Atherosclerosis, Immune Mediated Inflammation and Hypoestrogenemia in Young Women

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A. Introduction

Premature CVD is the leading cause of death in young women, yet traditional risk factors underestimate CVD risk. While observational epidemiological study has identified ovulatory function and estrogen levels as potentially playing a role in CVD in women, specific study using well-phenotyped subjects and accurate measures of atherosclerosis has not been performed. The loss of estrogen after menopause is associated with an unfavorable shift of CVD risk factors and is believed to contribute to increased rates of CVD. Young premenopausal women can have hypoestrogenemia due ovulatory cycle disruption. Functional hypothalamic amenorrhea (FHA) is the cessation of the menstrual cycle due to suppression of the hypothalamic gonadotropin-releasing hormone (GnRH) resulting in severe hypoestrogenemia. It is estimated that 1.62 million women between the ages of 18 and 44 years in the US and 17.4 million women worldwide have functional hypothalamic amenorrhea (FHA) resulting in premenopausal hypoestrogenemia (HypoE).

We hypothesize that a chronic state of premenopausal HypoE is associated with more adverse CVD risk factors and inflammatory biomarkers, and is associated with pre-clinical CVD. Prior research has found an adverse association in young women with irregular menses and CVD events; young women with Turner's syndrome, a genetic defect resulting in severe hypoestrogenemia from birth have premature deaths due to accelerated CVD; and hypoestrogenemia of hypothalamic origin in premenopausal women has been associated with premature obstructive coronary artery disease (CAD).

Furthermore, the health impact of premenopausal HypoE in young amenorrheic woman has also been established on bone health resulting in early osteoporosis paralleling the changes seen after menopause estrogen loss, approximately 2.5% bone loss per year. Several epidemiological studies have found low bone mineral density has been associated with subclinical atherosclerosis and CVD mortality.[1-3] While CVD and osteoporosis are both diseases of aging, more recent data suggests a common physiologic mechanism linking these two conditions beyond age, such as inflammatory markers and cytokines.[4] To date, however, the impact of low estrogen in young premenopausal and the relation between bone mass and subclinical CVD unknown.

Both the American Academy of Pediatrics and the American College of Obstetrics and Gynecology have advocated that the menstrual cycle should be considered a "vital sign" due to the importance of estrogen on tissues throughout the body. To date, however, there has been no study assessing the relationship between HypoE, CVD risk factors, bone mass and subclinical CVD. The overarching goals of this research proposal are to test whether 1) Premenopausal HypoE is associated with CVD risk factors and inflammatory biomarkers; 2) Premenopausal HypoE is a marker of subclinical CVD. In order to achieve our goals, we will compare Premenopausal HypoE women to regularly cycling women (normal controls) and women who are recently menopausal (<3 years from last menses) not on hormone replacement therapy ; 3) To determine that estrogen improves CVD inflammatory biomarkes and markers of subclinical CVD in premenopausal HypoE women. In a randomized, double-blind placebo-controlled trial in premenopausal HypoE women we will test 12 weeks of transdermal estradiol followed by 2 weeks of estradiol plus progesterone on CVD biomarkers and subclinical CVD versus placebo. 4) to Results of the larger study could have readably translatable implications – use of hormonal contraceptive therapy provides sustained and safe levels of estrogen in premenopausal women and is a strategy that has been evaluated in a primate animal model. Hormonal contraceptive therapy could be tested as a next step on measures of atherosclerosis should the study results be as hypothesized.

Specific Aim 1: Is premenopausal HypoE associated with CVD risk factors and biomarkers?
To establish whether CVD risk factors and biomarkers are more adverse in premenopausal women with functional hypothalamic amenorrhea (FHA) resulting in premenopausal hypoestrogenemia (HypoE) compared to regularly cycling women and recently postmenopausal woman (<3 yrs) not on hormone replacement therapy. CVD risk factors will include systolic blood pressure (SBP), diastolic blood pressure (DBP), low density lipoprotein cholesterol (LDL) and high density lipoprotein cholesterol (HDL), triglycerides, body mass index (BMI), and inflammatory biomarkers such as high sensitivity C-reactive protein (hsCRP).

Specific Aim 2: Is premenopausal HypoE associated with relatively endothelial dysfunction?
To evaluate whether endothelial dysfunction is greater in women with premenopausal HypoE compared to regularly cycling women and recently postmenopausal women using peripheral arterial tonometry (PAT).

Specific Aim 3: To determine that estrogen improves CVD inflammatory biomarkers and markers of subclinical CVD in premenopausal HypoE women. In a randomized, double-blind placebo-controlled trial in premenopausal HypoE women we will test 12 weeks of transdermal estradiol on inflammatory biomarkers and subclinical CVD versus placebo at baseline and 12 weeks followed by 2 weeks of estradiol plus progesterone for endometrial safety.

B. Background and Significance

B.1. CVD is the leading killer of young women. Although significant advancements have been made in cardiovascular research, CVD remains the leading killer of women at all ages.[5] Traditional CVD risk factors are not adequately predictive of CVD mortality in young women and a significant amount remains unexplained. Notably, young, premenopausal women have a 2-4 fold increased risk or mortality with CVD compared to age-matched males, despite adjustment for CVD risk factors and comorbidity.[6] In the US each year: 1) approximately 480,000 women will die of CVD, 2) one in three women will die of heart disease regardless of race or ethnicity, 3) more women die of CVD than men, and 4) more women will die of CVD than the top 6 cancers combined.[7] CVD remains a dominant contributor to the nation's morbidity and health care expenditures in women.

B.2. Pre-clinical atherosclerosis. The risk of CVD significantly increases in the 5th decade of a woman's life; however the development of atherosclerosis represents a process that begins much earlier. In the Bologusa Heart Study, there is evidence that the pre-clinical fatty atherosclerotic streaks form in the teenage years, followed by fibrous atherosclerotic plaques in the 20's and 30's, and eventually clinical atherosclerosis resulting in CVD events.[8] Autopsy data from this study showed that by age 30, the prevalence of a fibrous-plaque lesion is 60% in both the aorta and the coronary artery.[8] In the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study, an autopsy study on men and women, age 15-34 years, approximately one-third of women had atherosclerosis by age 34.[9]

B.3. CVD occurs later in women than in men. Women typically develop CVD 10 years later than men.[10] (see figure 1) As shown in figure 1, the equivalent rate of myocardial infarction (MI) in women occur 10-years later than men, as illustrated by the dashed line. After the age of 50, CVD risk factors shift unfavorably for women; which coincides temporally with estrogen loss and natural menopause. In women who undergo surgical removal of the ovaries, oophorectomy, the CVD risk increases. An increased risk of CVD is also observed among young women undergoing premature menopause, compared to age-matched women.[11]

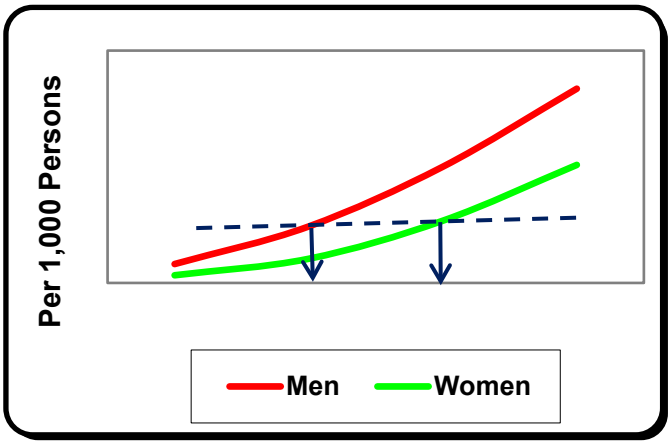


Figure 1: Annual Rate of First Heart Attacks by Age and Sex

B.4. Women with premature estrogen loss have early death and increased CVD mortality. Whether menopause is natural or surgical, common sequelae of the low estrogen environment are worsening CVD risk factors including dyslipidemia and hypertension. Postmenopausal women in the Framingham study had significantly worse lipid profiles than same-aged premenopausal women. A shift to an increase in total cholesterol, LDL and triglycerides and decrease HDL has been shown to occur just 6 months after cessation of the last menses.[12] Compared to women who go through natural menopause, women who go through early menopause at or before age 46 have overall worse CVD risk factors, CVD mortality[13-15]and death[16,17] Early menopause has also been associated with accelerated subclinical atherosclerosis by carotid IMT as compared to women who go through later menopause.[18,19] In the Nurse's Health Study, women who underwent surgical oophorectomy and were not treated with estrogen replacement therapy prior to the age of 50 were found to be at increased risk for coronary heart disease (HR 1.98, 95% CI 1.18-3.32), stroke (HR 2.19, 95% 1.16-4.14) and all-cause death (HR 1.40, 95% 1.01-4.33).[20]

Turner's syndrome (TS), 45 X, and primary ovarian insufficiency (POI), 46,XX are both conditions that result in severe estrogen deficiency in young women. TS is an X-chromosomal defect that results in ovarian dysgenesis and lifelong estrogen deficiency. These women have a reduced life expectancy with the main cause of death being cardiovascular complications including a seven-fold higher rate of CVD.[21] Additionally, women with TS have higher rates of CVD risk factors including hypertension, hyperlipidemia, diabetes, and obesity. Another model of severe estrogen deficiency in young women is 46, XX primary ovarian insufficiency (POI) resulting in primary amenorrhea. In a cross-sectional study of women in their early 30's with estrogen deficiency, either TS or POI, a statistically significantly thicker cIMT (mm) was found in TS and POI women compared to normal controls of similar ages. (0.61 $\pm$ 0.07 mm; 0.60 $\pm$ 0.05, and 0.55 $\pm$ 0.06, $p<0.001$, respectively).[22]

B.5. CVD risk in women with premature estrogen loss is attenuated by estrogen therapy. Estrogen therapy reduces oophorectomy-related and POI CVD risk,[23-28,29] Estrogen therapy in young TS and POI results in improved high density lipoprotein cholesterol (HDL-C), serum glucose and cIMT. These important observational findings suggest that estrogen modulates CVD in young women with HypoE.

B.6. Premenopausal HypoE due to Functional Hypothalamic Amenorrhea (FHA) may contribute to CVD risk. HypoE in premenopausal women is most often due to FHA; up to one third of adult-onset amenorrhea is due to estrogen disruptions.[30] FHA is defined as no menses

due to low estrogen due to suppression of the hypothalamic-pituitary-ovarian axis and excludes pathologic diseases such as those related to a systemic disease or associated with a disorder of the reproductive tract. FHA is due to suppression of the hypothalamic gonadotropin-releasing hormone (GnRH) pulsatility resulting in lower levels of follicle stimulating hormone, luteinizing hormone, and a state of estrogen deficiency. There are 3 types of FHA associated with stress, weight loss, or excessive exercise.

Animal data suggest that premenopausal cardiovascular disease is strongly determined by estrogen status characterized by hypoestrogenemia of hypothalamic origin.[31] Specifically, when young premenopausal monkeys are subjected to environmental stress (due to frequent cage rotation and constant re-establishment of social hierarchical position); they develop a stress reaction that is characterized by a reduction of the central brain hormones that stimulate the ovaries.[32] Menstrual cycling becomes irregular or ceases, fertility is low or absent, and estrogen levels fall due to ovarian shut down. The monkeys have abnormally low follicle stimulating hormone (FSH) and luteinizing hormone (LH), as well as low estrogen (E2) and progesterone (PO) levels.[32] These young female monkeys develop cardiovascular disease. Female monkeys that maintain normal ovarian function and estrogen levels are protected from cardiovascular disease. These results suggest strongly that environmental stress is a cause of infertility among monkeys, and that the resulting estrogen deficiency can cause cardiovascular disease. In addition, in premenopausal monkeys administering oral contraceptive therapy for 2-year period was found to be protective against the development of atherosclerosis in at-risk monkeys.[33] The relevance of these findings to human females, however, is unknown.

Menstrual cycle irregularities have been studied as a marker for cardiovascular disease. In a cohort of 82,439 nurses aged 20-35yrs, compared to women with regular menses, women with usually irregular or very irregular menses have a significantly increased risk for CVD. (RR 1.22, 95% CI 1.04-1.44, and RR 1.53, 95% CI 1.24-1.90, respectively).[28] This 50% increased risk for CVD included nonfatal MI or fatal CHD adjusted for risk factors such as BMI, hormone use, smoking status and suggests that irregular menses may be a marker for increased risk of later CVD. Menstrual cycling irregularities have also been associated with uterine atherosclerosis.[34] This suggests that amenorrhea and cycling irregularity may be a metabolic marker useful to predict women at risk for developing CVD.

Menstrual cycle irregularities are associated with hypoestrogenemia. In the NHLBI-sponsored Women's Ischemia Syndrome Evaluation (WISE) study, 95 premenopausal women with coronary risk factors underwent coronary angiography for evaluation of suspected cardiovascular disease.[35] Thirteen premenopausal women with cardiovascular disease had significantly lower estradiol, bioavailable estradiol, and follicle-stimulating hormone (FSH) (all $p < 0.05$), compared to the 82 premenopausal women without angiographic cardiovascular disease, even after controlling for age. Estrogen deficiency of hypothalamic (central brain) origin, defined as estradiol <50 pg/ml, FSH <10 IU/L, and luteinizing hormone (LH) <10 IU/L, was significantly more prevalent among the women with cardiovascular disease compared to those without (69% vs 29%, respectively, $p=0.01$). This estrogen deficiency was the most *powerful predictor of angiographic cardiovascular disease* in statistical modeling.

B.7. Meaures of pre-clinical CVD. Peripheral arterial tomometry (PAT) measures coronary and peripheral endothelial function and is a functional expression of atherosclerotic risk.[36] PAT has been found to correlate in patients with coronary microvascular dysfunction,[37] ischemic heart disease,[38] and in menopausal women indicates an independent component of CV risk.[39] WISE studies have demonstrated that the PAT index has a sensitivity of 85% and specificity of 80% for detection of endothelial dysfunction in CAD patients.[36]

C. Research Design and Methods

C.1. Specific Aim 1: Is premenopausal HypoE associated with CVD risk factors? To test the hypothesis that CVD risk factors are more adverse in premenopausal HypoE compared to regularly cycling women and recently postmenopausal woman (<3 yrs) not on hormone replacement therapy.

C.1.1. Recruitment: Women with HypoE, normal controls and recently postmenopausal woman will be recruited from several different locations including the Women's Hormone and Menopause Program at the Barbra Streisand Women's Heart Center, Center for Fertility and Reproductive Medicine in the Department of Obstetrics and Gynecology at Cedars-Sinai Medical Center (CSMC) and CSMC OB/GYN staff physician's offices by posting the study flyer in waiting rooms. We also plan to recruit women from the community by posting our study flyer on community bulletin boards at local college campuses, gyms and yoga studios as well as expand the social media posting to ~~social~~ media. I will also contact student health centers and obtain permission to post the study flyer at these locations. (see study flyers) To determine if a self-identified woman has HypoE, we will provide screening laboratories: FSH, LH, estradiol, progesterone, TSH, Free T4, prolactin, testosterone, free testosterone and androstenedione. Beta-HCG by urine or serum to ensure negative pregnancy prior to enrollment. These screening labs will be performed after a woman has signed the consent form. If a woman has already seen a physician and has had these laboratory results performed, we plan to use that report and not perform additional labs. For women who qualify as HypoE, they will return within 7 days for a repeat estradiol blood draw to confirm HypoE. Regularly cycling women will be (age- and BMI-match) defined as having self-reported menstrual cycle lengths of 24 to 38 days not on hormone therapy, and confirmation of one ovulation by either urinary LH surge or day 22-26 progesterone > 3 ng/ml. If ovulation is not confirmed that month, i.e. progesterone < 3 ng/ml then we would rescreen the subsequent month. Beta-HCG by urine or serum to ensure negative pregnancy prior to enrollment. For recently postmenopausal women will be determined by FSH>30 and 12 months of amenorrhea,[40] within 3 years of final menstrual period with natural menopausal not on hormone therapyFinally, we will offer a one-time $50 stipend to normal control and menopausal women, for the HypoE women up to $300 stipend will be offered for their research visit and time commitment. I have developed a pre-screening questionnaire which includes questions regarding the inclusion and exclusion criteria (see below). For this project, I will recruit 30 women with premenopausal HypoE, 30 regularly cycling women and 30 postmenopausal women.

C.1.2. Eligibility/Inclusion Criterion: *Entry criteria* for HypoE and normal controls will include premenopausal by WISE criteria currently not on hormone therapy, English speaking (for the purposes of complete psychosocial assessment), able to give informed consent, a gynecological age (age since menarche) > 10 and < 25 years, and chronological age > 18 years. Within 90-110% of ideal body weight as determined by the 1983 Metropolitan height and weight table for women. All patients with hypothalamic amenorrhea will be diagnosed based on exclusion of other etiologies for their amenorrhea, including pregnancy, thyroid dysfunction, hyperprolactinemia, POI, and polycystic ovary disease. For recently postmenopausal women will be determined by FSH>30 and 12 months of amenorrhea,[40] within 3 years of final menstrual period with natural menopausal not on hormone therapy, English speaking, able to give informed consent.

Exclusion criteria for normal control and postmenopausal groups are current use of hormone contraceptive or any estrogen or progestin therapy, smoking, hypertension, hyperlipidemia, diabetes, medical, neurological, or ophthalmologic disease except acuity problems, a major Axis I disorder other than depression, parturition in the last 12 months and/or lactating in the last 6

months or the patient has any concurrent disease or condition that in the opinion of the investigator would make the patient unsuitable for participation in the study.
For HypoE women, exclusion criteria includes current use of hormone contraceptive or any estrogen or progestin therapy, allergy to adhesive or tape allergy, or the patient has any concurrent disease or condition that in the opinion of the investigator would make the patient unsuitable for participation in the study.
For postmenopausal women exclusions also include surgical or chemotherapy induced menopause or premature ovarian failure.

C.1.3. Data Collection: All laboratory data will be collected and analyzed according to our published WISE Core Lab methods.[41] Data collection will occur at the Cedars-Sinai Women's Heart Center. Collection will include: reproductive hormone assays (estradiol, progesterone, FSH, LH, bioavailable testosterone, fasting lipid panel (LDL, HDL, Trigylcerides) and metabolic and inflammatory markers (glucose, cortisol, hsCRP). Blood pressure, pulse and body mass index computed as weight in kg/height in m^2 will be collected.

C.2. Specific Aim 2: Is premenopausal HypoE associated with relatively endothelial dysfunction? To establish whether subclinical atherosclerosis is greater in premenopausal HypoE compared to regularly cycling women and recently postmenopausal women using peripheral arterial tonometry (PAT).

C.2.1. Data Collection: *Measure of Vascular Function:* **PAT** is a proven, non-invasive measure of vascular function is method of detecting endothelial function by Endo PAT 2000 (Itamar® Medical Ltd), which we and others including the Framingham Study have successfully used.[42] The device consists of a finger probe to assess digital volume changes accompanying pulse-waves. A pressure of 40-70mmHg is applied by the probe to the index fingers of both hands to eliminate venous stasis and arterial pulse amplitude is recorded. Device calculates a reactive hyperemia peripheral arterial tonometry (RH-PAT) index, which reflects peripheral vascular function.

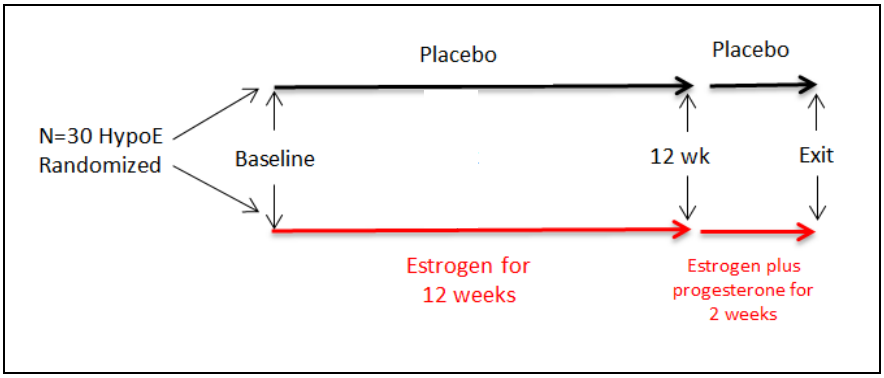
C.3. Specific Aim 3: To determine that estrogen improves CVD inflammatory biomarkers and markers of subclinical CVD in premenopausal HypoE women. In a randomized, double-blind placebo-controlled trial in premenopausal HypoE women we will test 12 weeks of transdermal estradiol on inflammatory biomarkers and subclinical CVD versus placebo at baseline and 12 weeks followed by 2 weeks of estradiol plus progesterone for endometrial safety.

30 HypoE women will be randomized to transdermal estrogen for 12 wks followed by transdermal estrogen plus oral progesterone for 2 weeks. We will compare markers of endothelial function and inflammatory response at baseline and 12wks in women randomized to hormone therapy vs placebo.

C.3.1. Rationale. Estrogen treatment has been shown to improve vascular function in women with premature menopause and it is well established that estrogen has immune-modulating effects resulting in lower pro-inflammatory cytokines in postmenopausal women. We will use our PAT index as a vascular function measure of pre-clinical CVD. We will use a dose of transdermal estradiol 0.1 mg/day weekly patch for 12 wks, which will result in a mean serum estradiol level of 70 pg/ml.[43] We are working with the manufacturer to obtain placebo patches as has been done in previous double-blinded studies.[44] Patches will be applied by the subject to the lower abdomen twice weekly, alternating sides. The dose and 12 week duration of unopposed transdermal estradiol was selected based on prior study to maintain serum around 75 pgram and to minimize the risk of endometrial hyperplasia.[45] The use of unopposed estradiol at higher doses and longer duration can result in endometrial hyperplasia in postmenopausal women after one to three years. Our 12 week treatment of estrogen will be followed by 2 weeks of estrogen and progesterone to allow sloughing of the endometrium and reversal of the estrogen effect on the endometrium.[46] This dose is equivalent to an average serum estradiol level in an ovulating woman which ranges from 50 up to 400 pg/ml.[47] We selected a 0.1mg/day dose of transdermal estrogen

to provide a more physiologic estradiol level compared to oral estradiol which is rapidly convert to estrone.[48] After 12 wks of transdermal estradiol, we will continue estradiol and add oral micronized progesterone 200mg for 14 days to mimic the luteal phase and will allow us to evaluate the additional impact of estrogen plus progesterone. Progesterone 200mg is a peanut based product and for patients with a peanut allergy we will replace this with a synthetic progestin at an equivalent dose, medroxyprogesterone 10mg. This is an FDA-approved medication and has the same indication.

C.3.2. Hypothesis. We hypothesize that estradiol treatment will improve vascular endothelial function, and markers of inflammation in premenopausal HypoE women.



C.3.3. Experimental Protocol and Data Collection: Baseline serum hormones, vascular measures, and immune markers will be collected from aims 1 and 2 in the 30 HypoE women. Subjects will then be randomized to 0.1mg/day transdermal estradiol patch or placebo for 12 weeks followed by 2 weeks of estradiol patch with progesterone or placebo pills with placebo patch for 12 weeks +/- 1 week and 2 weeks +/- 3 days after completing estrogen and progesterone (**Figure**). One week after completing the patch and pill, study staff will contact participant and ask if they had their menstrual period. If needed, 1 patch and 3 more pills will be dispensed during the week 12 visit to account for patient schedules if they cannot come in exactly at day 14, as the exit visit on study drug is needed as suggested by the pharmacist.

C.3.4. Data Collection: Serum hormone evaluation: After 7 days of transdermal 17-beta estradiol and 12 weeks we will measure serum E2 levels and hormone panel to assess blood levels and compliance. Vascular measures will be tested at baseline and 12 week. Detailed method collection described under Aim 1A.

C.3.5. Data Analysis. Statistical method: Associations between change in serum estrogen, change in PAT, change in inflammatory marker levels will be examined. Pearson correlations will be examined between continuous variables. If the distributions are non-normal Spearman correlations will be used. A linear mixed model will be used on the change in PAT at week 12 to examine the association while adjusting for within subject correlation. In addition to choosing an appropriate correlation structure, other model building and diagnostics will be carried out as described in Aim1A. A Tukey-Kramer or Bonferroni adjustment for multiple pair-wise comparisons will be used as appropriate.

Power considerations: Pilot data indicated a baseline SD of 0.35 for PAT in the HypoE group and 0.44 in the control group. Assuming the larger SD at baseline and 4 weeks, a conservative estimate of the SD for the change in PAT assuming no correlation, would be the square root of the sum of the variance estimates at both time points, which is about 0.622. A sample of 15 women in the placebo group and 15 in the treatment group for change in PAT using a two sample t test with 80% power at a significance level of 0.0167 would allow detection of a difference of at least 0.78 units when the sample SD is 0.622. The significance of 0.0167 was chosen by dividing 0.05 by three for the number of comparisons between groups. Increasing correlation above zero between pre and post PAT values will give more precision and allow detection of smaller differences (e.g. a correlation of 0.8 allows detection of a difference of at least 0.35 units).

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