



Randomized Clinical Trial of RANKL Inhibition with Denosumab on Mammographic Density in High Risk Premenopausal Women with Dense Breasts (TRIDENT)

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Modality

Surgery
Medicine
Pathology
Biostatistics
Medicine
Surgery
Medicine
Radiology
Surgery

CONFIDENTIAL

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SCHEMA

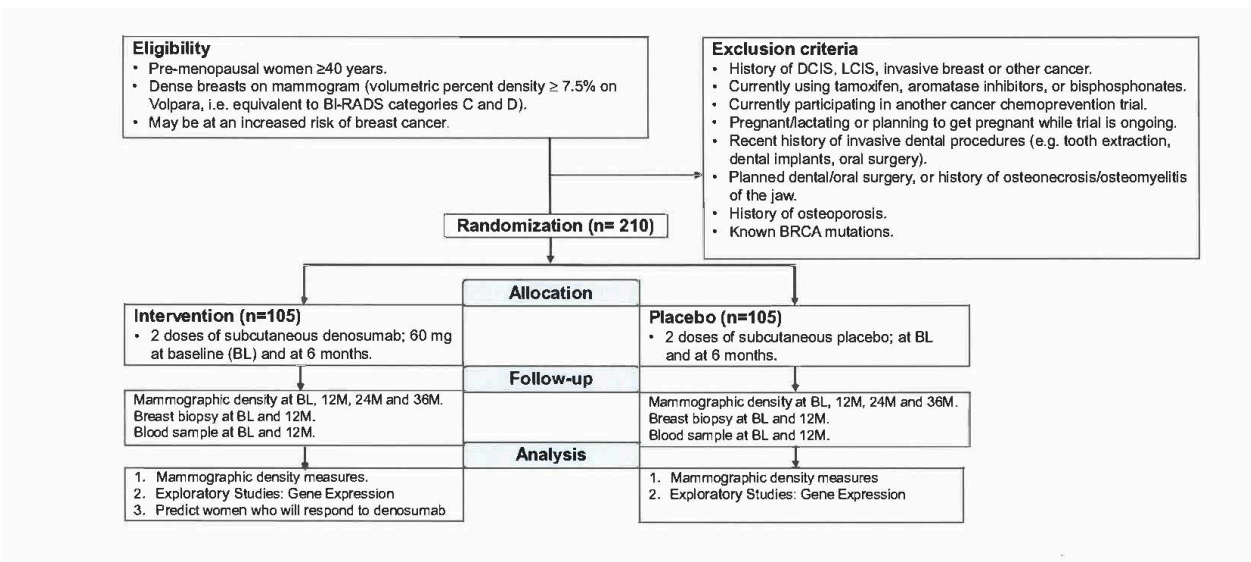


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1 BACKGROUND AND RATIONALE

1.1 Breast Cancer Prevention in High-Risk Premenopausal Women

Close to 25% of invasive breast cancer cases are diagnosed in premenopausal women¹, but lifestyle related factors to reduce breast cancer development, and chemoprevention options in premenopausal women are limited. Apart from reproductive factors and alcohol consumption, most lifestyle factors that can be modified to reduce breast cancer risk (e.g. obesity, menopausal hormone use) are only relevant to disease among postmenopausal women². Tamoxifen is the only approved chemoprevention agent in premenopausal women, but uptake is very low due to risk of serious side effects^{3, 4}. Therefore, additional chemoprevention options with fewer adverse side effects among high-risk premenopausal women is an urgent priority⁵.

1.2 Mammographic Density

Mammographic breast density, which reflects the amount of epithelial and stromal tissues in relation to adipose tissue in the breast⁶, is one of the strongest risk factors for breast cancer. Women with dense breasts have a 4- to 6-fold increased risk of breast cancer⁷⁻⁹. It is estimated that 39% of breast cancer cases in premenopausal women are attributable to having dense breasts compared to 8.7% for having a positive family history of breast cancer¹⁰. Mammographic breast density and genetic risk scores are both established and independently related to risk for breast cancer¹¹.

1.2.1 Decreasing Mammographic Density

A reduction in mammographic density translates to a reduction in breast cancer incidence¹²⁻¹⁵. In the International Breast Cancer Intervention Study (IBIS) I, a trial of tamoxifen for breast cancer prevention, there were significant differences in the mean breast density between the tamoxifen and placebo groups within the first 12-18 months of treatment¹⁴. The mean breast density reduced by 7.9% in the tamoxifen group compared to 3.5% in the placebo group. These differences were sustained till 54 months (13.7% in the tamoxifen group vs. 7.3% in the placebo group). Women assigned to tamoxifen who experienced a $\geq 10\%$ reduction in breast density at their 12-18-month mammogram had a 63% reduction in breast cancer incidence at 54 months, but those who took tamoxifen and experienced a $<10\%$ reduction in breast density had no reduction in breast cancer incidence¹⁶.

Therefore, interventions to reduce dense breasts could prevent many breast cancers and identifying a pathway that can be targeted to reduce density and breast cancer risk among women who have dense breast tissue, with fewer side effects, is crucial. Building on data from our team and others showing that the receptor activator of nuclear factor- κ B (RANK) pathway is associated with mammographic density and breast cancer risk, we propose to perform a randomized clinical trial to quantify the effects of a RANK ligand (RANKL) inhibitor on mammographic density and breast tissue markers associated with breast cancer risk in high-risk premenopausal

women with dense breasts.

1.3 The RANK Pathway in the Breast

The RANK pathway, which includes RANK, RANK ligand (RANKL), and the decoy receptor osteoprotegerin (OPG), are members of the tumor necrosis factor (TNF) and TNF-receptor superfamily and are essential for bone health^{17, 18}. RANK is the signaling receptor for RANKL while OPG competes with RANK for RANKL signaling, thereby blocking RANK activation¹⁹. The RANK pathway regulates the development of the lobulo-alveolar mammary structures during pregnancy and the formation of lactating mammary glands²⁰⁻²².

RANK overexpression in the mammary epithelia impairs alveolar differentiation and lactation²³. RANK and RANKL knock out mice display a complete defect in the formation of lactating mammary glands due to decreased proliferation and survival of mammary epithelial cells, similar to that observed in progesterone receptor B knockout mice^{17, 20, 24}. RANKL signaling also activates downstream signaling cascades such as NF-κB and cyclin D1^{19, 25}, which are key pathways involved in breast cancer development

Progesterone is a proliferative hormone in the breast; it increases breast density, and breast cancer incidence, independent of estrogen²⁶⁻³³. Combined estrogen+progestin hormone therapy is classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC)³³. Thus, targeting progesterone signaling could be a viable strategy to reducing breast density and breast cancer incidence. Progesterone has direct and indirect effects on mammographic density³¹. Preclinical studies have shown that RANKL signaling is the major mediator of progesterone induced mammary proliferation, and progesterone-driven expansion of mammary stem cells. We propose that one of the major mechanisms through which progesterone impacts breast density and breast cancer risk is via its effect on RANKL signaling.

1.3.1 RANK Pathway, Mammographic Density, and Breast Cancer Risk

Emerging data indicate that the RANK pathway is associated with mammographic density and breast cancer risk. We recently provided the first evidence that both breast tissue RANKL gene expression³⁹ as well as circulating RANK biomarkers (RANK and soluble RANKL – sRANKL)⁴⁰ are positively associated with mammographic density in premenopausal women. Two studies recently investigated the associations of sRANKL levels with breast cancer risk^{41, 42}. The first study, within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, showed that higher sRANKL concentrations were positively associated with risk of ER+ disease, although there was evidence suggestive of an inverse association with ER-/PR- disease⁴¹. RANKL signaling activates NF-κB and canonical NF-κB pathways are most active in ER+ breast cancers⁴³. The second study reported a five-fold increased risk of breast cancer for elevated RANKL among women with high progesterone levels⁴². These findings support the potential utility of inhibiting RANKL signaling in primary breast cancer prevention in well-

defined groups of women. Of note, well-tolerated RANKL neutralizing antibody (denosumab) is already in clinical use in women without cancer but is yet to be applied to breast cancer prevention in high-risk premenopausal women with dense breasts.

1.4 Denosumab

Denosumab has a better safety profile than the current chemoprevention option for premenopausal women. Denosumab is a fully human, IgG2 monoclonal antibody that binds to RANKL with high affinity, preventing its interaction with RANK in a way similar to that of OPG. It is approved for the treatment of postmenopausal osteoporosis and preventing bone loss and fractures associated with cancer therapies and bone metastases⁴⁴⁻⁴⁷. Long-term (10-year) safety has been evaluated in clinical trials and the 60 mg dose is well tolerated with minimal side effects^{48,49}. Fracture risk and osteonecrosis of the jaw are two potential rare side effects that have garnered attention. In the Adjuvant Denosumab in Breast Cancer (ABCSG-18) trial of 3,425 participants, denosumab treatment for 10 years was associated with very low rates of adverse events⁴⁸. Despite proactive adjudication of every potential osteonecrosis of the jaw by an expert panel, no cases of osteonecrosis of the jaw were reported⁴⁸. In the Fracture Reduction Evaluation of Denosumab in Osteoporosis Every 6 Months extension trial of 7,808 postmenopausal women, 13 cases of osteonecrosis of the jaw were reported in 10 years (5.2 per 10,000 participant-years). The cases of osteonecrosis of the jaw were mild. Eleven cases resolved completely while still enrolled in the trial and taking denosumab⁴⁹. Recent history of tooth extraction was a major factor associated with the development of osteonecrosis of the jaw.

1.5 Rationale

Data supporting a role for RANKL signaling in mammographic density and breast cancer development has begun to emerge, but clinical trial data providing definitive evidence that would allow the adoption of RANKL inhibition in primary breast cancer prevention are not yet available. Our hypothesis is that RANKL inhibition with denosumab will decrease mammographic density in high-risk premenopausal women with dense breasts. To address this, we have developed this clinical trial to quantify the impact of RANKL inhibition on mammographic density in high-risk premenopausal women with dense breasts and to determine the effect of RANKL inhibition on breast tissue RANK, progesterone-regulated pathway gene expression, and biomarkers associated with breast cancer risk. Successful demonstration that RANKL inhibition reduces mammographic density could open up additional approaches to primary breast cancer prevention in high-risk premenopausal women, who do not have dominant genetic predisposition.

2 OBJECTIVES

2.1 Primary Objective

Quantify the effect of RANKL inhibition with denosumab on mammographic density in

high-risk premenopausal women with dense breasts.

2.2 Exploratory Objectives

Perform gene expression and metabolomic analysis to determine the effect of RANKL inhibition on breast tissue markers as well as biomarkers associated with breast cancer risk.

1. Determine the effect of RANKL inhibition on breast tissue RANK, progesterone, metabolic, immune and inflammatory gene expression.
2. Determine the effect of RANKL inhibition on biomarkers of breast epithelial and stromal proliferation.
3. Determine the effect of RANKL inhibition on breast metabolome.
4. Evaluate correlations between gene expression within the breast tissues and within the blood.
5. Predict response to denosumab using the biomarkers evaluated in the above objectives.
6. Perform pilot spatial analysis in pre-and post-intervention breast tissue samples of women assigned to denosumab who have clinically meaningful decreases in mammographic density.

3 PARTICIPANT SELECTION

3.1 Inclusion Criteria

1. Female.
2. Premenopausal*.
3. At least 40 years of age.
4. Dense breasts on routine mammogram (BI-RADS Category C and D)
5. Able to understand and willing to sign an IRB-approved written informed consent document.

*When menopausal status is uncertain, we will measure follicle-stimulating hormone and estradiol to ascertain that a participant is premenopausal.

3.2 Exclusion Criteria

1. History of breast or any other invasive cancer except for DCIS (ductal carcinoma in situ) who received only lumpectomy, LCIS (lobular carcinoma in situ), atypical hyperplasia, non-melanoma skin cancer, carcinoma in situ of the cervix.
2. Current use of tamoxifen, aromatase inhibitors, bisphosphonates, or RANKL inhibitors.
3. Concurrent participation in another cancer chemoprevention trial (unless no longer receiving the intervention).
4. Pregnant or lactating or planning to get pregnant while the trial is ongoing.

5. Recent history of invasive dental procedure (e.g., tooth extraction, dental implant, oral surgery).
6. Unhealed and/or planned dental/oral surgery.
7. History of osteonecrosis/osteomyelitis of the jaw.
8. History of osteoporosis or severe osteopenia.

3.3 Inclusion of Women and Minorities

Women and members of all races and ethnicities will be recruited for this trial. Men will not be recruited.

4 REGISTRATION PROCEDURES

Participants must not start any protocol intervention prior to registration through the Siteman Cancer Center.

The following steps must be taken before registering participants to this study:

1. Confirmation of participant eligibility
2. Registration of participant in the Siteman Cancer Center database
3. Assignment of unique participant number (UPN)

4.1 Confirmation of Participant Eligibility

Confirm participant eligibility by collecting the information listed below:

1. Participant's race, sex, and DOB
2. Copy of signed consent form
3. Completed eligibility checklist, signed and dated by a member of the study team
4. Copy of appropriate source documentation confirming participant eligibility

4.2 Participant Registration in the Siteman Cancer Center OnCore Database

All participants must be registered through the Siteman Cancer Center OnCore database.

4.3 Assignment of UPN

Each participant will be identified with a unique participant number (UPN) for this study. All data will be recorded with this identification number on the appropriate CRFs.

4.4 Randomization

Eligible participants, stratified by age, will be randomized at a 1:1 ratio to receive two doses of subcutaneous denosumab (60mg) or subcutaneous placebo (packaged the same as denosumab) at baseline and at 6 months (+/-1 month). Stratified permuted block

randomized design will be used to generate the randomization table where permuted block randomization will be used for each stratum by age, with a varying block size of 4 and 6.

5 STUDY PROCEDURES

Participants randomized to the study intervention arm will receive a subcutaneous injection of denosumab at a dose of 60 mg at two time points: baseline and 6 months (+/- 1 month). Participants randomized to the placebo arm will receive a subcutaneous injection of the placebo at the same time points. Participants will also be instructed to take calcium and vitamin D supplements daily for 12 months between baseline examination and 12-month mammographic breast density examination. Mammographic breast density will be assessed in all participants at: baseline, 12 months, and 24 months. Passive follow-up for mammographic breast density assessment will also take place at 36 months. Biopsies and blood draws will occur for research purposes at baseline and 12 months.

5.1 Administration of Denosumab/Placebo

Denosumab/placebo will be administered at a dose of 60 mg at baseline and again at 6 months (+/-1 month). It will be given as a subcutaneous injection in the upper arm, upper thigh, or abdomen.

Participants' calcium, magnesium, phosphorus, serum creatinine, and bilirubin will be measured, and a pregnancy test will be administered to all women of childbearing potential before denosumab/placebo is administered at both baseline and 6 months (+/- 1 month). Denosumab/placebo administration must occur within 8 weeks of calcium, magnesium, phosphorus, creatinine, and total bilirubin blood tests. Denosumab/placebo will be withheld if:

- serum calcium < 8.0 mg/dL
- serum magnesium < 1.7 mg/dL
- serum phosphate < 2 mg/dL
- serum creatinine >1.1 mg/dL
- total bilirubin > 2 x IULN
- positive pregnancy test

5.2 Supplementation with Calcium and Vitamin D

Participants will be instructed to take calcium (at least 500 mg) and vitamin D3 (at least 400 IU) supplements daily for 12 months between baseline examination and 12-month mammographic breast density examination. These supplements will be provided by the study. All participants will be provided with a 6-month supply of vitamin D and calcium at baseline and at 6 months. We will ask participants to complete a drug diary to monitor their compliance to supplement use. For participants who are unwilling to complete a drug diary, we will contact them (email or phone call) every month to ask about their compliance. Participants will also be asked to bring their supplement containers to 6-month and 12-month follow ups to further assess compliance.

If participants experience constipation from calcium, and constipation does not improve with dietary modifications, participants will be allowed to take high calcium diets containing calcium elements, at least 500 mg/day while continuing to take vitamin D supplement. Examples of high-calcium diets are in the appendix.

5.3 Assessment of Mammographic Breast Density

In addition to the clinical Breast Imaging Reporting and Data System (BI-RADS) assesment, Volpara software will be used to assess mammographic density in all study participants at baseline. The main outcome measure is change at 12 months. We will follow up with mammographic breast density at 24 months and an optional assessment at 36 months to evaluate if any changes observed are sustained till 36 months.

Volpara has been validated in many studies⁵⁰⁻⁵⁴, including by our team^{40, 55, 56}. Volpara provides three volumetric measures of density (volumetric percent density, dense volume and non-dense volume), of which volumetric percent density is the strongest predictor of breast cancer risk⁸. Thus, our main density measure for this trial is volumetric percent density. Volpara volumetric percent density measures range from 0.5–34.5%. In BI-RADS, these translate to (a) <3.5, (b) ≥3.5 and <7.5, (c) ≥7.5 and <15.5, and (d) ≥15.5%.

5.4 Specimen Acquisition

5.4.1 Core-Needle Biopsy

Core-needle biopsy will be performed on study participants at the Joanne Knight Breast Health Center, Siteman Cancer Center, and other Washington University or Siteman Cancer Center affiliate hospitals. The biopsy will be completed by using real time ultrasound guidance for gene expression analyses at baseline and around 12 months after the routine 12-month screening mammography as a standard of care.

Core needle breast biopsy will be performed under real time ultrasound guidance by the breast radiologist or by fellows in the breast radiology department or surgeons at Washington University or Siteman Cancer Center affiliate hospitals. Areas of mammographic dense tissue will be located by ultrasound and core needle biopsies will be extracted from these areas. Employing sterile technique, under local anesthesia, a 12-gauge or 14-gauge spring loaded core needle biopsy device will be used to obtain 3-4 core samples. A biopsy clip will be placed at the biopsy site. Clips are required in the event the biopsy site requires subsequent excision. Breast tissue samples will be processed within 1 hour of biopsy and stored at the Tissue Procurement Core (TPC). A biopsy sample will also be fixed in formalin and sent to pathology to be reviewed by the study pathologist. Tissue collection for this research proposal would be used for research purposes only and would not inform participant care. In the event that an abnormality that requires clinical care, such as a breast tumor is detected, the Chief of anatomic pathology will be contacted to discuss the path forward.

If participants have the mammogram results showing BI-RADS category 3-4A at baseline or 12 months and need a further breast biopsy as part of the standard of care, we may collect normal breast tissue as part of the study in the same procedure, if possible. The biopsy could be a core needle breast biopsy under real time ultrasound guide or a stereotactic breast biopsy at the discretion of the radiologist or surgeon.

5.4.2 Blood Collection

Twenty to 30 mL of fasting whole blood will be collected at baseline and 12 months for biomarker analyses. Samples will be processed and stored at the TPC.

5.5 Specimen Analysis

Total RNA will be extracted from the breast core biopsies using AllPrep DNA/RNA Kit (Qiagen). We will use the Qubit Fluorometer (Invitrogen) to determine total RNA concentrations. Gene expression analyses, using the NanoString nCounter platform, will be performed at the McDonnell Genome Institute (MGI).

5.6 Evaluability

Participants must undergo the 12-month assessment of mammographic breast density in order to be considered evaluable for the study outcomes. Participants who do not undergo the 12-month imaging will be replaced.

5.7 Women of Childbearing Potential

Women will not be enrolled if they are pregnant or plan to get pregnant while on the trial. Pregnancy tests will be performed on women of childbearing potential within 14 days prior to each administration of denosumab/placebo. Denosumab/placebo will be withheld if there is a positive pregnancy test.

5.8 Duration of Intervention

If at any time the constraints of this protocol are considered to be detrimental to the participant's health and/or the participant no longer wishes to continue on study, participation should be discontinued and the reason(s) for discontinuation documented in the case report forms.

5.9 Survey

Participants will be asked to complete a one-time questionnaire at the time of enrollment. The survey should not take longer than 30 minutes to complete, and all questions are voluntary.

6 REGULATORY AND REPORTING REQUIREMENTS

The entities providing oversight of safety and compliance with the protocol require reporting as outlined below.

The Washington University Human Research Protection Office (HRPO) requires that all events meeting the definition of unanticipated problem or serious noncompliance be reported as outlined in Section 6.2.

6.1 Definitions

6.1.1 Adverse Events (AEs)

Definition: any unfavorable medical occurrence in a human subject including any abnormal sign, symptom, or disease.

Grading: the descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be utilized for all toxicity reporting. A copy of the CTCAE version 5.0 can be downloaded from the CTEP website.

Attribution (relatedness), Expectedness, and Seriousness: the definitions for the terms listed that should be used are those provided by the Department of Health and Human Services' Office for Human Research Protections (OHRP). A copy of this guidance can be found on OHRP's website:

<http://www.hhs.gov/ohrp/policy/advevntguid.html>

6.1.2 Serious Adverse Event (SAE)

Definition: any adverse drug experience occurring at any dose that results in any of the following outcomes:

- Death
- A life-threatening adverse drug experience
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity (i.e., a substantial disruption of a person's ability to conduct normal life functions)
- A congenital anomaly/birth defect
- Any other experience which, based upon appropriate medical judgment, may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above

6.1.3 Unexpected Adverse Experience

Definition: any adverse drug experience, the specificity or severity of which is not consistent with the current investigator brochure (or risk information, if an IB is not required or available).

6.1.4 Life-Threatening Adverse Experience

Definition: any adverse drug experience that places the subject (in the view of the investigator) at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.

6.1.5 Unanticipated Problems

Definition:

- unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;
- related or possibly related to participation in the research (“possibly related” means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

6.1.6 Noncompliance

Definition: failure to follow any applicable regulation or institutional policies that govern human subjects research or failure to follow the determinations of the IRB. Noncompliance may occur due to lack of knowledge or due to deliberate choice to ignore regulations, institutional policies, or determinations of the IRB.

6.1.7 Serious Noncompliance

Definition: Noncompliance that materially increases risks, that results in substantial harm to subjects or others, or that materially compromises the rights or welfare of participants.

6.1.8 Protocol Exceptions

Definition: A planned deviation from the approved protocol that are under the research team’s control. Exceptions apply only to a single participant or a singular situation.

Pre-approval of all protocol exceptions must be obtained prior to the event.

6.2 Reporting to the Human Research Protection Office (HRPO) at Washington University

The PI is required to promptly notify the IRB of the following events:

- Any unanticipated problems involving risks to participants or others which occur at WU, any BJH or SLCH institution, or that impacts participants or the conduct of the study.
- Noncompliance with federal regulations or the requirements or determinations of the IRB.
- Receipt of new information that may impact the willingness of participants to participate or continue participation in the research study.

These events must be reported to the IRB within **10 working days** of the occurrence of the event or notification to the PI of the event. The death of a research participant that qualifies as a reportable event should be reported within **1 working day** of the occurrence of the event or notification to the PI of the event.

6.3 Reporting to the Quality Assurance and Safety Monitoring Committee (QASMC) at Washington University

The PI is required to notify the QASMC of any unanticipated problem occurring at WU or any BJH or SLCH institution that has been reported to and acknowledged by HRPO as reportable. (Unanticipated problems reported to HRPO and withdrawn during the review process need not be reported to QASMC.)

QASMC must be notified within **10 days** of receipt of IRB acknowledgment via email to a QASMC auditor.

6.4 Timeframe for Reporting Required Events

Immediate adverse events will be tracked from date of signed consent through 1 week after the last study procedure. After administering the intervention at baseline and 6 months, we will contact participants twice by email or phone (1-2 weeks, and 4-5 weeks post-intervention) to evaluate for adverse events. We will also contact participants to evaluate for any potential adverse events related to breast biopsy 1 week after their 12-month biopsy visit. Long-term adverse events (fracture risk) will be tracked at 24-month after baseline intervention. For the purposes of this protocol, adverse events collected and documented on CRFs are those considered at least possibly related to study interventions (administration of denosumab, specimen collection) and not those related to other procedures or therapies the participant may undergo during her participation.

7 PHARMACEUTICAL INFORMATION

7.1 Denosumab

7.1.1 Denosumab Description

Denosumab is indicated for: (1) treatment of postmenopausal women with osteoporosis at high risk for fracture; (2) treatment to increase bone mass in men with osteoporosis at high risk for fracture; (3) treatment of glucocorticoid-induced osteoporosis in men and women at high risk for fracture; (4) treatment to increase bone mass in men at high risk for fracture receiving androgen deprivation therapy for non-metastatic prostate cancer; (5) treatment to increase bone mass in women at high risk for fracture receiving adjuvant aromatase inhibitor therapy for breast cancer; (6) prevention of skeletal-related events in Participants with multiple myeloma and Participants with bone metastases from solid tumors; (7) treatment of adults and skeletally mature adolescents with giant cell tumor bone that is unresectable or where surgical resection is likely to result in severe morbidity; (8) treatment of hypercalcemia of malignancy refractory to bisphosphonate therapy.

Denosumab is a human IgG2 monoclonal antibody with affinity and specificity for human RANKL. It has an approximate molecular weight of 147 kDa and is produced in genetically engineered mammalian (Chinese hamster ovary) cells.

7.1.2 Clinical Pharmacology

Denosumab binds to RANKL, a transmembrane or soluble protein essential for the formation, function, and survival of osteoclasts, the cells responsible for bone resorption. It prevents RANKL from activating its receptor, RANK, on the surface of osteoclasts and their precursors. Prevention of the RANKL/RANK interaction inhibits osteoclast formation, function, and survival, thereby decreasing bone resorption and increasing bone mass and strength in both cortical and trabecular bone.

7.1.3 Pharmacokinetics and Drug Metabolism

In a study conducted in healthy male and female volunteers (n = 73, age range: 18 to 64 years) following a single subcutaneously administered Prolia dose of 60 mg after fasting (at least for 12 hours), the mean maximum denosumab concentration (C_{max}) was 6.75 mcg/mL (standard deviation [SD] = 1.89 mcg/mL). The median time to maximum denosumab concentration (T_{max}) was 10 days (range: 3 to 21 days). After C_{max}, serum denosumab concentrations declined over a period of 4 to 5 months with a mean half-life of 25.4 days (SD = 8.5 days; n = 46). The mean area-under-the-concentration-time curve up to 16 weeks (AUC_{0-16 weeks}) of denosumab was 316 mcg·day/mL (SD = 101 mcg·day/mL).

7.1.4 Supplier

Denosumab is commercially available and will be provided at no cost to participants.

7.1.5 Dosage Form and Preparation

Denosumab is available as 1 mL of a 60 mg/mL solution in a single-use prefilled syringe or as 1 mL of a 60 mg/mL solution in a single-use vial. Denosumab 60 mg/mL, 1 mL, will be transferred into a syringe to look identical to placebo to maintain the blind.

7.1.6 Placebo

One mL placebo (normal saline) will be drawn up into a syringe that looks identical to denosumab 1 mL to maintain the blind. The label for every syringe (denosumab and placebo) will read “denosumab 60 mg or placebo” to maintain the blind. These processes will be undertaken by the pharmacist and the study team will be blinded to these processes.

7.1.7 Storage and Stability

Store in a refrigerator at 2°C to 8°C (36°F to 46°F) in the original carton. Do not freeze. Prior to administration, denosumab may be allowed to reach room temperature in the original container. Once removed from the refrigerator, denosumab must not be exposed to temperatures above 25°C/77°F and must be used within 14 days.

Protect from direct light and heat. Avoid vigorous shaking.

7.1.8 Administration

Refer to Section 5.1

7.1.9 Special Handling Instructions

None.

8 STUDY CALENDAR

	Screening	Baseline	6 Month (+/-1 month)	12 Month	24 Month	36 Month
Informed consent	X					
Review of medical history	X					
Pregnancy test ¹		X	X			
Serum calcium, magnesium, phosphorus, creatinine, total bilirubin		X	X			
Mammographic density measurements (Volpara)		X		X	X	X ²
Denosumab/placebo		X	X			
Calcium and vitamin D		Daily for 12 months				
Survey		X				
Call or email to assess supplement compliance ³		Every month for 12 months				
Core-needle biopsy		X		X		
Research blood draw		X		X		
Adverse event assessment		Refer to Section 6.4				

1. Women of childbearing potential only

2. Optional

3. For participants who are unwilling to complete a drug diary, we will call/email them monthly to assess their compliance. Study participants will also be asked to bring their supplement containers to 6-month and 12-month follow ups to further assess compliance.

9 DATA SUBMISSION SCHEDULE

Case report forms with appropriate source documentation will be completed according to the schedule listed in this section.

Case Report Form	Submission Schedule
Original Consent Form	Prior to registration
On-Study Form	Prior to starting treatment
Survey	Baseline
Mammographic Density Measurements	Baseline, 12 Month, 24 Month, 36 Month (optional)
Denosumab Administration Form	Baseline, 6 Month
Specimen Collection Form	Baseline, 12 Month
Adverse Event Form	Continuous

10 DATA AND SAFETY MONITORING

An independent Data and Safety Monitoring Board (DSMB) will be convened. A DSMB will consist of no fewer than 3 members including 2 clinical investigators and a biostatistician. DSMB members will be employed by Washington University, Barnes-Jewish Hospital. DSMB members are subject to the Washington University School of Medicine policies regarding standards of conduct. Individuals invited to serve on the DSMB will disclose any potential conflicts of interest to the trial principal investigator and/or appropriate university officials, in accordance with institution policies. Potential conflicts that develop during a trial or a member's tenure on a DSMB must also be disclosed.

The DSM report for the DSMB will be prepared by the study team with assistance from the study statistician, will be reviewed by the DSMB, and will be submitted to the QASM Committee. The DSMB must meet at least every six months beginning six months after study activation at Washington University. This report will include:

- HRPO protocol number, protocol title, Principal Investigator name, data coordinator name, regulatory coordinator name, and statistician
- Date of initial HRPO approval, date of most recent consent HRPO approval/revision, date of HRPO expiration, date of most recent QA audit, study status, and phase of study
- History of study including summary of substantive amendments; summary of accrual suspensions including start/stop dates and reason; and summary of protocol exceptions, error, or breach of confidentiality including start/stop dates and reason
- Study-wide target accrual and study-wide actual accrual
- Protocol activation date
- Average rate of accrual observed in year 1, year 2, and subsequent years
- Expected accrual end date and accrual by cohort
- Objectives of protocol with supporting data and list the number of participants who have met each objective
- Early stopping rules with supporting data and list the number of participants who have met the early stopping rules
- Summary of toxicities and separated by cohorts with the number of dose-limiting toxicities indicated
- Abstract submissions/publications
- Summary of any recent literature that may affect the safety or ethics of the study

The study principal investigator and coordinator will monitor for serious toxicities on an ongoing basis. Once the principal investigator or coordinator becomes aware of an adverse event, the AE will be reported to the HRPO and QASMC according to institutional guidelines.

Refer to the Washington University Quality Assurance and Data Safety Monitoring Committee Policies and Procedures for full details on the responsibilities of the DSMB. This is located on the QASMC website at <https://siteman.wustl.edu/research/clinical-research-resources/protocol-office-prmcqasmc/>.

Early Stopping Rules

Initially, we plan to perform futility analyses when 2/3rds of women enrolled have completed 12-month follow-up to determine if the intervention is likely to achieve its primary aim.

However, we have decided not to perform a futility analysis at this stage. This decision is based on the fact that we have nearly completed enrollment, with 200 out of 210 participants already enrolled. Additionally, our primary outcome measure is breast density, not the incidence of breast cancer. As such, stopping the trial based on a futility analysis would not deprive participants of the potential benefits of the treatment. Moreover, conducting a futility analysis with only 140 participants may not yield significant benefits or conclusive results. Therefore, we will proceed with the trial as planned, without conducting a futility analysis.

11 STATISTICAL CONSIDERATIONS

11.1 Study Design

This is a double-blinded randomized clinical trial where eligible participants, stratified by age (< 45 vs 45~50 vs. ≥ 50 years), will be randomized at a 1:1 ratio to receive two doses of subcutaneous denosumab (60mg) at BL and at 6 month (6M) or 2 doses of subcutaneous placebo at BL and 6M. Stratified permuted block randomized design will be used to generate the randomization table where permuted block randomization will be used for each stratum by age, with a varying block size of 4 and 6. Randomization table will be generated using the R package “blockrand.”

11.2 Endpoints

11.2.1 Primary Endpoint

The primary hypothesis to be tested is the difference in change in mammographic breast density, from baseline to 12 months, between the treatment arm and the control arm:

H0: VPD percent change at 12M from baseline ≥ 0 versus H1: VPD percent change at 12M from baseline < 0.

We will use volumetric percent density (VPD) as the primary mammographic breast density measure. The primary endpoint is VPD percent change at 12M from baseline. The VPD percent change at 12M from baseline will be calculated as volumetric percent density at 12 months divided by baseline volumetric percent density minus 1.

11.3 Secondary Endpoints

The secondary endpoint is VPD percent change at 24 M from baseline and from previous time point (24 M vs. 12 M). The VPD percent change at a post-intervention time point

from baseline (or a prior time point) will be calculated as volumetric percent density at a post-intervention time point divided by baseline (a prior time point) volumetric percent density minus 1. Among women who have 36 M mammograms available, we will also evaluate VPD percent change at 36 M (from baseline and 36 M vs. 24 M).

11.4 Exploratory Study Endpoints

The exploratory study endpoints include

- Breast tissue RANK, progesterone, metabolic, immune and inflammatory pathway gene expression
- Biomarkers of breast epithelial and stromal proliferation
- Breast metabolome

11.5 Sample Size Estimate

We analyzed data on 34 premenopausal women with dense breasts without any chemoprevention treatment, recruited as part of our study (CCR15332379) to obtain information for sample size calculation. The mean volumetric percent density change at 12 months from baseline is estimated at approximately -7.24% with a standard deviation (SD) of 0.17. We assumed that the VPD percent change in the placebo arm had the same mean/SD as observed in our preliminary data and the VPD percent change in the denosumab arm would show a less than doubled decrease to -13.9% , both with a SD of 0.17. We require 210 evaluable participants, 105/arm to achieve 80.4% power to detect the difference of -6.66% in VPD percent change between the denosumab and the placebo arm, based on two-sided two sample t-test at a 5% level. We estimate that 10% of study participants might not return for repeat biopsy, hence, to compensate for the attrition, we plan to enroll 232 participants in our trial (116/arm).

11.6 Statistical Analysis for Primary Endpoint

The primary endpoint for this trial is mammographic breast density, assessed as volumetric percent density. The baseline volumetric percent density of a participant at enrollment will serve as the control of the post-intervention 12 months volumetric percent density of the same participant. The volumetric percent density (VPD) at each time point will be summarized per treatment arm using descriptive statistics including mean, median, range, and standard deviation. The volumetric percent density change will be similarly summarized using descriptive statistics by arm. The volumetric percent density change will be compared between the two arms using two-sample t-test and the results will be reported as the primary analysis results. Linear regression will be applied to volumetric percent density change to estimate the effect of denosumab relative to placebo, adjusting for potential confounders. Our randomized controlled study design will help to ensure a balanced distribution of reproductive factors, and other potential confounders that may be associated with mammographic density in both arms of the trial. We also will collect information on phase of menstrual cycle; i.e. average menstrual cycle length, date of onset of their last menstrual period and the date of the predicted onset of their next menses to allow tight adjustment for menstrual cycle in statistical analyses. The random coefficients

linear mixed effects model, which allows us to include the participants with missing measurements at 12 months will be applied to volumetric percent density at baseline and 12 months to estimate the rate of change difference in the two arms, without and with adjustment for confounders.

11.7 Statistical Analysis for Secondary Mammographic Breast Density Endpoints

VPD at 24M and 36 M as well as VPD percentage change at 24M and 36M from BL and from their respective prior time point will be similarly analyzed as described above for the primary endpoint. We will also use linear mixed effects model for repeated measures to integratively analyze VPD across all time points given sufficient data points at later time points.

11.8 Statistical Analysis for Exploratory Objectives

Gene expression data will be analyzed as raw count data and as normalized data according to the manufacturer's manual and data analysis guide. The gene expression data will be examined for batch effect by principal component analysis and graphical plotting. In presence of batch effect, batch effect will either be removed using ComBat or BioConductor LIMMA or be incorporated into downstream statistical analyses of gene expression data with batch variables. Unsupervised hierarchical clustering analysis will be performed on the normalized gene expression data for potential participant subgroups within arms and across arms. Normalized gene expression at each time point and gene expression difference will be subjected to principal component analysis to visualize potential separation by arm or by other factors. The raw count data of gene expression at baseline and 12-month will be analyzed by the negative-binomial distribution-based modeling using the BioConductor package DEseq2 to identify differential genes for volumetric percent density change while normalized gene expression data will be analyzed using the BioConductor LIMMA package for the effect of denosumab. Potential covariates and batch variables will be considered to evaluate the independent effect of the markers for volumetric percent density. Arm-specific mean expression and log fold change accompanied with unadjusted raw p-values and false discovery rate (FDR) adjusted p-values will be reported. Significantly differential genes will be identified at a pre-specified FDR value, e.g., 5%. Volcano plots will be generated to visualize the significance testing of differential expression and the log fold change between the two arms. Normalized gene expression change between baseline and 12 months will be compared between the two arms using two-sample t-test or Wilcoxon rank sum test as appropriate. Further, gene expression at each time point and baseline-12 months expression change will be correlated with volumetric percent density at each time point and baseline-12 months volumetric percent density change, within each arm and across both arms, as quantified by Pearson or Spearman correlation coefficient. MKI67 gene expression will be compared between the two arms using Wilcoxon rank sum test or two sample t-test as appropriate. Pearson or Spearman correlation will be calculated between the IHC and gene expression measurements. Linear regression will be fitted between IHC and gene expression of Ki-67 for the shape of the relationship. For all biomarker analyses, permutations will also be performed to calculate the permutation-based p-values to address multiple testing issues

while accounting for correlation between markers. Pearson or Spearman correlation between VPD change at two time points with gene expression change at corresponding time points will be calculated.

We will identify a biomarker signature using all the measured exploratory biomarkers that predicts women who will have clinically important reductions in mammographic density ($\geq 10\%$ decrease), thus, more likely to benefit from chemoprevention.

Outcome: Among women in the denosumab arm, responders will be defined as those who achieve a $\geq 10\%$ decrease in volumetric percent density from baseline to 12 months. Those who do not achieve a $\geq 10\%$ decrease in volumetric percent density will be defined as non-responders. We chose a $\geq 10\%$ decrease in volumetric percent density because data from the IBIS trial showed that women who took tamoxifen and had a $\geq 10\%$ reduction in breast density had a 63% reduction in breast cancer risk, but those who took tamoxifen but experienced a $< 10\%$ reduction in breast density had no reduction in breast cancer risk. We estimate a 30~40% response rate. We will use the exploratory markers to construct a response signature. The logistic regression modeling and receiver operating characteristics (ROC) analysis will be performed for each marker separately for the binary volumetric percent density outcome and area under ROC (AUC) will be calculated to gauge the overall discriminative ability of a marker's expression for response to denosumab. **Cross-validation.** Due to the relatively small sample size, we will perform 5-fold internal cross-validation (CV) for training and validation. During one CV, 80% of the samples will be used as training samples to estimate parameters in a response signature while the rest of the samples will be left out as test samples to have their response predicted by the trained signatures later. After all CVs, the performance of the signatures will be evaluated by tabulating the predicted response status versus the true response status of the left-out samples based on the performance metrics.

Sample Size Justification: We target an AUC of at least 0.65 in a range of 0.65~0.8 for the multi-marker based response signature. The 95% CI lower and upper limit on the estimated AUC for varying response rate between 30% and 40% and target AUC can be calculated. A sample size of 105 allows us to estimate the AUC with a lower limit of the 95% CI being above 0.5 (with true AUC=0.65, lower limit =0.532/0.537/0.541 corresponding to a response rate=0.3/0.35/0.4, respectively), performing better than random chance.

11.9 Missing Value Handling

In presence of missing data, we will develop appropriate missing data models based on the data missingness mechanism. We will first check if the data are missing completely at random and if not, as is likely, will examine which variables are associated with missingness pattern and adjust for observed variables related to missingness using, if possible, longitudinal fixed or random effects models, assuming the mean and covariance structures are known. If considerable data are missing on key predictors, we will use multiple imputation. As a sensitivity analysis, we will compare to complete case analysis

results and will also analyze differences between arms at each time point imputing a change of zero for those missing follow-ups as the worst scenario.

11.10 Analysis Population

The primary efficacy analysis uses the intent to treat population with both baseline and 12M VPD measurements available, regardless of treatment received. Sensitivity analysis will be conducted using the per protocol population, referring to the participants who complete the study without deviation from treatment allocation.

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High Calcium Foods

The list below is an example of high calcium foods, including, but not limited to:

1. **Seeds:** Chia, poppy, sesame, celery
1 tablespoon (9 grams) of poppy seeds packs 127 mg of calcium, or 10% of the recommended daily value. Sesame seeds contain 7% of the daily value for calcium in 1 tablespoon (9 grams);
2. **Cheese:** Parmesan cheese has the most, with 242 mg- or 19% daily value- per ounce (28 grams). Soft cheeses tend to have less, for instance, 1 ounce (28 grams) of brie only delivers 52 mg, or 4% of the daily value;
3. **Yogurt:** one cup (245 grams) of plain yogurt delivers 23% of the daily value for calcium. Low fat yogurt may contain higher calcium levels, with 34% of the daily value in 1cup (245 grams). Greek yogurt has less calcium than regular yogurt;
4. **Sardines and canned Salmon:** A 3.75 ounce (92 grams) can of sardines delivers 27% of the daily value and 3 ounces (85 grams) of canned salmon with bones has 19%;
5. **Beans and lentils:** White beans, with 1 cup (179 grams) of cooked white beans provides 12% of the daily value. Other varieties offer 3-4% of the daily value per cup.
6. **Almonds:** Of all nuts, almonds are among the highest in calcium. 1 ounce (28 grams) of almonds, or 23 nuts, delivers 6% of the daily value.
7. **Whey protein:** protein found in milk, exceptionally rich in calcium- a 1.2 ounce (33 gram) scoop of whey protein powder isolate contains approximately 160 mg, or 12% of the daily value;
8. **Leafy greens:** Collard greens, spinach and kale- 1 cup of cooked collard greens has 268 mg of calcium or about 21% of the amount that you need in a day;
9. **Rhubarb:** 105 mg of calcium per cup (122 grams) of raw rhubarb, or about 8% of the daily value;
10. **Fortified foods:** cereals- some types of cereal can deliver up to 1000 mg (100 % of the daily value) per serving and that's before adding milk. (Note that your body can't absorb all that calcium at once and it's best to spread your intake throughout the day). Flour and cornmeal may also be fortified with calcium.

11. **Edamame and tofu:** one cup of cooked edamame packs 8% of the daily value for calcium. Tofu that has been prepared with calcium also has high amounts with over 66% of the daily value for calcium in just half a cup.
12. **Fortified drinks:** One cup of soy milk has 23% of the daily value. Nondairy milks and orange juice may be fortified with calcium. 1 cup of fortified orange juice can have 27% of the daily value.
13. **Figs:** They have more calcium than other dried fruits. Dried figs provide 5% of the daily value for calcium in a 1.4 ounce (40 gram).
14. **Milk:** one cup of cow's milk has 306-325 mg, depending on whether it's whole or nonfat milk. Goat's milk is also a good source, providing 327 mg per cup.

14 ADDENDUM

Randomized Clinical Trial of RANKL Inhibition with Denosumab on Mammographic Breast Density in High-Risk Premenopausal Women with Dense Breasts (TRIDENT): Addendum for the Memorial Hospital Shiloh, IL Site

This study aims to investigate the impact of RANKL inhibition with denosumab on mammographic breast density in high-risk premenopausal women with dense breasts. We will also perform gene expression analyses in breast tissue and blood samples to determine the effect of RANKL inhibition on the expression of RANK pathway genes, progesterone receptor and progesterone-regulated pathway genes, and other biomarkers associated with breast cancer risk. Successful demonstration that RANKL inhibition reduces mammographic density could open up additional approaches to primary breast cancer prevention in high-risk premenopausal women who do not have dominant genetic predispositions.

We propose to continue building and expanding the TRIDENT study to the Memorial Hospital Shiloh, IL, to reach premenopausal women with dense breasts, especially racial minorities (non-Hispanic Black).

Eligible and Interested Participants

Note: Research coordinators are employed by Washington University

The research coordinators will review in Epic BJC/WUSTL the medical records of potential participants who have scheduled their annual screening mammogram at the Memorial Hospital Shiloh, IL. For potentially eligible participants, staff and physicians at the Memorial Hospital Shiloh will contact them and ask whether they are interested in the study and allow research coordinators to contact them. If potential participants give permission, the research coordinator will mail a study invitation letter and brochure to them and follow up with a phone call for a phone screen. If they are initially eligible for the study based on dense breasts (BI-RADS Category C or D) on the latest mammogram result and medical records in Epic BJC/WUSTL, they will be consented remotely and enrolled in the study. The research coordinator will send the potential participants a digital informed consent to review at least 24 hours before the remote consent is scheduled.

All participants will be reminded that participation in the study is entirely voluntary and will not affect their care. They are at liberty to withdraw from the study at any time. Also, participants will have sufficient time to review the informed consent document and decide whether they want to enroll in the study.

Study activities and Participant-Research Coordinator interaction

All the onsite study activities may be performed at the Memorial Hospital Shiloh, IL except denosumab/placebo injections. The onsite study activities include pregnancy tests, blood tests (serum calcium, magnesium, phosphorus, creatinine, and total bilirubin), denosumab/placebo injections, research blood draws, and breast core-needle biopsy (See Study Procedures and Locations below and 8. Study Calendar). All study procedures will be performed according to the

protocol (See 5.4 Specimen Acquisition). The collected biospecimens, including blood for research and breast tissue, will be transported to Washington University using courier services.

The research coordinators will meet the participants onsite up to four times over the first 12 months. The research coordinators will enter the laboratory and biopsy orders in Epic and contact participants to schedule study appointments. The research coordinators will work closely with Memorial Hospital staff and physicians to ensure that all study procedures and records follow the protocol.

STUDY PROCEDURES AND LOCATIONS

Study Visit	Baseline Screening	Baseline	6 Month	12 Month	24 Month	36 Month (optional)
Procedures	Safety Test*	Breast Biopsy	Safety Test*	Mammogram	Mammogram	Mammogram
	Blood for research	Denosumab/Placebo	Denosumab/Placebo	Breast Biopsy		
	Mammogram	Injection	Injection	Blood for research		
Location	Memorial	BJC-WU	BJC-WU	Memorial	Memorial	Memorial

*Safety tests include blood tests for serum calcium, magnesium, phosphorus, creatinine, total bilirubin and pregnancy test (blood or urine)
Blood for research and Breast tissue will be transported to Washington University.