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Background: Inflammation is implicated in many aging-related disorders. In animal models, menopause leads to increased gut permeability and inflammation. Our primary objective was to determine if gut permeability increases during the menopause transition (MT) in women. Our exploratory objectives were to examine whether greater gut permeability is associated with more inflammation and lower bone mineral density (BMD).Methods: We included 65 women from the Study of Women's Health Across the Nation. Key measures were markers of gut permeability (gut barrier dysfunction [fatty acid binding protein 2 [FABP2]) and immune activation secondary to gut microbial translocation (lipopolysaccharide binding protein [LBP], soluble CD14 [sCD14]); inflammation (high-sensitivity CRP); and lumbar spine (LS) or total hip (TH) BMD. Results: In our primary analysis, FABP2, LBP, and sCD14 increased by 22.8% (P = 0.001), 3.7% (P = 0.05), and 8.9% (P = 0.0002), respectively from pre- to postmenopause. In exploratory, repeated measures, mixed-effects linear regression (adjusted for age at the premenopausal visit, body mass index, race/ethnicity, and study site), greater gut permeability was associated with greater inflammation, and lower LS and TH BMD. Conclusions: Gut permeability increases during the MT. Greater gut permeability is associated with more inflammation and lower BMD. Future studies should examine the longitudinal associations of gut permeability, inflammation, and BMD.Funding: NIH, Department of Health and Human Services, through the National Institute on […]

TITLE

Gut permeability, inflammation, and bone density across the menopause transition

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CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

ABSTRACT

Background: Inflammation is implicated in many aging-related disorders. In animal models, menopause leads to increased gut permeability and inflammation. Our primary objective was to determine if gut permeability increases during the menopause transition (MT) in women. Our exploratory objectives were to examine whether greater gut permeability is associated with more inflammation and lower bone mineral density (BMD).

Methods: We included 65 women from the Study of Women's Health Across the Nation. Key measures were markers of gut permeability (gut barrier dysfunction [fatty acid binding protein 2 [FABP2]) and immune activation secondary to gut microbial translocation (lipopolysaccharide binding protein [LBP], soluble CD14 [sCD14]); inflammation (high-sensitivity CRP); and lumbar spine (LS) or total hip (TH) BMD.

Results: In our primary analysis, FABP2, LBP, and sCD14 increased by 22.8% (p=0.001), 3.7% (p=0.05), and 8.9% (p=0.0002), respectively from pre- to postmenopause. In exploratory, repeated measures, mixed-effects linear regression (adjusted for age at the premenopausal visit, body mass index, race/ethnicity, and study site), greater gut permeability was associated with greater inflammation, and lower LS and TH BMD.

Conclusions: Gut permeability increases during the MT. Greater gut permeability is associated with more inflammation and lower BMD. Future studies should examine the longitudinal associations of gut permeability, inflammation, and BMD.

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INTRODUCTION

Inflammation contributes to the pathogenesis of numerous medical disorders that commonly affect older adults, including osteoporosis, diabetes mellitus, cardiovascular disease, and dementia (1-10); this is often referred to as "inflammaging" (3, 11). Beyond chronological aging, the postmenopausal state may independently contribute to greater inflammation in older women. In animal models, chemical or surgical menopause leads to down-regulation of epithelial junction proteins, gut barrier dysfunction and increased gut permeability (12). This, in turn, permits the translocation of microbes from the intestinal lumen into the subepithelial space, triggering immune cells to produce pro-inflammatory cytokines (12). Whether an increase in gut permeability accompanies the menopause transition (MT) in humans is uncertain. If gut permeability does increase, whether it is associated with inflammation and end organ manifestations is also unknown.

The overarching goals of this pilot study were to determine if gut permeability increases during the MT, and if such an increase were confirmed, to explore whether gut permeability is associated with inflammation and bone mineral density (BMD). In this report, subsequent use of the term "gut permeability" refers to its indirect assessment using these blood markers. We used bone as a model end-organ system because increased gut permeability mediates inflammation and hypogonadal BMD loss in rodents, and probiotics that reduce gut permeability (12) or inflammation (13-15) can decrease bone resorption (12-15) and prevent bone loss (12, 15). Our primary hypothesis was that gut permeability increases from pre- to postmenopause. Our exploratory hypothesis was that greater gut permeability is associated with greater inflammation

(assessed by high-sensitivity CRP [hs-CRP]) and lower BMD (assessed by dual-energy X-ray absorptiometry [DXA]).

The parent study for this pilot was the Study of Women's Health Across the Nation (SWAN), a multi-racial/ethnic, longitudinal cohort study of the MT. We obtained two stored plasma samples for each study participant from the SWAN Repository: one from premenopause (operationalized as 3-5 years before the final menstrual period [FMP]), and one from postmenopause (operationalized as 3-5 years after the FMP). To assess gut permeability, we assayed indirect markers of gut barrier dysfunction (fatty acid binding protein 2 [FABP2]) and immune activation secondary to gut microbial translocation (lipopolysaccharide binding protein [LBP], soluble CD14 [sCD14]). FABP2 is considered a marker of gut epithelial cellular dysfunction because it is expressed by enterocytes, and released into the circulation when gut epithelial cells are damaged (16, 17). LBP and sCD14 are deemed markers of immune activation secondary to gut microbial translocation because they are produced by hepatocytes and monocytes/macrophages, respectively, in response to lipopolysaccharide (a product of gram negative bacterial, of which the gut is the predominant source) (18, 19).

RESULTS

Participant Characteristics in Pre- to Postmenopause

We included a total of 65 women. At the premenopausal visit, subjects were, on average, 49.9 years of age, and 3.8 years before their FMP. At the postmenopausal visit, mean age was 57.7 years and mean time after the FMP was 4.0 years (Table 1).

Results for Primary Objective: Change in Gut Permeability during the Menopause Transition

From pre- to postmenopause, median E2 decreased from 51.7 to 15.5 pg/ml ($p<0.0001$, Wilcoxon signed-rank test), and FSH increased from 14.8 to 84.3 mIU/ml ($p<0.0001$, Wilcoxon signed-rank test) (Table 1). E2 and FSH had skewed distributions, so were log-transformed for analyses. During the same period, FABP2, LBP, and sCD14 increased from 1,298 to 1,595 pg/ml, 5892 to 6,112 ng/ml, and 948 to 1,032 ng/ml, respectively.

In our primary analysis, we used the paired t-test at a two-sided alpha of 0.05 to determine if the rise in each gut permeability marker was statistically significant. Using this approach, the increases in FABP2 ($p=0.001$), LBP ($p=0.05$), and sCD14 ($p=0.0002$) were considered statistically significant. We further examined the associations of these markers with E2 and FSH (log transformed), using repeated measures, mixed effects linear regression (Table 2). Adjusted for chronological age at the premenopausal visit, race/ethnicity, BMI, and study site, lower E2 and greater FSH (tested separately) were associated with greater FABP2 and sCD14. Each 50% decrement in E2 was associated with 77 pg/ml greater FABP2 ($p=0.02$), and 28 ng/ml greater sCD14 ($p=0.001$). Analogously, each two-fold increment in FSH was associated with 113 pg/ml

greater FABP2 (p=0.001), and 34 ng/ml greater sCD14 (p<0.0001). Neither E2 nor FSH was significantly associated with LBP.

Results for Exploratory Objective: Associations of Gut Permeability with Inflammation and Bone Mineral Density

Median hs-CRP increased from 1.4 mg in premenopause to 1.6 mg/L in postmenopause, but this increase did not reach statistical significance (p=0.06, Wilcoxon signed-rank test). The distribution of hs-CRP was skewed. During the same period, mean LS and TH BMD decreased from 1.104 to 0.986 and from 0.964 to 0.901 g/cm^2, respectively (p<0.00001 for both sites). Scatter plots of FABP2, LBP, and sCD14 vs. hs-CRP are presented in Figure 1. Figure 2 contains scatter plots of FABP2, LBP, sCD14 and hs-CRP vs. BMD; we present plots for LS only, as those for FN were similar (data not shown).

To test whether greater FABP2, LBP, or sCD14 is associated with greater hs-CRP, we used repeated measures, mixed effects linear regression (Table 3). hs-CRP was log transformed for these analyses because of skewed distributions. Adjusted for age at the premenopausal visit, race/ethnicity, BMI, and study site, each SD increment in LBP and sCD14 was associated with 2.32- (p<0.001) and 1.44- (p=0.001) fold greater hs-CRP, respectively. FABP2 was not associated with hs-CRP.

To examine the associations of FABP2, LBP, sCD14 and hs-CRP with BMD, we again used repeated measures, mixed effects linear regression. Adjusted for the same covariates listed above, each SD increment in FABP2 was associated with 0.021 and 0.017 g/cm^2 lower LS

(p=0.05) and TH BMD (p=0.02), respectively. Similarly, each SD increment in sCD14 was associated with 0.051 lower LS BMD (p=0.0001) and 0.025 g/cm^2 lower TH BMD (p=0.004). LBP was not associated with BMD. In contrast, each two-fold increment in hs-CRP was associated with 0.024 g/cm2 lower LS (p<0.0001) BMD; hs-CRP was not associated with TH BMD.

DISCUSSION

The primary objective of this longitudinal, pilot study was to determine if gut permeability (assessed non-invasively using markers of gut barrier dysfunction [FABP2] and immune activation secondary to microbial translocation [LBP, sCD14]) increases during the MT. Upon observing this increase, our exploratory objective was to examine whether greater gut permeability was associated with greater inflammation and lower BMD. Our primary analysis supports the hypothesis that, in humans, gut permeability increases from pre- to postmenopause. Exploratory analyses suggest that greater gut permeability (LBP and sCD14) is associated with greater inflammation (hs-CRP), and both greater gut permeability and inflammation (FABP2, sCD14, hs-CRP) are associated with lower BMD.

Several prior studies have reported the effects of aging, but not the MT, on gut permeability in humans. One cross-sectional analysis found that gut permeability was higher in older vs. younger men and women (20). Subsequently, the longitudinal Cardiovascular Health Study determined that sCD14 increased by 0.4% per year in men and women over 65 years ($p<0.0001$) (21). This is the first human study, to our knowledge, to demonstrate that gut permeability (assessed indirectly) increases during the MT. This finding is additionally supported by data that lower E2 and greater FSH are associated with greater FABP2 and sCD14.

In rodent models, increased gut permeability underlies an MT-related increase in inflammation. Chemical or surgical menopause leads to decreased expression of epithelial junction proteins (e.g., claudins 1, 2, 3, and Jam3), and increased gut permeability (12). This, in turn, permits the translocation of microbes from the intestinal lumen into the subepithelial space, triggering

immune cells to produce pro-inflammatory cytokines (12). Our exploratory analyses suggest that immune activation from gut microbial translocation (LBP and sCD14) is associated with greater inflammation (hs-CRP). The implications are that a mechanism that contributes to inflammation in rodents may similarly be present in humans, and that MT-related changes in gut physiology warrants further research as a potential therapeutic target for preventing inflammation.

The physiologic importance of increased gut permeability and inflammation is highlighted in rodent studies in which probiotics that reduce gut permeability (12) or inflammation (13-15) can decrease bone resorption (12-15) and prevent bone loss (12, 15). In humans, a prior SWAN study reported that greater inflammation (assessed by hs-CRP) is associated with lower BMD (cross-sectional), lower hip strength (cross-sectional), and more future fractures (4). We also have ongoing analyses using the full SWAN Bone Cohort to examine the associations of change in hs-CRP and IL-6 with change in BMD. Here, we report that greater FABP2 and sCD14 are associated with lower LS and TH BMD, and greater hs-CRP is associated with lower LS BMD. While these findings suggest a link between gut permeability, inflammation and BMD, this study was not sufficiently powered to explicitly relate changes in FABP2, LBP, sCD14, and hs-CRP to concurrent change in BMD. We also could not discern whether inflammation vs. circulating bacterial products contribute more to lower BMD.

Gut permeability can be assessed by various methods. Histologic and electron microscopic evaluation of biopsy samples provides direct information on gut barrier integrity and mucosal tight junctions (22). Alternatively, enteral administration of non-digestible markers affords a functional assessment of gut permeability (22-24). However, neither methodology is well-suited

to community-based cohort designs. For this pilot study, we indirectly assessed gut permeability using markers of gut barrier dysfunction (FABP2) and immune activation secondary to gut microbial translocation (LBP, sCD14). These markers are associated with adverse outcomes in pathologic conditions characterized by increased gut permeability (e.g., liver cirrhosis, inflammatory bowel disease, HIV, and sepsis) (18, 25-28). That FABP2, LBP, and sCD14 increase from pre- to postmenopause suggests that they are sensitive and precise enough to examine within-individual changes in gut physiology during the MT. Ideally, prior to large-scale implementation of these blood markers in observational research, their correlation with functional measurements of gut permeability using non-digestible markers should be determined.

While FABP2 is specific to the intestine (17), LBP and sCD14 are not; they are produced by hepatocytes and immune cells, respectively (18, 19, 22). We presume that increases in LBP and sCD14 reflect immune activation from gut microbial translocation because the gut is the primary source of gram negative microbes (29). However, the vaginal flora becomes more gram negative during postmenopause (30). Thus, gram negative microbial translocation could also occur across the vaginal epithelium. Future studies should consider whether postmenopausal loss of epithelial integrity contributes to a greater inflammatory burden.

The primary strength of this pilot is that we examined longitudinal changes in markers of gut permeability in a well-characterized sample of women transitioning through the MT. In addition, in exploratory analyses, we related these gut permeability markers to a marker of inflammation and BMD. This study is a critical first step in elucidating whether a mechanism that contributes to inflammation and disease in animal models is similarly present in humans.

The principal limitation of this study is the modest sample size of 65 women, which was, nonetheless, powered to our primary objective: to determine if gut permeability increases during the MT. Our primary analysis upheld that all tested markers of gut permeability increase from pre- to postmenopause. While our exploratory analyses suggest a link between gut permeability, inflammation and BMD, not all predictor-outcome associations were significant. In addition, this study was not sufficiently powered to explicitly examine the longitudinal associations between within-woman changes in exposures and outcomes. Nonetheless, our pilot data provide a strong rationale to conduct future studies of these questions using larger study samples. Specifically, future investigations should explore the trajectories of change in gut permeability across the MT, and relate the longitudinal changes in gut permeability and inflammation to bone loss and other end-organ manifestations. Lastly, we measured a limited number of gut permeability markers. Future work should also assay markers such as zonulin and caludin 3, which reflect gut epithelial paracellular integrity (31, 32), since decreased estrogen down-regulates gut epithelial junction proteins (12).

In summary, our findings support that gut permeability increases from pre- to postmenopause, and exploratory results suggest a relation between gut permeability, inflammation, and BMD, that should be further investigated in larger study samples. To our knowledge, this is the first demonstration of a MT-related "gut leak" in humans. If, indeed, increased gut permeability during the MT leads to a state of immune activation and inflammation with negative health consequences, the clinical implications could be substantial: interventions that target gut

physiology during and after the MT could lessen inflammation and multiple disorders that plague older adults.

METHODS

SWAN, the parent study for this pilot, is a multi-center, longitudinal cohort study of the MT in 3,302 ambulatory, multi-racial/ethnic (Black, Chinese, Japanese, White) women. The SWAN cohort has been described in detail (33). In brief, at SWAN baseline, participants were in pre- (no change in menstrual bleeding in the past year) or early perimenopause (less predictable menstrual bleeding at least once every 3 months), and between 42 to 52 years of age. After 2 decades of observational follow-up, consisting of 17 serial visits, all SWAN women have transitioned to postmenopause. To access BMD data, we selected our pilot sample from the SWAN Bone Cohort, a subset of 2,365 participants, in whom BMD was measured at each study visit. The SWAN Repository provided previously collected and frozen plasma samples to measure the various markers of gut permeability (33, 34).

Study Sample

Eligibility: To be included in the pilot, participants were required to: 1) have undergone natural menopause; 2) have a known final menstrual period (FMP) date; 3) have at least one visit in premenopause (operationalized as having occurred 3-5 years prior to the FMP) and one visit in postmenopause (operationalized as having occurred between 3-5 years after the FMP) from which previously collected plasma could be accessed through the SWAN Repository to measure FABP2, LBP, and sCD14; 4) have pre-existing measures of E2, FSH, hs-CRP and BMD corresponding to the plasma sample times; and 5) have not used sex steroid medications or bone modifying agents in between their pre- and postmenopausal pilot study visits. We selected the timing of the pre- and postmenopausal samples (relative to the FMP date) based on the

trajectories of change in estradiol across the MT: estradiol does not decreases significantly until 3 years pre-FMP and nadirs at about 3 years after the FMP (35).

Sample Derivation: Figure 3 depicts the derivation of the analysis sample. In total, 621 women met our eligibility criteria. From this potential pool of subjects, the SWAN Repository randomly selected 65 women for this pilot study (see Analysis for sample size considerations).

Phlebotomy

Every effort was made to perform phlebotomy fasting, and before 10:00 AM during the early follicular phase (EFP, between days 2 and 5) of a spontaneous menstrual cycle (prior to postmenopause). If an EFP sample could not be obtained after 2 attempts, a random fasting sample was taken within a 90-day window of the anniversary of the baseline visit. Collected specimens were initially stored between -20 to -80 degrees Celsius at individual study sites for up to 30 days, and then shipped to a central lab (either Medical Research Lab [Highland Heights, KY] or CLASS Laboratory at the University of Michigan [Ann Arbor, MI]). Once at the central lab, samples were stored at -80 degrees Celsius.

Marker of Gut Permeability

To assess gut permeability, we measured markers of gut barrier dysfunction (FABP2) and immune activation secondary to microbial translocation (LBP, sCD14) using citrated plasma samples that were accessed from the SWAN Repository. FABP2 was measured using the Quantikine Human FABP2/I-FABP ELISA (R&D Systems). The lower limit of detection (LLD) for this assay is 6.21 pg/mL. The intra-assay coefficient of variation (CV) was <4.1%. LBP and

sCD14 were assayed in multiplex, using the Luminex platform with custom-made panels (R&D Systems). This platform uses microparticles that are pre-coated with analyte-specific antibodies and incubated with diluted plasma samples, followed by a biotin antibody, and lastly by a streptavidin-phycoreythin conjugate. The fluorescence intensity of each analyte's microparticles are then quantified using a Bioplex 200 (Luminex) System Analyzer, and the data analyzed using Bioplex Manager software. The LLD for the LBP is 0.0839 ng/ml, and the intra-assay CV was <2.9%. The LLD for sCD14 is 0.0366 ng/ml, and the intra-assay CV was <2.9%.

Markers of Ovarian Function

To examine whether reduced ovarian function is associated with greater gut permeability across the MT, we used previously measured E2 and FSH. Serum E2 was measured in duplicate with a modified, off-line ACS:180 (E2–6) immunoassay using an ACS:180 automated analyzer (Bayer Diagnostics Corp., Tarrytown, New York). The average between duplicates was recorded in the dataset and used in analyses. The LLD was 1.0 pg/ml, and the intra-assay CV was 6.4%. Serum FSH was measured in singlicate with a 2-site chemiluminometric assay (Bayer Diagnostics Corp., Tarrytown, New York). The LLD was 1.05 mIU/ml, and intra-assay CV was 6.0%. For this pilot, we only included women whose premenopausal E2 and FSH measurements were obtained in the EFP of a spontaneous menstrual cycle.

Marker of Inflammation: High-sensitivity C-reactive Protein (hs-CRP)

To assess whether the associations of gut permeability with inflammation, and of inflammation with BMD, we used hs-CRP. CRP was measured at SWAN baseline, and at follow-up visits 1, 3-7, 9, 10, 12, and 15. Owing to the long duration of follow up, the laboratories that measured CRP

and the assays employed changed with time. SWAN used 3 CRP assays and a fourth, hs-CRP assay as the calibration standard to harmonize all assays. The fourth assay was also used to obtain results for analytes that had been below the LLD on each of the 3 original assays. From baseline to follow-up visit 7, CRP was measured by Medical Research Laboratories. CRP assays for samples collected at follow-up visit 9 and later were conducted by the CLASS Laboratory at the University of Michigan (Ann Arbor, MI) using the Alfa Wassermann ACE analyzer. Assays and their calibration are described below.

CRP assay 1: From baseline to follow-up visit 7, CRP was measured using an ultrasensitive rate immunonephelo-metric method, with a LLD of 0.3 mg/L (BN100; Dade-Behring, Marburg, Germany). The intra-assay CV at CRP concentrations of 0.5 and 22.0 mg/L were 10–12% and 5–7%, respectively. CRP assay 2: For follow-up visit 12, CRP was assayed using the ACE *Ultra*Wide Range assay, a latex-enhanced turbidimetric *in vitro* immunoassay (Alfa Wassermann, West Caldwell, NJ). The LLD was 0.1 mg/L, and the intra-assay CV at CRP concentrations of 0.5 mg/L and 9.8 mg/L were 5.7-7.0% and 1.2%, respectively. CRP Assay 3: Samples from follow-up visit 9, 10 and 15 were assayed using a high sensitivity immunoassay (Alfa Wassermann, West Caldwell, NJ). The LLD was 0.1 mg/L, and the intra-assay CV at CRP concentrations of 0.5 and 9.8 mg/L were 5.7-7.0% and 1.2%, respectively. CRP assay 4: For approximately 25% of all samples run between baseline and follow-up 15, CRP was below the LLD of the original 3 assays outlined above. In these instances, an additional sample was retrieved from the Repository and CRP was measured using the Human High Sensitivity CRP ELISA (R&D Systems, DCRP00), a quantitative sandwich enzyme immunoassay. The lower LLD for this assay was 0.10 pg/ml. The remaining 75% of results (those above the LLD for the 3

original assays) were calibrated to the high sensitivity ELISA by simultaneously assaying 600 paired samples (representing the full range of results from each of the original 3 assays) and the high sensitivity assay; thus, there were 200 paired samples for each original vs. high sensitivity ELISA. After calibration, correlations between the first 3 CRP assays and the fourth, high sensitivity ELISA were $\geq$0.94.

Bone Mineral Density

BMD at the lumbar spine (LS) and total hip (TH) BMD was measured by DXA. At study inception, the Pittsburgh and Oakland sites used the Hologic QDR 2000 machine, and the Boston, Los Angeles, and Michigan sites used the Hologic QDR 4500A machine. At follow-up visit 8, Pittsburgh and Oakland upgraded to the 4500A models. Boston and Los Angeles upgraded to the Hologic Discovery model at follow-up visit 13. To develop cross-calibration regression equations, each site obtained duplicate scans using the old and new hardware within 90 days in at least 40 volunteers (N=40 for Oakland, Pittsburgh; N=41 for Boston; and N=50 for Los Angeles). To determine the short-term *in vivo* precision error, each study site measured LS and TH BMD twice in 5 women with complete subject repositioning between duplicate scans. Using the root mean square SD approach, the precision error in SWAN was 1.4% at the LS and 2.2% at the TH. An anthropomorphic spine phantom was circulated between sites for cross-site calibration. Standard quality control phantom scans were conducted before each BMD measurement session. If necessary, these were used to adjust for longitudinal machine drift.

Additional Measures and Variables

The following were included in analyses as covariates: age (years), race/ethnicity (Black, Chinese, Japanese, White), and body mass index (BMI [weight in kilograms/(height in meters)^2]), based on height and weight measured using standardized protocols and with participants wearing light clothing and no shoes.

Statistical Analyses

Sample Size: The sample size of 65 subjects was predicated on funding constraints and the power to detect change in our primary outcomes. Because there are no published cross-sectional or longitudinal studies examining whether gut permeability (assessed directly or indirectly) increase across the MT, we relied on change in sCD14 with aging published in the Cardiovascular Health Study (CHS) (21). In CHS, sCD14 increased by 0.4% per year (21), which would result in a gain of 2.4 to 4.0% over a 6 to 10-year period (the approximate time interval between our pre- to postmenopausal time points). A sample size of 65 yielded 90% power at a 2-sided alpha of 0.05 to detect 4.5%, 4.4%, and 2.4% changes in FABP2, LBP, and sCD14, respectively, using the inter-assay CV for each analyte.

Data Analyses: We generated descriptive statistics for all variables and assessed the distributions of continuous variables for normality. E2, FSH, and hs-CRP had skewed distributions, and were thus log-transformed for relevant analyses. For all analyses, a p-value of $\leq$0.05 was considered to be statistically significant.

To assess our primary hypothesis, we used the paired t-test at a 2-sided alpha of 0.05 to test the mean within-person change in FABP2, LBP, and sCD14 from pre- to postmenopause against the

null hypothesis of zero change. We considered a p-value of 0.05 and lower to be statistically significant. To additionally examine whether ovarian function is associated with gut permeability, we used repeated measures, mixed effects linear regressions with E2 or FSH as continuous primary predictors, and FABP2, LBP, or sCD14 as continuous outcome measures (with each primary predictor-outcome pair modeled separately). Models included a random intercept at the individual level to account for clustering. Covariates were chronologic age at the premenopausal visit, BMI, race/ethnicity, and SWAN study site.

To test our secondary hypothesis, we used repeated measures, mixed effects linear regression with FABP2, LBP, sCD14, or hs-CRP as continuous primary predictors, and hs-CRP or LS or TH BMD as continuous outcomes variables. As above, each primary predictor-outcome pair was modeled separately, and all models included a random intercept at the individual level. Covariates were chronologic age at the premenopausal visit, BMI, race/ethnicity, and study site.

For all relational analyses stipulated above, we used mixed effects, repeated measures linear regression because this approach is best suited for detecting predictor-outcome associations in small samples. This is because this type of model accounts for the associations of both between-women and within-woman differences in the predictor with corresponding differences in the outcome.

Human Study Approval

Each SWAN clinical site obtained Institutional Review Board approval, and all participants provided written informed consent.

AUTHOR CONTRIBUTIONS

Participant recruitment for the parent SWAN study was contributed by GAG. Data management and cleaning were contributed by AS, ASK, and GAG. Performance of gut permeability assays was contributed by ME. Analytic design and statistical analysis were contributed by AS, ASK, and GAG. Primary manuscript drafting by contributed by AS and GAG. Critical review and revision of manuscript were contributed by AS, ME, ASK, and GAG.

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FIGURE AND FIGURE LEGENDS

Figure 1

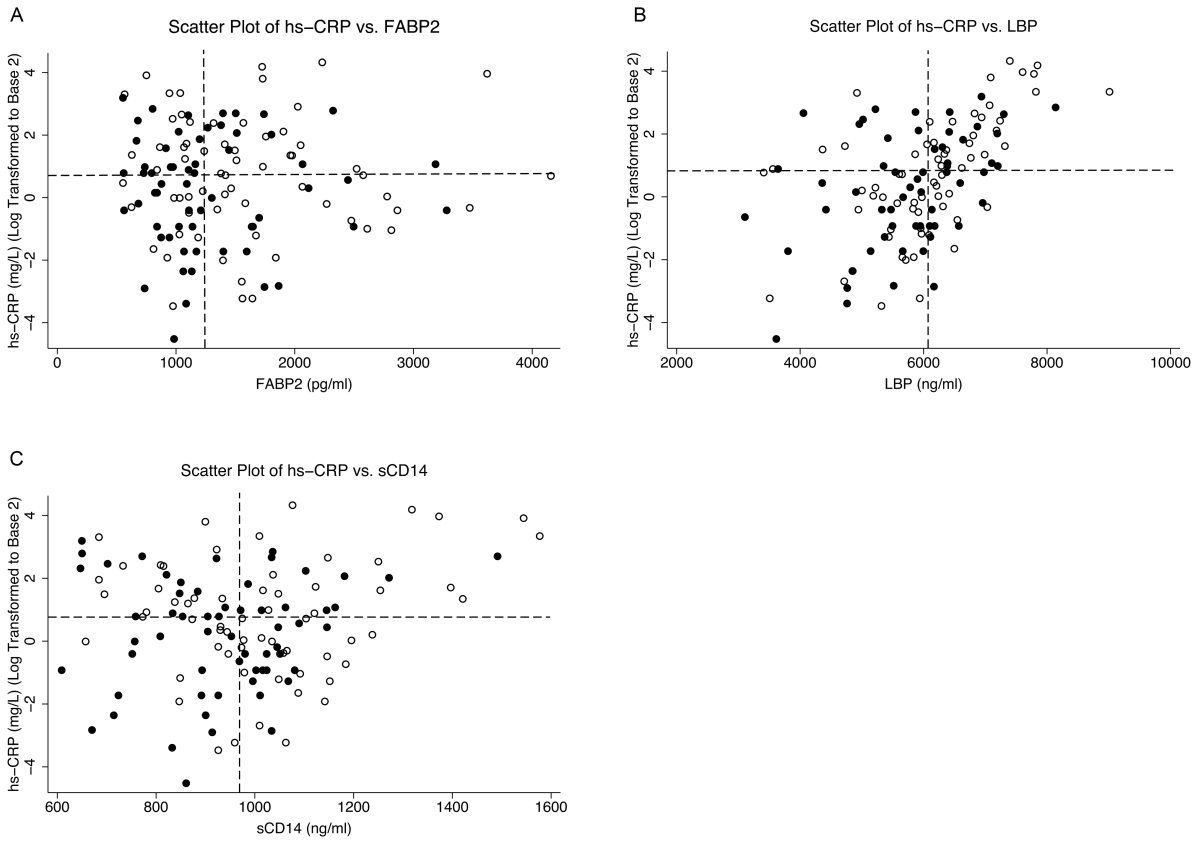

Figure 1 Legend. Scatter plots of gut permeability markers vs. hs-CRP. Plots of paired measures of FABP2 (panel A, gut barrier dysfunction marker), LBP (panel B, immune activation/gut microbial translocation marker), or sCD14 (panel C, immune activation/gut microbial translocation marker) vs. hs-CRP. Paired measures were obtained in premenopuase (closed circles, 3-5 years before the final menstrual period) and postmenopause (open circles, 3-5 years after the final menstrual period). Vertical dashed lines indicate the median gut permeability marker values, and horizontal dashed lines mark the median hs-CRP value. A total of 65 subjects were included.

Figure 2

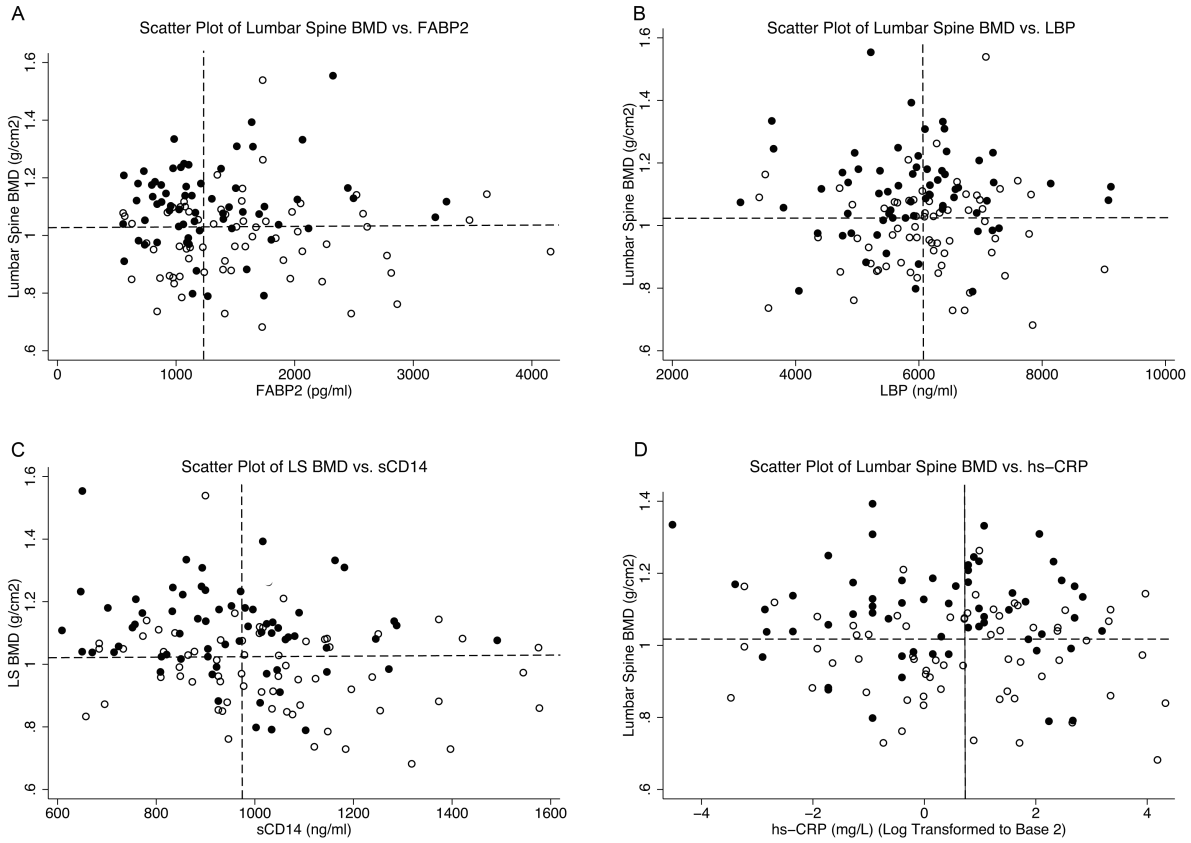

Figure 2 Legend. Scatter plots of gut permeability and inflammatory markers vs. lumbar spine bone mineral density. Plots of paired measures of FABP2 (panel A, gut barrier dysfunction marker), LBP (panel B, immune activation/gut microbial translocation marker), sCD14 (panel C, immune activation/gut microbial translocation marker), or hs-CRP (panel D, inflammatory marker) vs. lumbar spine bone mineral density. Paired measures were obtained in premenopuase (closed circles, 3-5 years before the final menstrual period) and postmenopause (open circles, 3-5 years after the final menstrual period). Vertical dashed lines indicate median gut permeability or inflammatory marker values, and horizontal dashed lines mark the median lumbar spine bone mineral density value. A total of 65 subjects were included.

Figure 3

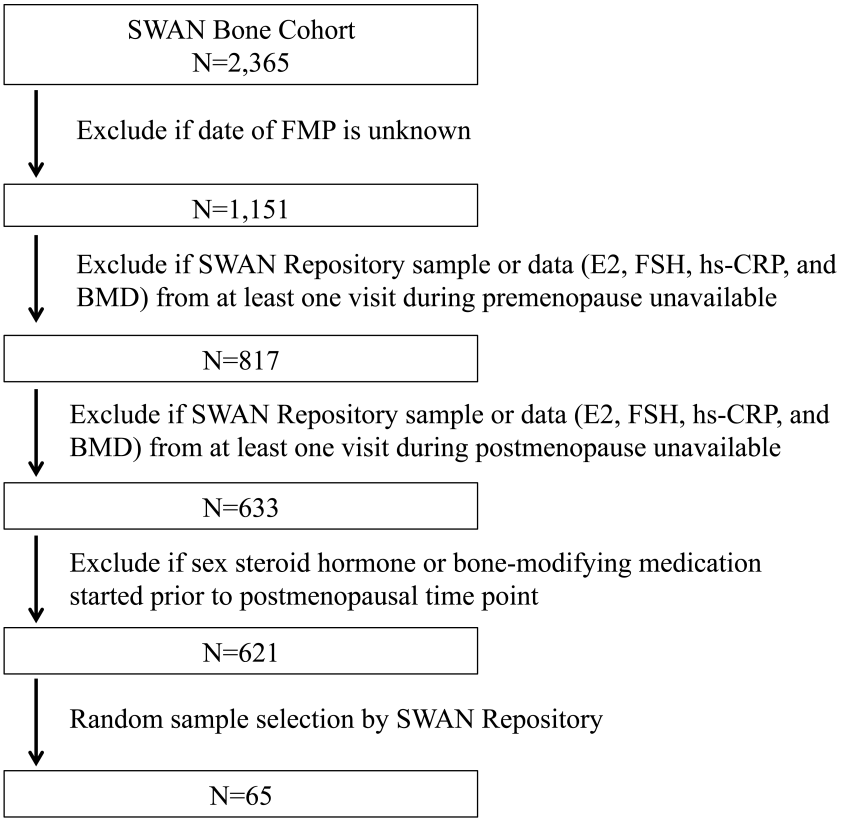

Figure 3 Legend. Analysis sample derivation. This flow chart shows the derivation of the analysis sample. In order to be included in the study, participants needed to meet the following criteria: 1) have undergone natural menopause; 2) have a known final menstrual period (FMP) date; 3) have at least one visit in premenopause and one visit in postmenopause from which previously collected plasma could be accessed through the SWAN Repository to measure FABP2, LBP, and sCD14; 4) have pre-existing measures of E2, FSH, hs-CRP and BMD corresponding to the plasma sample times; and 5) have not used sex steroid medications or bone modifying agents in between their pre- and postmenopausal pilot study visits. A total of 621 women met these criteria. From this potential pool of subjects, the SWAN Repository randomly selected 65 women for this pilot study.

573 **TABLES**
574
575 **Table 1**

576 **Descriptive statistics in pre- vs. postmenopause for analytic sample (N=65): Study of**

577 **Women's Health Across the Nation (SWAN)**

	Premenopause[a]	**Postmenopause[b]**	**p-value**
Race/ethnicity			
Age (years)[c]	49.9 (1.9)	57.5 (1.8)	<0.0001
BMI (kg/m^2)[c]	27.6 (5.6)	28.9 (6.1)	
Time from final menstrual period (years)[c]	-3.8 (0.6)	+4.0 (0.6)	<0.0001
Ovarian function markers			
E2 (pg/ml)[d]	51.7 (32.3, 86.6)	15.5 (10.7, 20.1)	<0.0001
FSH (mIU/ml)[d]	14.8 (10.0, 21.8)	84.3 (64.1, 108.2)	<0.0001
Gut permeability markers			
FABP2 (pg/ml)[c]	1,298 (577)	1595 (754)	0.001
LBP (ng/ml)[c]	5,892 (1,139)	6,112 (1,037)	0.05
sCD14 (ng/ml)[c]	948 (176.2)	1,032 (212)	0.0002
Inflammation			
hs-CRP (mg/L)[d]	1.4 (0.5, 3.3)	1.6 (0.8, 3.3)	0.06
Bone mineral density			
Lumbar spine (g/cm^2)[c]	1.104 (0.139)	0.986 (0.142)	<0.0001
Total hip (g/cm^2)[c]	0.964 (0.123)	0.901 (0.124)	<0.0001

578

a Data obtained in premenopause, operationalized as a study visit from three to five years before the final menstrual period (before ovarian function decreases)

b Data obtained in postmenopause, operationalized as a study visit from three to five years After the final menstrual period (after ovarian function plateaus at its nadir)

c Variables with normal distribution, reported as mean (standard deviation). Within-individual change from pre- to postmenopause assessed using the paired t-test.

d Variable with skewed distribution, reported as median (interquartile range). Within-individual change from pre- to postmenopause assessed using the Wilcoxon rank-sum test.

Table 2

Associations of estradiol (E2) or follicle stimulating hormone (FSH) with gut permeability[a]

	Increment in FABP2 (pg/ml), LBP (ng/ml), or sCD14 (ng/ml) per 2-fold decrement in E2 or 2-fold increment in FSH (95% CI)[a]	**p-value**
FABP2		
E2	77 (10, 143)	0.02
FSH	113 (47, 179)	0.001
LBP		
E2	33 (-41, 110)	0.3
FSH	21 (-55, 98)	0.5
sCD14		
E2	28 (12, 44)	0.001
FSH	34 (19, 50)	<0.0001

a Gut permeability assessed by FABP2 (barrier dysfucntion), LBP and sCD14 (immune activation secondary to gut microbial translocation).

b Associations assessed by repeated measured, mixed effect linear regression with FABP, LBP, or sCD14 as continuous outcome variable, and E2 or FSH (log transformed) as continous primary predictors (each outcome/primary predictor pair modeled separately). Analyses were adjusted for age at premenopausal time point, BMI, race/ethnicity, and SWAN study site.

Table 3

Associations of gut permeability[a] with a marker of inflammation[b]

	***"X"*-fold increment in hs-CRP per SD increment in FABP2, LBP, or sCD14 (95% CI)[a]**	**p-value**
Gut barrier integrity or immune activation markers		
FABP2	-0.05 (-0.02, +0.02)	0.5
LBP	+2.32 (+1.87, +2.87)	0.0001
sCD14	+1.44 (+1.15, +1.80)	0.001

a Gut permeability assessed by FABP2 (barrier dysfucntion), LBP and sCD14 (immune activation secondary to gut microbial translocation).

b Inflammation assessed by hs-CRP.

c Associations assessed by repeated measured, mixed effect linear regression with hs-CRP as continuous outcome variable, and FABP2, LBP, or sCD14 as continous primary predictors (each modeled separately). Analyses were adjusted for age at premenopausal time point, BMI, race/ethnicity, and SWAN study site.

Table 4

Associations of gut permeability[a] with bone mineral density

	Increment in lumbar spine or total hip bone mineral density (g/cm^2) per SD increment in FABP2, LBP and sCD14, or per 2-fold increment in hs-CRP (95% CI)[a]	**p-value**
FABP2		
Lumbar spine	-0.021 (-0.043, 0.000)	0.05
Total hip	-0.017 (-0.031 -0.003)	0.02
LBP		
Lumbar spine	-0.018 (-0.047, 0.010)	0.2
Total hip	-0.005 (-0.239, 0.015)	0.6
sCD14		
Lumbar spine	-0.051 (-0.076, -0.026)	0.0001
Total hip	-0.025 (-0.041, -0.008)	0.004
hs-CRP		
Lumbar spine	-0.024 (-0.038, -0.011)	<0.0001
Total hip	-0.008 (-0.018, 0.002)	0.1

a Gut permeability assessed by FABP2 (barrier dysfucntion), LBP and sCD14 (immune activation secondary to gut microbial translocation).

b Associations assessed by repeated measured, mixed effect linear regression with bone mineral density at the lumbar spine or femoral as continuous outcome variables, and FABP2, LBP, or sCD14 as continous primary predictors (each modeled separately). Analyses were adjusted for age at premenopausal time point, BMI, race/ethnicity, and SWAN study site.