The proposed competitive renewal of our grant, Bone Strength Through the Menopausal Transition: Trabecular Bone Score, builds upon the Study of Women's Health Across the Nation (SWAN) Bone Project. SWAN is a multi-site, multi-racial/ethnic longitudinal study designed to characterize the physiological and psychosocial changes that occur during the menopausal transition (MT). One of the key physiological consequences of ovarian aging that was specifically targeted in SWAN was loss in skeletal mass.

In SWAN, areal bone mineral density (aBMD) loss begins 2 years before the final menstrual period (FMP) at both the lumbar spine (LS) and femoral neck (FN) sites. Cumulative rates of bone loss are greatest from 1 year before through 2 years after the FMP, termed the transmenopause. Bone loss 2-5 years after the FMP slows. Germane to this application, transmenopausal BMD loss is greater at the LS than at FN, concordant with the higher proportion of trabecular bone in the LS. We also found that LS, but not FN, aBMD was associated with incident fractures across the MT, again emphasizing the potential dominant role of trabecular bone. However, in SWAN we are currently limited to dual-energy x-ray absorptiometry (DXA) measures of aBMD which cannot distinguish trabecular and cortical bone.

Trabecular bone score (TBS) is a new index of trabecular bone structure, obtained from LS DXA scans. Microarchitecture of trabecular bone is a key determinant of bone strength. Both thinning of trabeculae and loss of trabecular connectivity substantially undermines structural integrity and weakens bone. TBS is associated with other measures of trabecular bone structure and predicts fracture in postmenopausal women.

Our proposal offers an unprecedented cost-efficient opportunity to investigate trabecular bone structure longitudinally in the SWAN cohort. No other study has LS aBMD serially for a 20-year time span that includes the entire MT in a large multiracial sample. By reanalyzing SWAN spine DXA scans to obtain TBS we have the unique opportunity to isolate trabecular microarchitecture and test several novel hypotheses.

We propose the following specific aims:

Aim 1: Determine the direction and magnitude of ethnic difference in TBS at baseline when all participants were either premenopausal or early perimenopausal and so at or near their peak BMD.

a. We hypothesize that Black women will have higher TBS scores than will White and Japanese women; Japanese women will have lower TBS scores than White women. *These observations will be independent of aBMD and bone geometry (FNAL, FNW) and composite strength indices.*

Aim 2: Examine the rate of longitudinal change in TBS with aging and with the MT

a. We hypothesize that declines in TBS will mirror those of aBMD, specifically they will be minimal in the pre-transmenopause (5 years to 1 years before the FMP), accelerate transmenopause (1 year before to 2 years after FMP), decelerate in the postmenopause period (2 to 5 years after FMP) and stabilize in the later postmenopausal period (>5 years after FMP). *We will also examine whether there are racial differences in TBS declines during the MT. We hypothesize that Black women will experience slower declines and Japanese, faster declines in TBS compared to Whites.*

b. We will test the hypothesis that longitudinal declines in TBS will be related to higher levels of follicle stimulating hormone (FSH) and lower levels of sex steroid hormones (estradiol (E2)). Sex steroids and gonadotropins were measured annually in SWAN.

Aim 3: Examine the association between baseline TBS and longitudinal declines in TBS with incident fractures in midlife women.

a. We hypothesize with lower TBS at baseline *and greater loss in TBS* will be associated with incident fractures, independent of aBMD, *FNAL, FNW and composite indices of bone strength.*

Aim 4: Examine the effect of diabetes and insulin resistance on TBS at baseline and longitudinally as women traverse the MT.

a. We hypothesize that diabetic women will have lower TBS than non-diabetics. In addition, we hypothesize that there will be an inverse association between insulin resistance and TBS in non-diabetics.

b. We hypothesize that declines in TBS with MT will be greatest in women with diabetes mellitus and among the non-diabetics, those with greater insulin resistance, the primary pathology of type 2 diabetes.

The central tenet of our work is "beyond aBMD". This proposal builds on our initial grant where we made substantial contributions showing that hip structural, biomechanical and geometric elements improves risk classification. We now propose to focus on LS trabecular microarchitecture and test whether the rapid transmenopausal loss preferentially effects trabecular bone which could lead to irreparable structure damage. Our long term objective is to substantially improve our understanding of aging, menopause and skeletal strength.

B. BACKGROUND AND SIGNIFICANCE

B.1. Osteoporosis: Public Health Impact and Risk Assessment of Osteoporosis: Osteoporotic fractures are a major public health problem (1). It is estimated that 30 million US women age 50 + have low bone mass or osteoporosis. About one-third of US women age 50+ with bone mineral density (BMD) in low bone mass range are candidates for pharmacology therapy (2). This number is expected to reach 41 million in 2020 (3). In 2005, more than 2 million incident fractures were reported in the US with a total cost of $17 billion (4). This number will increase dramatically as the number of women with low bone mass and osteoporosis increases. Although fracture rates are lower in non-White women, compared with Whites (5-7), the consequences of fracture may be greater in minority women. Mortality (8, 9) and disability (10, 11) after a hip fracture may be greater among Black women. The absolute number of fractures exceeds the combined number of cases of breast cancer, myocardial infarction and stroke in US women (12), irrespective of race/ethnicity.

The development of areal BMD (aBMD), a 2 dimensional estimate of the amount of bone per unit area and the demonstration that current aBMD predicts future fracture were landmark advances in the field of osteoporosis almost 3 decades ago (13-15). However, we have since learned that aBMD falls far short in capturing the entire variation in fracture risk. The majority of fractures occur in non-osteoporotic women (16) and only 15% of the reduction in fracture risk associated with antiresorptive therapy can be accounted for by BMD (17). The proportion of fractures attributed to osteoporosis (defined by BMD T-score, <-2.5) is modest ranging from <10% to 44% (18). Therefore, the pursuit of non-invasive, inexpensive methods for improving fracture risk prediction remains a high priority. Because aBMD does not capture bone structure well (neither macroarchitecture nor microarchitecture) and because structural characteristics independently contribute to strength (resistance to fracture), many investigators including our team are working extensively on the relationship between bone structure and fracture (19-21).

In our first funding period, we developed the concept of DXA based integrated measures of hip strength which go beyond aBMD using 2 approaches. One approach incorporated bone size and the impact that bone must sustain during trauma (e.g., a fall) and the second was based on hip geometry (see Progress Report). Our results strongly support that adding these structural, biomechanical and geometric elements improves risk classification. In this application we explore a different domain of bone strength, specifically trabecular microarchitecture. *To do so, we will make trabecular bone score (TBS) measures using archived lumbar spine (LS) DXA scans, examine whether baseline TBS differs by race/ethnicity, quantify longitudinal decline in TBS during MT and test whether these declines differ by race/ethnicity, describe factors related to this decline, study the association of TBS measured at baseline and serially to fracture risk across MT and test whether these associations are independent of aBMD, bone geometry and strength.*

B.2. Menopause and Skeletal Health: In SWAN, menopause transition (MT) stage assignment was based on annual reports about menstrual bleeding. Women were classified as “premenopausal” if they had experienced at least 1 menstrual period in the last 3 months with no change in the regularity of their menses during the last year; “early perimenopausal” if they had experienced at least 1 menstrual period in the last 3 months with some change in the regularity during the last year; “late perimenopausal” if they had experienced no menstrual periods in the last 3 months but at least 1 menstrual period during the last 11 months; “postmenopausal” once they had experienced at least 12 consecutive months of amenorrhea.

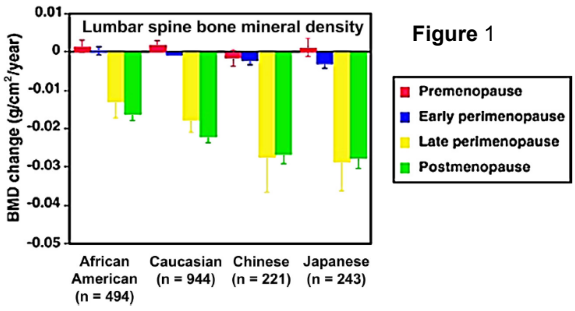


Figure 1

Results using these menstrually based MT stages showed little loss in either hip or LS BMD in the premenopausal or early perimenopausal periods. The annual rate of bone loss accelerated in the late-perimenopausal and postmenopausal period, **Figure 1** (22). Patterns were similar in women of all ethnicities although absolute annual rates of decline were slowest in Black women and fastest in Asian women.

However, menstrually defined MT stages, are imprecise predictors of when the final menstrual period (FMP) will occur. Women who are in early or late perimenopause may be more or less proximal to their FMP, and rates of BMD loss may therefore differ within menstrually defined stages. Similarly, the time at which bone loss decelerates after the FMP cannot be discriminated using menstrually classified MT stages. The inference when using menstrually classified MT stages is that the later the transition stage, the closer a woman is to her FMP. However, we have shown that during the year before the FMP, 68% of women were still classified as

early perimenopausal based on bleeding patterns. Even in the year after the FMP, 30% of women were still classified as early perimenopausal according to bleeding patterns (23).

In SWAN, using the FMP approach, we analyzed aBMD trajectories in 242 Black, 384 White, 117 Chinese and 119 Japanese women for whom an FMP date could be determined. BMD loss began 1 year before the FMP and decelerated (but did not cease) 2 years after the FMP, at both the LS and femoral neck (FN) sites (23). During the 10-year observation period, at each bone site, the rates and cumulative amounts of bone loss were greatest during years after the FMP, termed the transmenopause. Cumulative, 10-year LS BMD loss was 10.6%; 7.38% was lost during the transmenopause. Cumulative FN loss was 9.1%; 5.8% was lost during the transmenopause. Black heritage was related to slower loss rates, whereas the opposite was true of Japanese and Chinese women. Faster rates of LS BMD loss compared to FN BMD loss across the MT were also reported in White women enrolled in the Michigan Bone Health Study (24).

Greater BMD loss in the LS may reflect the greater proportion of trabecular bone at the LS. Riggs, Khosla, and Melton originally proposed that accelerated, early postmenopausal bone loss affected trabecular bone to a greater degree than it affected cortical bone and that the subsequent, slower rate of BMD loss was similar in both bone compartments (25). The initial, accelerated phase was ascribed to the loss of a tonic estrogen effect on bone turnover. Newer, CT based studies still find a menopausal acceleration of trabecular bone loss (more pronounced at the LS than at the distal tibia or radius) but newly report that trabecular loss begins in White women during their 20s, whereas tibial and radial cortical bone losses do begin at the MT (26). We did not observe aBMD loss before the transmenopause, likely because of the lesser sensitivity of DXA compared with CT. The MT (and concomitant change in estradiol and other factors) appears to play a major role in onset of cortical bone loss and the amplification of trabecular bone loss in midlife women (27, 28). A cross-sectional study of 638 Chinese women age 20-80 from Hong Kong also confirmed that trabecular volumetric BMD (vBMD) started to decline in women in their 30s but accelerated after the age of 50. Cortical vBMD did not start to decline until age 50 (29). To our knowledge, nothing is known about these changes in other racial groups, specifically, Blacks or other Asian groups.

B.3. Follicle stimulating hormone, E2 and MT bone loss: Using our 3-phase model of aBMD decline in relation to the FMP described above, we examined the relations between sex steroids and gonadotropins and menopause-related BMD loss (30). Higher levels of FSH (but not E2) predicted faster rates LS aBMD loss in the pre-transmenopausal and transmenopausal phases. During the third phase, starting 2 years postmenopause, lower levels of E2 (but not FSH) predicted higher rates of LS loss. Sex steroids and gonadotropins did not predict aBMD loss during any phase at the FN site. Our finding that higher FSH and lower E2 were related to LS but not femoral bone loss could be a due to statistical power – the ability to detect associations is greater at the LS, because the rate of bone loss is 50% higher than that at the FN (23). Or, it may be that the LS, due to the predominance of trabecular bone, is more sensitive to the effects of changing hormones during the MT (31). TBS may be even more responsive than aBMD to sex hormone changes, because TBS is a more direct index of trabecular bone.

Observations from SWAN that FSH (and not E2) is related to perimenopausal bone loss have fueled the debate about whether FSH (vs. E2) is the primary determinant of bone loss, especially in the early MT (30, 32). Investigations into the role of FSH and bone loss using mouse models produced disparate results. One group reported that FSH receptor null (FORKO) mice are hypogonadal, but have normal bone mass (33). However, these mice also have high testosterone levels, which may be bone-protective. Others found that FORKO mice do have reduced bone mass and that bilateral oophorectomy dropped their testosterone levels, leading to bone mass values similar to oophorectomized control mice (34). A randomized controlled trial in which postmenopausal women were randomized to leuprolide acetate or placebo (along with an aromatase inhibitor in each group) found that FSH suppression did not affect bone turnover markers (35). It may be that FSH is a more sensitive marker of declining E2 levels early in the MT, especially in studies such as SWAN that measure annual sex steroids during the early follicular phase, when estrogen is at its cyclic nadir. This project proposes to examine the longitudinal relations between change in FSH and E2 and TBS, but the interpretation of these associations must be done in the context of the challenges of capturing declines in E2 in the early transition.

B.4. Fracture Risk at Menopause: The rapid aBMD loss that occurs during the MT, could place women at a higher risk for fractures. SWAN is the first large scale multiethnic longitudinal cohort to assess BMD, bone resorption and fractures across the MT (36). After a mean of 7.6 years of follow-up, 184 (8.7%) women experienced an incident fracture. Women who experienced an incident fracture had significantly lower LS aBMD at baseline (premenopausally), 1.05 (0.14) g/cm^2 than women without a fracture, 1.08 (0.14) g/cm2, p=0.005. In contrast, premenopausal total hip aBMD was similar in women with and without a fracture. A

one-standard deviation (SD) decrease in LS aBMD was associated with a hazard ratio (HR) of fracture, HR=1.50; 95% confidence interval (CI), 1.28-1.68. In summary, LS BMD was associated with an increased risk of fracture across menopause, and the magnitude of the association was similar to what has been reported for older women and men (18, 37). Notably, hip aBMD was not associated with fractures across the MT.

B.5. Importance of Microarchitecture of Bone: Osteoporosis is defined as a combination of diminished bone mass and altered bone quality resulting in decreased bone strength and an increased risk of fractures (38). While aBMD by DXA is the gold standard clinical tool for osteoporosis detection, its short comings are acknowledged (39). For example, aBMD's 2-dimensional (D) estimate of density systematically underestimates the actual 3-dimensional density of smaller bones. Individuals with smaller bones will have lower BMD by DXA. This systematic bias in aBMD due to differences in bone size accounts for some of the well-known disparities between aBMD and fracture risk. Specifically, Asian women have aBMD that is similar or lower than Whites (40) but have lower fracture risk (41).

Trabecular bone microarchitecture is an important predictor of fracture independent of bone mass. Animal and human studies show that thinning of trabecular and loss of connectivity substantially diminishes the load bearing capacity of bone (42-44). Until now, microarchitecture could only be measured by bone biopsies, high resolution x-ray imaging or MRI. The development of TBS makes epidemiological and clinical evaluation of bone microarchitecture feasible and enables us to pursue the proposed investigation using archived DXA scans (45).

B.6. Trabecular Bone Score: Hans and colleagues have developed an algorithm to analyze the LS scans to determine the TBS. The methods and validation of TBS were published in 2008 (45). TBS was developed using 3D μCT images of trabecular bone specimens from human cadavers. TBS is an **index** of bone microarchitecture obtained from 2D DXA images, not a direct measure of bone microarchitecture (45, 46). TBS is, however, correlated with micro-computed tomography measures of bone volume fraction, connectivity density, trabecular number and trabecular separation and with vertebral mechanical behavior in ex-vivo studies (46). These correlations remained significant after adjustment for aBMD. The TBS analyzes pixel gray scale variations from 2D LS DXA images and is related to bone microarchitecture and fracture risk, providing information independent of aBMD (47-50). A dense healthy trabecular network, associated with greater mechanical bone strength, produces a projection image with many gray-level texture variations of small amplitude and therefore a steep variogram slope with a high TBS value (better bone strength), **Figure 2A column**. In contrast a low TBS value indicates fewer gray-level texture variations of larger amplitude and therefore a lower slope (worse bone structure), **Figure 2B column**.

Figure 2: Trabecular Bone Score: focal variations of change in trabecular bone density

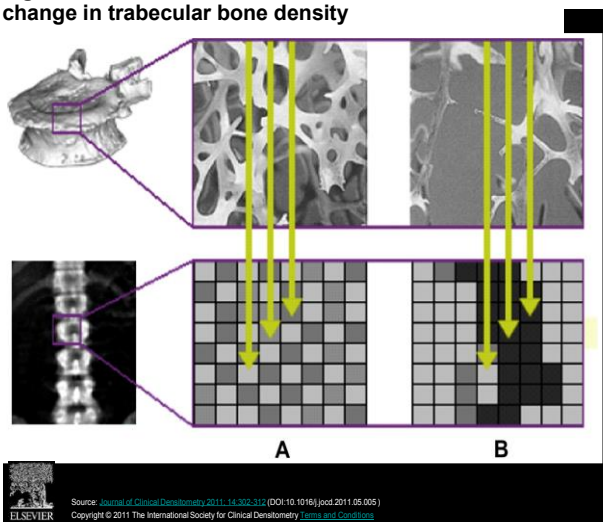
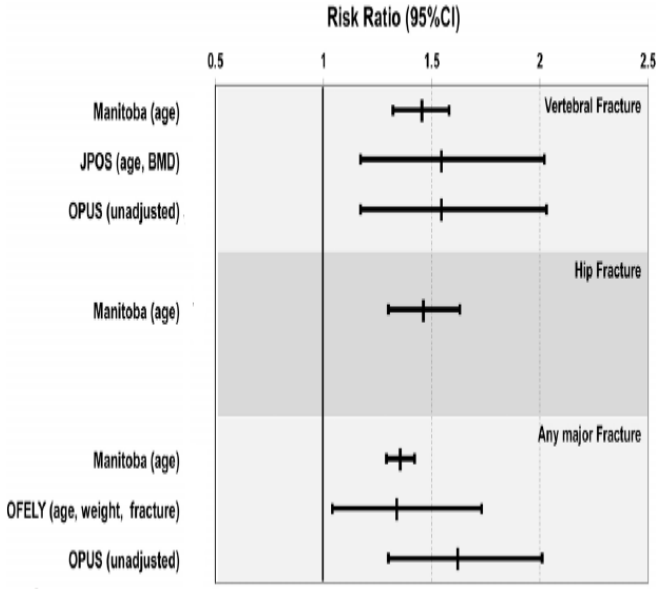


Figure 3: Risk Ratio (95% CI) per SD decrease in TBS



Case control studies showed that TBS was significantly lower in subjects with fractures compared to controls (51-57). The magnitude of the TBS difference between those with a fracture versus control were similar for vertebral, hip or any major fracture (58).

A model with both aBMD and TBS significantly increased sensitivity (28% increase) with modest improvements in specificity (9% increase) (52). The net reclassification index was 36%; thus, more than one-third of women were reclassified correctly as a fracture case or control based on a combination of TBS and aBMD as compared to either measure alone.

A recent review summarized the published prospective

studies of TBS and fracture (48, 59-62), ***Figure 3****. This review (48) and others (46) concluded that TBS holds promise as an emerging technology that could become a valuable clinical tool in diagnosis of osteoporosis and fracture risk assessment.* The risk ratio for each SD decline in TBS was associated with 1.3-1.6-fold increase in vertebral, hip and any major fracture. In the largest study from Manitoba, Hans et al studied 29,407 women (mean age 65 years) for a mean follow-up of 4.7 years (61). For every SD decline in TBS, there was an increased risk of clinical spine fracture, HR=1.29; 95% CI, 1.17-1.42), hip fracture (HR=1.40; 95% CI, 1.24-1.56) and any major osteoporotic fracture (HR=1.25; 95% CI, 1.19-1.31) (61). These associations were independent of total hip, FN or LS aBMD and age. The combined AUC for BMD of the FN and TBS was 0.73, clinical spine fracture; 0.81, hip fracture and 0.69, any major osteoporotic fracture. There was significant improvement in AUC when TBS was added to the corresponding model for aBMD. Similar results were reported in the Ofely cohort (mean age 67.5 years) (60) and in a prospective study of Japanese women (mean age 64 years) (59). In the European Osteoporosis and Ultrasound Study (OPUS) of 1007 women (mean age 65.9 years) (62), the AUC for TBS was similar to BMD and the combinations with TBS with BMD. *More recently, Leslie et al updated the Manitoba results and compared prediction models using the WHO Fracture Risk Assessment tool (FRAX) and TBS (63). In models adjusting for FRAX (clinical risk factors and FN aBMD), LS TBS remained a significant predictor of major osteoporotic fracture (HR=1.18 (95% CI, 1.12-1.23). TBS was also associated with an increased mortality but the association with fracture remained even after adjusting for competing mortality. Thus, TBS provides additional risk information above and beyond FRAX and aBMD. It is well established that most fractures occur in women with low bone mass (T-score -1 to -2.5). A recent study of 429 women, age 71.3 showed that TBS identified 66-70% of women with fracture who were not classified as osteoporosis by BMD alone (64).*

Of importance, the mean age of the women in these prior studies was approximately 65 years. Nothing is known about TBS in midlife women who are experiencing the MT (Aim 2), nor do we know whether TBS and fracture are related during midlife (Aim 3). *Attempts have been made to create a reference database of TBS in US non-Hispanic White women (65). This cross-sectional study of 619 US women, age 30-90 showed that there was little change in TBS from age 30 to 45. Between age 45-65, sharp declines in TBS were noted; after 65, the decline was much slower. This observed pattern mimics what has been observed for aBMD but is limited in its cross-sectional design and lack of information on menopause.* Finally, it has been shown that hip BMD is somewhat more strongly related to all types of fractures than spine aBMD at least in older women (18). Thus, as part of Aim 3, we will test whether the association between TBS and fracture is independent of either aBMD of LS or hip *as well as hip geometry and strength*.

B.7. Independence of TBS from aBMD and Associations with other Measures of Trabecular Microarchitecture: Correlations between TBS and aBMD are modest ranging from r=0.28 (49, 64) to r=0.52 (54), suggesting that TBS provides additional information above and beyond that afforded by aBMD. In one study, the correlation between TBS and LS BMD in women age 45-55 years was 0.41 (66). Notably, the correlation between TBS and aBMD was even weaker in older women age 75-88 years, r=0.31, consistent with the expected loss of trabeculae that occurs with age (66). Indeed, TBS was 14.5% lower in women aged 85 compared to those in their 40's, for an estimated "decline" of 0.36% per year. *TBS correlated with microstructural parameters of bone evaluation by HRpQCT in a random sample of 72 premenopausal women aged 20-40 suggesting that TBS is an index of microarchitecture in young premenopausal women (67).* The correlation between TBS and microarchitectural parameters using excised vertebral have been reviewed (48). TBS was correlated with vertebral trabecular number (r=0.68 to 0.82), trabecular spacing (r= -0.64 to -0.72) and connectivity (r=0.71 to 0.87), but not with trabecular thickness. But, the correlations varied widely across bone sites and studies varied in microarchitectural parameters and designs. An ex-vivo study showed the combination of TBS, trabecular thickness and bone mineral content explained 79% of variability in bone stiffness (49).

B.8. Ethnic Difference in TBS: There is minimal data on TBS differences by race/ethnicity. *A recent cross-sectional study of 4550 representative Japanese women showed that TBS was lower in Japanese women than Caucasian women across all ages with the differences increasing with age, especially from age 45 to age 65 (68). The results suggested that the effects of estrogen deprivation on trabecular bone microarchitecture may be greater in Japanese women than in Caucasian women and is consistent with observations from SWAN that both Japanese and Chinese women experienced greater declines in aBMD across the MT (22, 23).* HRpQCT studies from the Boston SWAN site support that skeletal cortical and trabecular bone microarchitecture is greater in Blacks (n=100) vs White women (n=173) (69). This cross-sectional comparison was carried out during the 11th and 12th SWAN follow-up visits at which time women were age 56-66 and 93% were postmenopausal. Comparison of trabecular microarchitecture at the radius showed greater trabecular

thickness and lower trabecular separation among Blacks compared to Whites. Trabecular thickness at the tibia was higher among Blacks but differences were attenuated with adjustment for clinical covariates. Of importance, this study could not capture worsening in bone structural characteristics in association with advancing MT. Pre- and postmenopausal Chinese women had smaller bones than White women but thicker, denser cortices and thicker trabeculae leading to greater bone strength at the radius and tibia using HRpQCT (70). Total appendicular vBMD was 10% higher in Chinese but trabecular vBMD did not differ because trabeculae were 7% fewer but 10.8% thicker (71). In another study using central CT scans, LS trabecular vBMD was 5.8% higher in Chinese vs White premenopausal women (72) but there was no difference in LS vBMD in postmenopausal women suggesting that decreases in vBMD with MT may be greater in Chinese than White women. In summary, little is known about race/ethnic differences in TBS or other measures of microarchitecture. For the most part, the sample sizes were small (≈ range 29-83). The proposed project will be the largest comparison of trabecular microarchitecture in women across 3 race groups, all recruited using the same inclusion/exclusion criteria and protocol. We hypothesize that while White and Japanese women have similar aBMD after adjustments for body weight and other covariates, Japanese women will have lower TBS than Whites. We hypothesize that Black women will have higher TBS than White and Japanese women.

B.9. TBS and Sex Steroids: A small (n=76) ancillary study to the Kronos Early Estrogen Prevention Study (KEEPS) compared transdermal and oral estrogen with placebo on cortical and trabecular microarchitecture (73). Menopausal hormone therapy (HT) preserved trabecular bone at spine but not radius for reasons unknown. To our knowledge, there is no data on effects of HT on TBS which could be an exploratory analysis of the current application. A small study compared the effects of tamoxifen (n=17) with exemestane (n=19), an aromatase inhibitor in women with estrogen receptor positive breast cancer (74). Women randomized to tamoxifen experienced a mean increase of TBS, 2.2%, 3.5% and 3.3% at 6-, 12- and 24-month treatment, respectively. Conversely, women randomized to exemestane showed decreases in TBS of -0.9%, -1.7% and -2.3% at 6-, 12- and 24-month treatment, respectively. Thus, TBS is clearly hormonally sensitive. Little is known about the association between endogenous gonadotropins, sex steroids and TBS.

B.10. Diabetes, Menopause and Skeletal Health: Meta-analyses have documented a higher risk of fracture in both Type 1 and Type II diabetes despite higher aBMD (75). Diabetic status has also been linked to faster rates of bone loss (76). In SWAN, diabetic women experienced menopause 3 years earlier than non-diabetic women and the risk of fracture was 2-fold higher in diabetic women compared to non-diabetic women, HR=2.20; 95% CI, 1.26-3.85) (77). Of importance to the current protocol, Leslie et al showed that while Type 2 diabetics had higher aBMD, their TBS was significantly lower suggesting that the higher fracture risk in diabetics may be due to impairments in microarchitecture (78). TBS predicted fractures in subjects with diabetes and captured a larger proportion of the diabetes associated fracture risk than aBMD.

B.11. Gaps in Current Knowledge: Trabecular bone microarchitecture was studied in 38 women with paired transilial biopsies specimens premenopausally and 12 months after their FMP (79). Bone volume fracture (-13%), trabecular number (-9%), volumetric density (-10%) declined significantly from pre to post-menopause and trabecular spacing (+7%) increased. These biopsies demonstrate that trabecular bone structure declines at menopause and are an important first step in our understanding of how menopause damages bone structure. A large US cross-sectional study of 619 US women aged 30 to 90 showed the greatest change in TBS between ages 45-65, suggesting that menopause may play a role in the decline in TBS with age (65). A small longitudinal study of 29 women who became menopausal after 2.9 years showed that TBS declined 4.6% (80). Taken together, these small studies suggest that TBS declines at menopause. But, they cannot tell us when the trabecular microarchitecture decline begins and whether the amount or rate of change in microarchitecture predicts fracture outcome. Limited data exists on ethnic differences. All TBS fracture analyses were carried out in much older women. This proposal offers a unique opportunity to address these important gaps in our understanding of the skeletal consequences of the MT.

C. INNOVATION

Innovation is often thought of as the *invention of a new idea or method*. But innovation also includes the *generation of new knowledge* and *taking the steps required to meaningfully advance an existing theory*; the proposed project will accomplish each of these latter two aspects of innovation. To date there is no other existing cohort that can address these research questions. It will:

- Newly describe the natural history of change (we hypothesize decline) in trabecular microarchitecture, assessed by TBS, during the MT and into the early-to-mid-to late postmenopausal period.
- Describe characteristics that may be associated with higher peak TBS (at baseline, prior to the MT), such as Black race.

- Study, for the first time, characteristics that may predict faster or slower loss of TBS, such as sex steroid levels or dysregulated glucose metabolism.
- Meaningfully advance the thesis that the rapid bone loss that characterizes the MT is particularly damaging to skeletal strength. For many years, investigators have asked whether the rapid BMD loss that happens during the MT has implications beyond the BMD decline itself. On average, in SWAN, the absolute quantity of BMD lost during the 3-year period bracketing the FMP, 7.4% at the LS and 5.3% at the FN, is unlikely to result in a BMD value sufficiently low to meet even the most conservative treatment thresholds. But absolute decline in BMD may be less critical than the rapid bone turnover that it signals. Rapid turnover may result in weakened trabeculae, loss of trabecular elements, and diminished trabecular connectivity (31). Concordant with this thesis, histomorphometry studies that did paired transilial biopsies in women in their mid 40's and again one year after the FMP demonstrated declines in trabecular number, enlargement of trabecular spacing and conversion of trabecular plates to rods, in direct correspondence with increases in activation frequency (31, 79). Our large sample of women and 13 serial TBS assessments would newly provide information on spinal trabeculae. *Moreover, it would allow us to describe the timing of onset and rates of TBS decline in relation to the FMP and to test whether thess trajectories mimic trajectories in aBMD, FN geometry and composite strength indices (CSI) and to evaluate greater declines in TBS are independently related to greater fractures risk.*
- Concern about irreparable architectural bone damage has led some to advocate for short-term anti-resorptive therapy during the MT in an attempt to prevent such damage (81). While we concur that the major import of transmenopausal accelerated bone loss may be its threat to microarchitecture, and indeed prevention may be warranted, we do not believe that the currently available data are sufficient to recommend treatment. Rather, we believe further characterization of this phenomenon is essential.
- In addition to newly quantifying the amount and temporal pattern of TBS loss during the MT, the proposed study will gauge the impact of such loss, independent of decline in LS and hip BMD, *hip geometry and CSI* by assessing whether TBS decline independently predicts fracture risk. If so, this would substantively advance the case for a prevention trial targeted to this period of rapid loss.

SPECIFIC AIMS

Our hypothesis is that there will be a combination of serum markers that can be used to define the biological progression of fracture healing and that we will be able to relate one or more of these markers to specific structural, functional and clinical radiological signs that define the progress of healing.

Aim 1: This aim will identify those proteins in the serum proteome that show quantitative changes in their levels of expression across the time course of fracture healing relative to unfractured bone.

a) Two different approaches will be used in this aim: a mass spectrometry approach and; a targeted novel aptamer-based multiplexed proteomic technology. The use of these two approaches will provide differing capabilities with Mass Spectrometry providing the ability to survey the entire proteome but having the limitation of nM sensitivity while the aptamer approach provides a more limited coverage of the serum proteome of ~700 proteins but having a greater dynamic range of sensitivity to ~pM levels. Time points that are indicative of the initial inflammatory response to injury, periods of cartilage formation, resorption and bone formation, tissue mineralization and bone remodeling (3, 7, 10, 14, 21, and 35 days post fracture) will be assessed in comparison to no fracture in normal healing of C57/B6 mice.

In this aim, we are using two methods to provide mutual cross confirmation. The aptamer approach was chosen to assess along with Mass Spectrometry since it has the potential to develop new aptamers and has easy multiplexing capability, with high sensitivity across a dynamic 9 log range at greater efficacy than an ELISA. It is our intent to use this approach in the second aim of the project, since its costs are comparable to developing novel antibodies and establishing and validating new ELISA assays. We also chose to use this method secondary to Mass Spectrophotometry since this method is easily adapted for human translation.

Aim 2: In aim 2, we will test a subset of 18 protein candidates identified in aim one, using a multiplex assay and test for their statistical correlation between these proteins and specific characteristics of callus tissue composition, tissue mineralization callus structural, biomechanical features and clinical radiological features by which progression of healing are defined.

a) In the first part of this aim, we will test for correlation between the modified Radiographic Union Score for Tibial (RUST) fracture healing score (Whelan *et al*., 2010)that is used to assess the progression of human long bone healing to the regain of specific callus structural and biomechanical functions. Using CCET and μCT measurements and biomechanical assessments, we will determine what features of callus composition and structure (cartilage, development and remodeling callus mineralization, callus volume) and callus biomechanical features (stiffness, strength, work to failure) correlate to the RUST score. This part of the aim is designed to identify what biomechanical and structure/ compositional features show the best correlation to the current clinical standard (RUST score) of assessing fracture healing.

b) In the second part of this aim, we will test the 18 protein subset identified in Aim 1 for their statistical correlation to the cartilage, development and remodeling callus mineralization, callus structural features, biomechanical features and RUST score assessments.

Outcomes: These comparisons in a step wise manner will allow us first identify the specific serum protein markers that show variations in expression that are associated to the biological progression of fracture healing. We will then determine the relational value of various markers to progression of healing as assessed by their correlation to structural and biomechanical features of fracture healing. We will then determine the degree to which a currently used radiological standard of human fracture healing correlates to specific structural and biomechanical features of healing and finally to specific protein markers. If successful, these study will identify a set of proteins that can be used in a human trial to follow key biological parameters that define the normal progression of fracture healing. We will identify those markers that show the strongest correlation to various biological and radiological features and those that might have greatest prognostic efficacy for delayed or failed healing.

RESEARCH STRATEGY

Significance Fractures are the most common large-organ traumatic injuries in humans, and osteoporosis-related fractures are the fastest growing health care problem of aging. Costs related to osteoporotic fractures approached $24 billion in 2004 with costs of treating injuries from falls projected to be $64 billion by 2020 (Stevens *et al*., 2006). (USDHHS Office of the Surgeon General, 2004, http://www.ncbi.nlm.nih.gov/books/NBK45513/) While the processes of fracture repair after surgery are usually optimal, the healing of up to 10% of the estimated ~8 to 10 million fractures that occur annually in the United States is delayed or impaired (Praemer *et al*., 1992). Almost all of these cases of poor healing require surgical intervention at significant cost and with considerable patient morbidity and represent about 1/3 of the cost of treating fractures.

Currently, radiographic assessment with reduction in healing complication validated patient reported out- comes of regain of pain free weight bearing and function are the primary diagnostic tools to assess the progression of fracture healing. These assessments are the primary surrogates that have regulatory acceptance in assessing medicinal compounds that promote fracture healing (Goldhahn *et al*., 2008). While there are now a number of radiological assessments for scoring the progression of healing and for defining non-union these are highly operator specific (Lane and Sandhu, 1987; Whelan *et al*., 2010). Assessing regain of function on patient based criteria is also very subjective. Finally, there is no one consensus on how to evaluate the progression of fracture healing (Axelrad and Einhorn, 2010; Goldhahn *et al*., 2008; Bhandari *et al*., 2002). This has made identifying those patients that might have problems in healing before it is necessary to do a revision and the assessment of therapeutics that could be used to enhance fracture healing very difficult. Radiographic and clinical measures of regain in function do not quantitatively measure specific underlying biological processes associated with the progression of healing. While plain film is the primary tool that is used for the diagnosis of delayed or failed healing, it does not identify those individuals at risk to develop non- union, nor does it define underlying biological conditions that might distinguish a non-union. Finally, due to the low sensitivity of radiographs and the subjective nature of validated patient reported outcomes human fracture healing studies are both more costly, more time consuming and need much larger numbers of patients to be enrolled in a Phase 2 or 3 clinical studies to reach meaningful outcomes. Finally current regulatory precedent is to have a separate study for every single fracture site for which a new therapeutic approach will have an approved use, has meant that each study has to be uniquely tailored to the bone that is being assessed with no one common set of factors that can be compared across studies.

Thus, there is an immense and immediate need to identify objective quantifiable biological markers that: relate to the underlying biological processes which define the progression of skeletal tissue healing: 2) that are indicative of the progression of skeletal tissue healing: 3) that would be prognostic of deficiencies in skeletal tissue healing. The development of an assay(s) would be an immense benefit and: 1): would be informative to the underlying causes for delayed and failed healing; 2) would be useful in clinical trials that assess the efficacy of biological or pharmacological therapies that promote bone healing; 3) potentially could identify those patients that would benefit from biological or pharmacological therapeutic interventions to promote bone healing. A biological assay that assesses fracture healing would also have immense benefit in assessing the pharma-cokinetics and levels of appropriate dosing for medicinal compounds (Fu *et al*. 2010; Regnier *et al*., 2010).

A review of the use of serum markers to predict fracture healing is presented in Pountos *et al.,* 2013. Over the last 10 years, a small number of animal and humans studies have been carried out using specific candidates markers found in serum that are indicative of bone formation (Cox *et al*., 2010; Stoffel *et al*., 2007; Ivaska *et al*., 2007), bone turnover (Stoffel *et al*., 2007; Ivaska *et al*., 2007; Seebeck *et al*., 2005), angiogenesis (Sarahrudi *et al*., 2009; Henle *et al*., 2005) and mineral metabolism (Goebel *et al*., 2009). The general consensus that has emerged from this small number of studies is that currently available bone formation markers commonly used to study osteoporosis including osteocalcin, BLK APase, collagen PINP have failed to detect variations in the progression of fracture healing. On the other hand, a number of studies now have shown that markers of bone turnover (Stoffel *et al*., 2007) and markers of angiogenesis are informative to the progression of healing, and even may have the potential to predict the development of non-union (Sarahrudi *et al*., 2009; Henle *et al*., 2005) or can be related to underlying biological processes of fracture healing (Wigner *et al*., 2012). The primary deficiencies in these studies have been that they are based small patient or animal sample sizes, focus usually on single candidate markers, are poorly controlled for the nature of the fracture or fixation methods used to repair the fracture and lack a robust set of measurable callus parameters that can be used to relate the underlying biological events in the callus back to the markers. *The paucity of well controlled*

studies in either animals or humans, the lack of identification of a broader set of uniquely expressed proteins associated with fracture healing, and the lack of a validated relationship of protein expression to various biological and temporal events of fracture healing has been a major impediment to developing serum based biological markers to follow the progression of fracture healing.

Innovation To date, there has been no systematic attempt to survey the serum proteome across the time course of fracture healing and systematically screen for detectable changes in serum markers that might relate to the biological processes that underlie the progression of healing. Our studies will be the first to do this and then attempt to develop correlations between serum markers to, tissue composition, structural and biomechanical features and clinical assessments that define the progression of fracture healing. If successful they will then enable us to relate how specific protein markers could be related to both quantifiable structural and functional metrics of the progression of fracture healing. We also feel the project is technically innovative. In this context we are using a novel cationic cartilage contrast agent that was developed by Dr. Mark Grinstaff (BU College of Engineering) which gives us accurate volumetric measurements of cartilage compositions in the callus from micro CT analysis. These measurements are correlated to the contrast agent's interactions with proteoglycan [(Hayward *et al*., 2012, 2013; Stewart *et al*., 2013; Bansal *et al*., 2010). This will allow us to develop accurate relational characteristics between progression of endochondral bone formation and potential serum markers that define cartilage formation or turnover. In the context of the proteomic assessment it is also technically innovative in that it will apply two state of the art proteomic approaches Mass Spectrometry and an Aptamer-Based Multiplexed Proteomic Technology available as a fee for service through Somalogics Inc (Gold *et al*., 2010).