of interest are defined according to standard procedures in Hologic's DXA manual. If correct definition of skeletal sub-regions prevents the correct definition of soft tissue sub-regions, priority is given to the former. A Hologic 4500 tissue bar is scanned separately once each week. Our short-term in vivo reproducibility for total body BMD measurements is 1.1%.

5.3.1.4 DXA body composition. Percentage fat body mass and percentage lean body mass are determined from the DXA total body scan using software version 11.2 as described previously (101).

5.3.2 Body composition by computerized tomography.

Body composition will be assessed by computerized tomography (CT) at the level of L4 and at the mid-femur with a GE Model 9800 scanner (General Electric Medical Systems; Milwaukee, WI) (137). Cross-sectional muscle area is a critical determinant of strength and overall functional status. Cross-sectional area of the thigh, thigh muscle area, and thigh fat area are determined as described previously (138). The midpoint of the femur is obtained using measurements from a scout image with extremities in a standard position. The leg is scanned with the knee fully extended and the foot perpendicular to the table. Cross-sectional area of

the thigh is determined from an outline of the thigh using image analysis software. Additional contours are identified for the anterior and posterior muscle groups. Cross-sectional areas for the anterior and posterior muscle groups are recorded and summed for the thigh muscle area. The standard error for thigh muscle area determination is ±1% by this method (138).

Cross-sectional areas of the abdomen, abdominal subcutaneous fat, intra-abdominal fat, and paraspinal muscles are determined at the level of the L4 vertebra as described previously (82, 101, 139). Briefly, total abdominal area is determined from an outline of the torso using image analysis software (General Electric Advantage Windows Workstation, Version 2.0). Two contours are identified: the body perimeter and deep fascia that delineates the back and abdominal wall musculature. The abdominal subcutaneous fat area is defined as the area between the two contours. Intra-abdominal fat is defined as the area within the inner contour comprising all pixels with attenuation coefficients between -50 and -250 Hounsfield units. Additional contours are identified for the psoas and erector spinae muscles. The total paraspinal area is defined as the sum of the cross-sectional areas for the psoas and erector spinae muscles. The paraspinal fat area is defined as the total paraspinal area comprising all pixels with attenuation coefficients between -50 and -250 Hounsfield units. The paraspinal muscle area is defined as the total paraspinal area minus the paraspinal fat area.

5.3.3.7 Total testosterone and estradiol levels.
The serum level of total testosterone at the final 2 study visits will be measured using liquid chromatography-tandem mass spectroscopy in the Brigham Research Assay Core Laboratory, a CDC-certified laboratory for measuring testosterone, and then averaged (20). The assay detection limit is 1.5 ng/dL.
Serum estradiol will be measured using liquid chromatography-tandem mass spectroscopy on a pool comprised of equal volumes of serum collected at weeks 4, 8, 12, and 16. The assay detection limit is 1.25 pg/mL.

5.3.3.8 **Serum CTX:** Serum CTX levels will be measured by ELISA (Crosslaps, Immunodiagnostic Systems)

5.3.3.9 Sex hormone-binding globulin.
SHBG will be measured by radioimmunoassay using a commercially available kit (Endocrine Sciences, Calabassas Hils, CA).

5.3.5 Muscle strength

After a 5-minute warm up on a stationary bike, effort-dependent lower extremity strength will be assessed on the basis of maximum weight lifted for one repetition (1-RM) using a leg press (Air 300 Leg Press; Keiser Corporation) (145). To minimize the confounding influence of the learning effect, testing is repeated after a two-day rest and greater of the two values is recorded as the 1-RM strength. If the two values differ by more than 5%, testing is repeated a third time and the greatest of the three values is recorded as the 1-RM strength.

5.3.6 Quality of life, fatigue, vasomotor symptoms, erectile function, and hypogonadism symptoms:

5.3.6.1 Quality of life. Quality of life will be assessed using a 30-item questionnaire that includes questions about activities of daily life, fatigue, pain, sexual functioning, interference with social life, and psychological distress (146-148). The questionnaire evaluates eight domains: pain, social functioning, emotional well being, vitality, activity limitation, bed disability, overall health, physical capacity, sexual interest, and sexual functioning. This 30-item questionnaire has been validated in previous studies in which men were rendered hypogonadal using androgen receptor blockade (146-148).

5.3.6.2 Fatigue. Fatigue will be evaluated using the Fatigue Severity Scale, a nine-item questionnaire. Scores range between 9 (indicating minimum fatigue) to 63 (indicating maximum fatigue). The Fatigue Severity Scale has been used successfully to evaluate fatigue in men receiving androgen deprivation therapy (149).

5.3.6.3 Vasomotor symptoms. Other symptoms of hypogonadism are evaluated using a questionnaire that includes questions about the frequency and severity of hot flashes (150, 151). The average daily hot flash scores are determined from a hot flash diary as described previously (150, 151).

5.3.6.4 Erectile function. Erectile function will be assessed using the International Index of Erectile Function (IIEF) (152). This is a well-validated, 15 items, self-administered scale that assessed 5 domains of male sexual function (erectile function, orgasmic function, sexual desire, intercourse satisfaction, and overall satisfaction). Subjects will be asked to keep daily logs of sexual activity for 7 consecutive days before each visit.

5.3.6.5 Hypogonadism symptoms.
In addition, making detailed assessments of individual symptoms of hypogonadism, we will administer the Androgen Deficiency in Aging Males (ADAM) questionnaire. ADAM is a 10-item scale that evaluates libido, potency, strength, mood, enjoyment of life, sleepiness work performance, and ability to play sports. In a group of 316 men aged 40-62, it had an 88% sensitivity and 60% specificity for detecting low bioavailable T levels. The reproducibility of the scale was 11.5% when administered twice at an interval of 2-4 weeks. Eighteen of 21 hypogonadal men treated with T had improvement in the ADAM scores (153).

5.3.7 Xtreme CT.

Xtreme CT (Scanco Medical AG, Basserdorf, Switzerland) is a new technology capable of assessing volumetric bone density as well as trabecular and cortical microarchitecture at the distal radius and distal tibia, with a nominal isotropic voxel size of 82 µm. The Xtreme CT system (Figure 1) employs a two-dimensional detector array in combination with a small-angle cone beam X-ray source (0.07 mm spot focal size), enabling the simultaneous acquisition of a stack of parallel CT slices with a nominal resolution (voxel size) of 82 µm (100 µm at 10% MTF). The X-ray tube employs an effective energy of 60 kVp, with a current of 95 mAs. The field of view is 130 mm and maximum scan length is 150 mm. The short-term reproducibility of measurements with this device is excellent, with precision errors of 0.7 to 1.5% for total, trabecular and cortical bone densities and 2.5 to 4.4% for trabecular architecture measures.

Scan acquisition: During scan acquisition, the arm or leg of the patient is immobilized in an anatomically formed carbon fibre shell. An anterior-posterior scout view is used to define the measurement region. At each

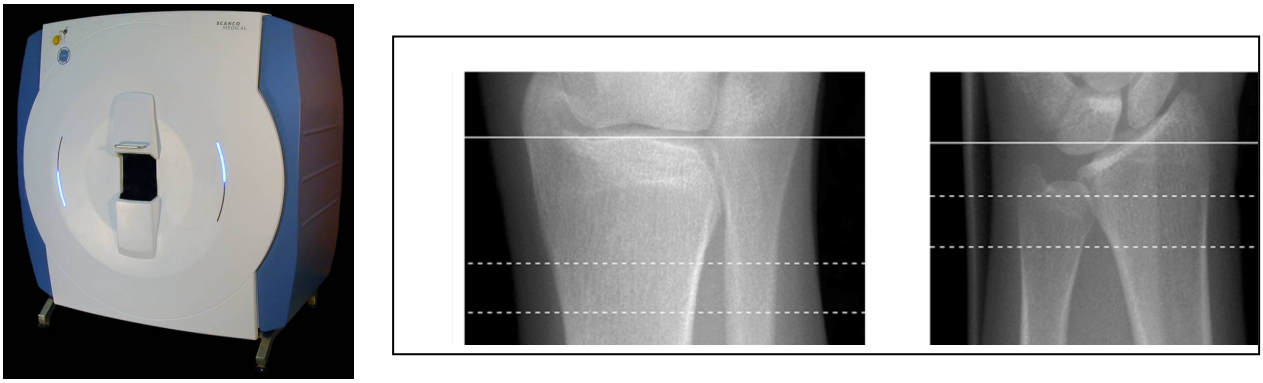

Figure 1. (left) Photo of Xtreme CT clinical imaging device, for assessment of trabecular and cortical bone microarchitecture at the distal radius and distal tibia. Image courtesy of Scanco Medical, AG. The middle and right images are standard antero-posterior scout views of the distal tibia (middle) and distal radius (right), showing the placement of a reference line (solid line) and the volume of interest where CT slices are acquired (between dashed lines).

skeletal site, 110 CT slices are acquired, thus delivering a 3D representation of approximately 9 mm in the axial direction. In standard analyses, a reference line is manually placed at the endplate of the radius and tibia, as shown in Figure 1 (middle and right). The first CT slice is acquired 9.5 mm and 22.5 mm proximal to the reference line for the distal radius and distal tibia, respectively (Figure 1).

5.3.8 Cognitive function tests

5.3.8.1 Rey Complex Figure Drawing. This is a test of spacial memory. The subject is given a complex figure and asked to draw it. The figure is then taken away and he is asked to draw it again from memory. Fifteen minutes later he is asked to draw it again from memory. A standardized scoring system is applied.

5.3.8.2 East Boston Memory Test. This is a test of verbal memory. The subject reads a brief story and is asked a series of questions immediately afterward and then again 15 minutes later.

5.3.9 Physical function tests in men 60 and older

5.3.9.1 Sit-to-stand speed, 40-foot walk speed, and grip strength. Men will be asked to participate in a series of standardized tasks to identify gradations in physical status and functioning and to characterize more precisely their physical status with respect to strength and mobility. These tests will include the time required to rise from sitting in a chair to standing 5 times, the time required to walk a distance of 40 feet, and an assessment of grip strength using a dynamometer. In both the timed sit-to-stand and the 40-foot walk tests, participants may use assistive devices should they chose to do so.

6. BIOSTATISTICAL ANALYSIS

6.1 Outcome measures for Specific Aim 1b

Bone Measures:

Serum CTX (primary outcome)
Serum P1NP
L4 trabecular BMD by QCT
DXA PA spine B(MD*
DXA total hip BMD*
DXA total body BMD*

Body Composition Measures:

Total body fat mass by DXA (primary outcome)
Total body lean mass by DXA
Subcutaneous fat area by CT
Intraabdominal fat area by CT*
Thigh fat and muscle area by CT

Sexual Function Measures

Sexual desire (using previously validated question)
Erectile function (using validated questionnaire)

Tests of physical function

Sit to stand speed*
40-foot walk speed*
Grip strength*
1 RM leg press strength*

Tests of cognitive function

Rey complex figure drawing*
East Boston memory test*

Questionnaire measures

Quality of life*
Fatigue*

Vasomotor symptoms*

Other blood measures

Lipids (total cholesterol, LDL, HDL, triglycerides)
HOMA-IR
Leptin

6.2 Power calculations for Specific Aim 1b.

All power calculations for Aim 1b are based on our findings, utilizing a virtually identical protocol, in 20-50-year-old men (1, 2). The primary end points will be changes in markers of bone resorption (serum CTX) and total body fat mass by DXA. Power calculations are based on those 2 outcome measures. In our prior study in men age 20-50, serum CTX increased 97$\pm$ 58% (mean + SD) in group 1, 35 $\pm$ 26% in Group 2, and 50 $\pm$ 47% in Group 3. Thus with 27 subjects per group we should have 80% power at a two-sided 5% alpha level to detect a change of 32.5% in serum CTX levels in group 1, 14.6% in group 2, and 26.3% in group 3. Similarly, total body fat mass increased 11.9 +12% in 10.4 + 6.4% in Group 2 and 3.0 + 6.6% in Group 3. Thus with 27 subjects per group we should have 80% power at a two-sided 5% alpha level to detect a change of 6.7% in total body fat mass levels in group 1, 3.6% in group 2, and 3.7% in group 3. These calculations were based on the assumption that the variability of these changes in this proposed study cohort would be the same, and the post-study effect size of each outcome that will be detected significantly with $p<0.05$ can be deviated from these estimates if the observed variability of this study is larger or smaller than that of the 20-50-year-old men.

6.3 Data analysis for Specific Aim 1b

The primary analysis will be a modified intention-to-treat analysis. Because we are assessing changes in outcome variables, participants who complete only the baseline visit will not be included in longitudinal analyses. Subjects who complete the first 3 study visits (through week 8), but who discontinue the study before week 16 will be permitted to undergo assessments planned for week 16 at their final visit. Because changes in body composition and BMD are unlikely to occur within the first several weeks of hormonal manipulation, subjects who complete less than 8 weeks of the protocol will not eligible for repeat imaging studies to avoid exposing participants to additional radiation when it appears unlikely that the results will be informative.

6.4 Statistical Analysis for Specific Aim 1b

Baseline characteristics for each of the 6 groups will be compared using analysis of variance. To determine if there is a statistically significant relationship between the testosterone dose and the outcome variables, we will test each outcome variable for a dose-dependent linear trend in mean changes using linear contrasts. If the contrast test is significant, we will then compare the changes in each of the first 5 groups with the change in Group 6 (the Double Placebo or Control Group) using Dunnett's test, for which the family-wise Type-1 error rate for multiple comparisons was set at 5%. All statistical tests are 2-sided.

6.5 Interpretation of results and potential limitations for Specific Aim 1b

It is unlikely that there is a distinct threshold above which men are normal and below which men experience undesirable changes in bone density, body composition, sexual function, and other outcomes that depend on sex steroids. Additionally, we previously reported that the increases in fat mass and bone resorption, and the decline in BMD and sexual function that occurs when testosterone levels are lowered in young men are due primarily, if not exclusively, to the decline in estradiol levels that invariably accompanies a decline in testosterone levels (18, 19). In contrast, changes in muscle mass and strength are regulated by androgens (18). Although the changes in CTX, L4 trabecular BMD, indices of body fat, and measures of sexual function in older men are also likely due to estrogen deficiency, in clinical practice changes in testosterone levels are often used as a "proxy" for the decline

in estradiol levels due to the difficulty in measuring estradiol levels accurately when they are very low, as in men.

7. RISKS AND DISCOMFORTS

7.1 Prior experience using these medications:

Most subjects with severe hypogonadism will experience side effects, mainly hot flashes and sexual dysfunction. These symptoms are likely to occur in the men randomized to Zoladex® alone and, possibly, those in the low dose T replacement groups. These symptoms are completely reversible when Zoladex® is discontinued and gonadal steroid levels return to normal. In addition, if the side effects are too bothersome, subjects will all be given the option of discontinuing the study protocol and starting treatment with testosterone until the effects of Zoladex® wear off. In our recent study (72), only 2 of 30 men treated with Zoladex® alone for 12 weeks dropped out of the study due to side effects (an additional 3 of these 30 men dropped out due to issues unrelated to the study). In the first phase of this protocol, 7 of 41 men (17%) who received Zoladex alone dropped out due to symptoms of testosterone deficiency. The overall dropout rate from this study was 20%. The current studies are powered on the basis of a 20% drop out rate.

7.2 GnRH agonist (Zoladex®):

Bone loss: Most, but not all, human studies report statistically significant bone loss after 6 months of GnRH agonist therapy but bone loss is generally not observed when GnRH analogs are administered for only three months. Because this study lasts only 16 weeks, bone loss is not a significant issue.

Decreased Libido/Fertility: A reversible decrease in sexual desire is expected in the group receiving the GnRH agonist alone. In men receiving Zoladex® for advanced prostate cancer, 21% reported sexual dysfunction and 18% reported decreased erections. These symptoms resolve after discontinuation of the drug and can be reversed immediately by administering testosterone, if necessary.

Miscellaneous: Hot flashes have been reported in 62% of patients receiving Zoladex for prostate cancer. Thus, we expect a significant number of the patients who receive Zoladex® alone to experience hot flashes. Hot flashes may also occur in men who receive Zoladex® plus very low doses of testosterone. Hot flashes resolve when Zoladex® is stopped and can be reversed immediately by administering testosterone, if necessary. On rare occasions, local reactions are observed at the administration site.

7.3 Testosterone (Androgel®)

Local Skin Reactions: In clinical studies with Androgel® the most common side effect was minor erythema at the site of application. Local skin reactions occur in about 5% of subjects. These skin reactions were generally mild and did not require drug discontinuation or treatment.

Prostate: Testosterone is necessary for prostate growth and is contraindicated in men with either benign prostatic hyperplasia or prostate cancer. Thus, subjects with a history of prostate disease (cancer or hyperplasia) will be excluded from the study. Careful histories will be taken to exclude any subjects with signs of urinary obstruction.

Hepatic: Liver disease has been reported with the use of orally active methylated testosterone preparations. When given in physiological doses (as in the current proposal), parenteral or topical testosterone preparations do not cause liver damage. In fact, in a study using pharmacologic doses of testosterone esters for 10 weeks in healthy men at doses that exceed the physiologic dosing proposed in this study by 3-4 fold, no significant changes in liver function tests, hematocrit, lipids, or PSA were seen. Still, LFTs will be monitored (see research methods).

Cardiovascular disease: A recent study (Basaria et al., NEJM 363L109-22, 2010) reported an excess of cardiovascular events in elderly, frail (i.e. difficulty walking 2 blocks on a level surface) hypogonadal men who were randomized to testosterone therapy. Specifically, 23 of 106 men treated with testosterone reported cardiovascular events vs 5 of 103 in the placebo group. The 23 events 2 myocardial infarctions, 2 episodes of chest pain, 1 revascularization procedure, 1 stroke, 2 syncopal episodes, 1 exacerbation of CHF, 1 LV strain pattern during exercise testing, 1 ST-segment depression during exercise testing, 2 subjects with HTN, 1 subject with tachycardia and fatigue, 1 subject with PVCs and couplets on ECG, 2 subjects with atrial fibrillation, 5 subjects with peripheral edema and 1 case of sudden death. In the control group, there was 1 case of syncope, 1 of tachycardia, 1 of HTN, 1 of ectopy on ECG, and 1 carotid bruit. At baseline the mean age of these subjects was 74, 85% had HTN, 45% were obese, 24% had diabetes, 63% had hyperlipidemia, and 74% were former or current smokers. Importantly, half of these subjects had known preexisting cardiovascular disease. The starting dose of testosterone gel was 10 g/day but the dose could be increased to 15 gm or reduced to 5 gm depending on the testosterone levels. The mean testosterone level AFTER ADJUSTMENT OF THE DOSE was 574 ng/dl (the authors do not state what the level was before dose adjustment). Cardiovascular events occurred in 4 of 14 (29%) men with testosterone levels > 1000 ng/DL during treatment, 5 of 21 (24%) men with levels of 500-1000 ng/dL and 7 of 46 (15%) with levels below 500 ng/dL. Because of the excess of cardiovascular events, this trial was stopped prematurely by the DSMB.

Note that a large (n=800) multi-center randomized clinical trial of testosterone in elderly hypogonadal men sponsored by the NIH is continuing.

7.4 Aromatase Inhibitor (Arimidex®)

Arimidex® is currently FDA approved for first-line treatment of postmenopausal women with hormone receptor-positive or hormone receptor-unknown locally advanced or metastatic breast cancer and also for treatment of advanced breast cancer in postmenopausal women with disease progression following tamoxifen therapy.

As with all medications, undesirable events are sometimes experienced.

In postmenopausal women with breast cancer, the most frequently experienced side effects of Arimidex® were hot flushes, asthenia (weakness/lack of energy), joint pain/stiffness, headache, vaginal dryness, diarrhea, thinning hair, and nausea.

Some of the other less frequently reported side effects were: anorexia (loss of appetite), vaginal bleeding (usually in the first few weeks of treatment only), sleepiness, increased cholesterol, constipation, vomiting, peripheral edema (water retention primarily in feet/ankles), depression, dizziness, cough increased, dyspnea (shortness of breath), pharyngitis (irritation of throat), rash, and dry mouth.

Very rarely, patients taking Arimidex® experienced severe skin reactions involving ulcers and lesions. Arimidex® may also increase the chance for bone fractures.

There is little information on the effects of aromatase inhibition in men and there is no documented risk in men in whom selective and reversible estrogen deficiency has been induced for a short period of time. We have given Arimidex® for 12 weeks to normal men in a previous study without any adverse events.

7.5 Radiation exposure

Each set of DEXA scans result in an effective radiation dose of approximately 0.5 mrem so that both sets results in an effective dose of approximately 1 mrem. The single slice CT of the thigh and abdomen result in an effective dose of 50 and 100 mrem, respectively. The Xtreme CT scan results in an effective radiation dose of <1 mrem. The total effective dose is therefore approximately 302

mrem. This amount of radiation is equal to the annual background radiation one is exposed to each year from the earth and the sky.

8. POTENTIAL BENEFITS

8.1 Potential benefits to participating individuals

Subjects will not experience any benefits to their personal health.

8.2 Potential benefits to society

At the present time, the degree of hypogonadism that places men at risk for bone loss, changes in body composition, lipid abnormalities or symptoms is unknown. The information from the study may help in the understanding of bone loss, body composition, lipid changes, and hypogonadal symptoms in men. This information may help clinicians to decide which men with low or borderline testosterone levels might benefit from testosterone therapy.

9. MONITORING AND QUALITY ASSURANCE

If subjects are unable to tolerate the symptoms of hypogonadism (i.e. low libido and possible hot flashes), Zoladex® therapy will be stopped. If necessary, subjects may be given testosterone therapy for a short period of time until the effects of Zoladex® on hormone production abate. Rotating sites of Androgel® administration can minimize local reactions to topical testosterone. The risks of phlebotomy will be minimized by careful attention to phlebotomy technique. Blood pressure, liver function tests, a complete blood count, and serum creatinine will be checked every 4 weeks. Specifically, if clinically significant abnormalities develop in any of these parameters appropriate measures will be taken. In the unlikely event that a patient experiences an adverse effect because of participation in this study, medical treatment will be available to treat that injury at the Massachusetts General Hospital at no charge.

If a subject's systolic BP is >160 or diastolic BP is >95 at any follow-up visit, he will be asked to return to have his blood pressure re-checked within 1 week. If his SBP is still > 160 or DBP is still > 95, he will be withdrawn from the study.

Similarly, if a subject's hematocrit is > 50 at any follow-up visit, he will be asked to return to have his hematocrit re-checked within 1 week. If his hematocrit is still > 50, he will be withdrawn from the study.

As noted above, all subjects will be informed of their rights under the Health Insurance and Portability and Accountability Act of 1996 as per federal law and hospital policy. The consent form will contain an IRB-approved template that describes what protected health information from the research study may be used or shared with others, why protected health information may be used or shared, who may use or share protected health information (within or outside of Partners HealthCare), the length of time that protected health care information may be used or shared with others, and a statement of privacy rights. Data collected strictly for research purposes will be stored in locked files and on computers with passwords required for access. When the data are published or presented, no names or other materials that allow identification of an individual will be used.

9.1 Independent monitoring of source data

Source data will be entered into a password-protected computerized database. The database is reviewed routinely by research assistants and by a study investigator. All assays are performed in duplicate. Assay data are reviewed first by the performing technician and then by one of the investigators. Entire assays are repeated if the standard curve or quality control values are not within range. Individual samples are re-run if duplicate values have coefficients of variation >10%. DXA and CT data are reviewed by experts in bone densitometry and a radiologist, respectively, who are blinded as to treatment assignment.

9.2 Safety monitoring

Although the study did not meet criteria for requiring a DSMB, a DSMB will now be convened to assist in safety monitoring. Because the major safety questions are related to cardiovascular disease, hormone effects, and prostate cancer, the DSMB will consist of 3 individuals including a cardiologist, an endocrinologist, and an oncologist. The DSMB will review the study no less than twice each year. The following safety measures will be assessed at each study visit:

1. Systolic and diastolic blood pressure
2. Complete blood count
3. Serum creatinine
4. Liver enzymes (AST and ALT)

The following procedures will be followed in response to these measures:

1. Blood pressure: If a subject's systolic BP is >160 or diastolic BP is >95 at any follow-up visit, he will be asked to return to have his blood pressure re-checked within 1 week. If his SBP is still > 160 or DBP is still > 95, he will be withdrawn from the study.
2. Hematocrit: Similarly, if a subject's hematocrit is > 50 at any follow-up visit, he will be asked to return to have his hematocrit re-checked within 1 week. If his hematocrit is still > 50, he will be withdrawn from the study
3. Creatinine: If a subject has a serum creatinine >2 mg/dL he will be asked to return to have his creatinine level re-checked within 1 week. If his creatinine is still > 2, he will be withdrawn from the study
4. Liver function tests: If a subject's liver enzymes (AST or ALT) are more than twice the upper limit of normal, he will be asked to return to have them re-checked within 1 week. If his AST or ALT is still more than twice the upper limit of normal, he will be withdrawn from the study.
5. Testosterone: If a subject's testosterone level rises above 1500ng/dL and either the subject's liver function tests are two times higher than the upper limit of the reference range or the hematocrit is >50%, the subject will be discontinued from the study. If a subject's testosterone level rises above 1500ng/dL but the subject's liver function tests are less than two times the upper limit and the hematocrit is less than or equal to 50%, the subject's dose will be lowered to the next highest dose and continued to be monitored at the lower dose. If the testosterone level continues to be >1500ng/dL at the subject's next visit after switching to the lower dose, he will be withdrawn from the study.

If questions arise as to the significance of a laboratory or clinical event, the information will be sent immediately to the DSMB and/or our local IRB for an impartial opinion. All adverse events will be reported to the IRB either immediately (in the case of serious adverse events) or at the time of the scheduled renewal process (for routine adverse events). The study physicians and the study staff will meet on a regular weekly basis to discuss any issues relating to adverse events, safety data and outcome data.

9.3 Outcomes monitoring

Because this is not a therapeutic trial, outcomes monitoring is not applicable.

9.4 Adverse event reporting

Serious adverse events will be reported within 24 hours of notification of a study staff member according to Partners HRT guidelines. Routine adverse events will be reported annually with the continuing review report.

Reporting of serious adverse events (language below is required by AstraZeneca)

Investigators and other site personnel must inform the FDA, via a Medwatch form, of any SAE that occurs according to the FDA reporting requirement timelines. The Investigator must also inform appropriate AstraZeneca representatives of any SAE that occurs in the course of the study within one day (i.e. immediately but no later than the end of the next business day) of when he or she becomes aware of it. Follow-up information of SAEs must also be reported by the investigator within

one day. A copy of the Medwatch report must be faxed to AstraZeneca at the time the event is reported to the FDA. It is the responsibility of the investigator to compile all necessary information and ensure that the FDA receives a report according to the FDA reporting requirement guidelines and to ensure that these reports are also submitted to AstraZeneca at the same time.

If a non-serious AE becomes serious, that and other relevant follow-up information must also be provided to AstraZeneca and the FDA within 1 day as described above. All SAEs have to be reported to AstraZeneca, whether or not considered causally related to the investigational product. All SAEs will be documented. The investigator is responsible for informing the IRB and/or the Regulatory Authority of the SAE as per local requirements.

The written SAE report will be sent to Astra Zeneca with a cover page indicating the following:

- Drug Name (Arimidex or Zoladex); this is an Investigator Sponsored Trial (IST)
- Research (Protocol) and AstraZeneca Tracking Number
- Principal Investigator's IND number assigned by the FDA
- Principal Investigator's full name and address
- Unblinding information (if applicable)

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