The Effects of Compounded Bioidentical Transdermal Hormone Therapy on Hemostatic, Inflammatory, Immune Factors; Cardiovascular Biomarkers; Quality-of-Life Measures; and Health Outcomes in Perimenopausal and Postmenopausal Women

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ABSTRACT

Menopause impacts 25 million women world wide each year, and the World Health Organization estimates 1.2 billion women will be postmenopausal by 2030. Menopause has been associated with symptoms of hot flashes, night sweats, dysphoric mood, sleep disturbance, and conditions of cardiovascular disease, depression, osteoporosis, osteoarthritis, depression, dementia, and frailty. Conventional hormone replacement therapy results in increased thrombotic events, and an increased risk of breast cancer and dementia as evidenced in large prospective clinical trials including Heart and Estrogen/Progestin Replacement Study I and the Women's Health Initiative. A possible mechanism for these adverse events is the unfavorable net effects of conjugated equine estrogens and medroxyprogesterone acetate on the hemostatic balance and inflammatory and immune factors. Physiologic sex steroid therapy with transdermal delivery for peri/postmenopausal women may offer a different risk/benefit profile, yet long-term studies of this treatment model are lacking. The objective of this study was to examine the long-term effects of compounded bioidentical transdermal sex steroid therapy including estriol, estradiol, progesterone, DHEA, and testosterone on cardiovascular biomarkers, hemostatic, inflammatory, immune signaling factors; quality-of-life measures; and health outcomes in peri/postmenopausal women within the context of a hormone restoration model of care. A prospective, cohort, closed-label study received approval from the Human Subjects Committee. Recruitment from outpatient clinics at an academic medical center and the community at large resulted in three hundred women giving signed consent. Seventy-five women who met strict inclusion/exclusion criteria were enrolled. Baseline hormone evaluation was performed along with baseline experimental measures. Following this, women received compounded transdermal bioidentical hormone therapy of BiEst (80%Estriol/20%Estradiol), and/or Progesterone for eight weeks to meet established physiologic reference ranges for the luteal phase in premenopausal women. The luteal phase hormone ratios were selected based on animal and epidemiologic studies demonstrating favorable outcomes related to traumatic, ischemic, or neuronal injury. Follow-up testing was performed at eight weeks and adjustment to hormone regimens were made including addition of androgens of DHEA and Testosterone if indicated. Experimental subjects were monitored for 36 months. Baseline, 2-month, and annual values were obtained for: blood pressure, body mass index, fasting glucose, Homeostasis Metabolic Assessment of Insulin Resistance (HOMA-IR), fasting triglycerides, total Factor VII, Factor VIII, fibrinogen, Antithrombin III, Plasminogen Activator Inhibitor-1(PAI-1), C-reactive protein (CRP), Interleukin-6 (IL-6), Matrix Metalloproteinase-9 (MMP-9), Tumor Necrosis Factor-α (TNF), Insulin-like Growth Factor (IGF-1), and sex steroid levels. Psychosocial measures included: Greene Climacteric Scale, Visual Analog Pain Scale, Hamilton Anxiety Scale, Hamilton Depression Scale, Holmes Rahe Stress Scale, Job Strain, and Home Strain. Health outcome measures included the number of prescribed medications used, number of co-morbidities, and endometrial thickness in postmenopausal women with intact uteri. Subjects receiving compounded transdermal bioidentical hormone therapy showed significant favorable changes in: Greene Climacteric Scale scores, Hamilton Anxiety Scale, Hamilton Depression Scale, Visual Analog Pain Scale, fasting glucose, fasting triglycerides, MMP-9, C-reactive Protein, fibrinogen, Factor VII, Factor VIII, Insulin-Like Growth Factor 1, and health outcomes of co-morbidities and a number of prescribed medications. Antithrombin III levels were significantly decreased at 36 months. All other measures did not exhibit significant effects. Administration of compounded transdermal bioidentical hormone therapy in doses targeted to physiologic reference ranges administered in a daily dose significantly relieved menopausal symptoms in peri/postmenopausal women. Cardiovascular biomarkers, inflammatory factors, immune signaling factors, and health outcomes were favorably impacted, despite very high life stress, and home and work strain in study subjects. The therapy did not adversely alter the net prothrombotic potential, and there were no associated adverse events. This model of care warrants consideration as an effective and safe clinical therapy for peri/postmenopausal women especially in populations with high perceived stress and a history of stressful life events prior to, or during the menopausal transition.

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INTRODUCTION

The menopausal transition effects over 25 million women world wide each year, and more than 1.2 billion women are estimated to be postmenopausal by the year 2030 according to the World Health Organization (WHO).[1] The increasing proportion of the aging female population and resulting physiological and patho-physiological changes induced by the menopausal transition is of clinical concern. Peri/postmenopausal women may experience symptoms of hot flashes, night sweats, dysphoric mood, and sleep disturbances; and conditions of cardiovascular disease (CVD), depression, osteoporosis, depression, dementia, and frailty have been linked to menopause.[2] Conventional hormone replacement therapy (HRT) of oral conjugated equine estrogens and medroxy-progesterone acetate results in increased adverse effects and outcomes as revealed in the Women's Health Initiative (WHI), yet animal studies and some epidemiologic data support benefits of hormone therapy.[3-7] Clinical therapy for menopausal symptoms remains a significant challenge.[8-10] Women as healthcare consumers are increasingly utilizing plant-derived human identical compounded hormone preparations, yet long-term effects of this treatment on cardiovascular biomarkers, thrombotic, inflammatory and immune signaling factors, and health outcomes have not been identified.[11]

Hormone Replacement Therapy and Hemostatic Factors

Observational studies, randomized clinical trials, and animal investigations have revealed a correlation between hemostatic and inflammatory factors, as well as pro-inflammatory cytokines and the risk of CVD.[12-20] Women become more vulnerable to CVD in the perimenopausal transition and postmenopause due to changes in the clotting cascade, including elevations in fibrinogen, PAI-1, Factors V, VII, and VIII; and decreases in Antithrombin III. Hemostatic factors interact with the cytokines, suggesting a novel relationship between the coagulation pathway and inflammatory pathways in midlife women contributing to endothelial dysfunction, intima media thickness, and macrovascular disease.[21-23] Emerging research has demonstrated an association between hemostatic Factor VII elevations and vasomotor symptoms physiology with proposed gender-specific etiological links to CVD, since midlife women with moderate to severe hot flashes in WHI, Hysterectomy Educational Resources and Services (HERS), and Study of Women's Health Across the Nation (SWAN) had correlation with endothelial dysfunction and cardiovascular events.[24,3,5]

Prothrombotic risk based on changes in the aforementioned factors has been studied in women receiving combinations of oral conjugated equine estrogens, synthetic estrogen, and synthetic progestins.[25-33] While studies of hemostatic factors during exogenous HRT demonstrate a consistent decrease in fibrinogen and PAI-1 levels that is suggestive of a reduced prothrombotic potential, the measured decrease in Antithrombin III levels and concomitant increases in factor V, factor VII and factor VII, and Factor VIII levels are more consistent with an increase in pro-thrombotic potential.

Hormone Replacement Therapy and Inflammatory and Immune Signaling Factors

Elevations of inflammatory factors and immune signaling factors and dysregulation of the crosstalk between these factors related to changes in sex steroid levels have been linked to CVD in midlife women.[34-36] In addition to the aforementioned effects of HRT on hemostatic factors, conjugated equine estrogens and medroxyprogesterone acetate (a synthetic progestin) have been shown to increase levels of C-reactive protein (CRP), an inflammatory marker that is considered an independent risk factor for CVD in healthy postmenopausal women.[37-41] CRP is a plasma protein, an acute-phase protein produced by the liver. It is thought to assist in complement binding to foreign and damaged cells and enhances phagocytosis. It is also believed to play an important role in innate immunity as an early defense system against infections. CRP is used mainly as a marker of inflammation. The American Heart Association recognizes high sensitivity CRP as a cardiology diagnostic test with levels correlating with CVD risk: low risk (<1mg/L); moderate risk (1 to 3 mg/L); and high risk (>3mg/L). Recent research suggests that patients with elevated basal levels of CRP are at an increased risk for diabetes, hypertension, metabolic syndrome, and cognitive dysfunction, in addition to CVD.[42-45] Healthy, non-obese women exhibit elevations in proinflammatory cytokines of Interleukin 6 and Tumor Necrosis Factor-α (TNF) alpha with menopause.[46] Estrogen deprivation likely enhances cytokine production resulting in increased levels which are closely related to oxidative stress. Several studies of HRT in postmenopausal women including the WHI have demonstrated an associated increase in Interleukin-6 (IL-6) in hormone users.[4,47-49] IL-6 is a cytokine derived from activated T lymphocytes that has many functions, including induction of B-cell growth; induction of B-cell differentiation and antibody production; induction of differentiation and proliferation of T cells; and induction of hepatocyte secretion of acute-phase inflammatory proteins. IL-6 is a proinflammatory cytokine that along with tumor necrosis factor-α (TNF-α) has both endocrine and immune functions and has been identified as an important regulator of estrogen synthesis in breast and adipose tissue.[50] Abnormal elevations of IL-6 and TNF-α promote tumor cell growth *in vitro* and *in vivo*.[51] Clinically, elevated levels are associated with CVD, frailty, cognitive dysfunction, obesity, cancer, depression, aging, osteoporosis, and Alzheimer's disease (AD).[52-54] IGF-1 is one of the most potent natural activators of the AKT signaling pathway, a stimulator of cell growth and multiplication, and a potent inhibitor of programmed cell death. In addition to the insulin-like effects, IGF-1 can also regulate cell growth and development, especially in nerve cells, as well as cellular DNA synthesis. Elevated levels of IGF-1 have

been identified in obese women with breast cancer, and HRT has been associated with increased IGF-1 levels in postmenopausal women.[55] Aging women exhibit dysregulation of the axis involving IL-6, TNF alpha, and IGF-1, with low IL-6/IGF-1 ratios correlating with frailty, a condition of grave concern in women 65 years of age and older.[56] The joint effects of IGF-I and IL-6 may be important targets for treatments to prevent or minimize age-related decline in women. Furthermore, HRT has been shown to increase the levels of Matrix Metalloproteinase 9 (MMP-9) in postmenopausal women, and such elevations are significant because MMP-9 accumulates in atherosclerotic plaques and is thought to contribute to the degradation of the extracellular matrix leading to plaque rupture in proposed gender-specific models of CVD in women.[57]

Perceived Stress, Mood and Hemostatic, Inflammatory, and Immune Signaling Factors in Women

Stress and mood in women have been associated with declining health status and increased vulnerability to CVD, the leading cause of death and disability in midlife and postmenopausal women.[58-62] The Women's Ischemia Syndrome Evaluation (WISE) studies revealed dysphoric mood to be an independent risk factor for CVD in postmenopausal women.[63-65] The menopausal transition and menopause are strongly associated with mood disorders, and one study has identified low levels of estrogen and progesterone contributing to suicide risk in women.[66-68] The ATTICA and Project for an Ontario Women's Health Evidence-Based Report (POWER) studies reveal that women with dysphoric mood have increased inflammatory and hemostatic factors.[69-70] Exposure to stressful working environments has been shown to affect psychological as well as physical health. Women have multiple roles and emotional demands both in the home and work environment which may impact their emotional and physical health and are more vulnerable than males to role overload and burnout, conditions associated with deterioration of physical and emotional health.[71-74] Studies reveal high home and/or work strain is associated with elevations in inflammatory factors; dysregulation of endocrine, immune, and metabolic pathways, and negative effects on cardiovascular reactivity.[75-78] Women with high job strain have been shown to exhibit increased cardiovascular reactivity.[79-81] Elevated home strain and caregiver status have been associated with dysregulation of immune pathways and elevated pro-inflammatory cytokines in women.[82-85]

Immunosenescence and Endocrinosenescence

The free radical theory of aging postulates that pathological aging is the result of the free radical damage of cells. Harman's hypothesis generally stated that aging is a free radical process and that degenerative diseases of aging (e.g., cancer, AD, arthritis, atherosclerosis, diabetes) involve free radical processes that cause a disease state.[86] The free radical theory of aging argues

that it is the accumulated oxidative damage with increasing age that causes the aging process. It is theorized that reactive oxygen species (ROS) may damage the DNA that plays a role in aging. The primary site of free radical oxidative damage is the mitochondria and the mitochondrial DNA.[87] The multiple hormone deficiency theory of aging put forth by Hertoghe states that the age-related decline of hormonal levels results in a range of illnesses because most hormones stimulate the formation of antioxidant systems.[88] The lack of adequate levels of hormones in the elderly negatively affects the endocrine system, causing metabolic changes and accelerating the aging process. Hertoghe asserted that curtailed endocrine levels, resulting from lack of hormones, alter intestinal cells in the elderly and thereby prevent the adequate absorption of antioxidant vitamins and trace elements. Low endocrine levels create a hypometabolic state that decreases the endogenous production of antioxidant enzymes, and automatically reduce antioxidant activity because many hormones are themselves strong antioxidants. Sex steroid hormones in women have been demonstrated to modulate inflammatory and immune signaling pathways.[89] As women age and hormone levels decline, disruption of immune/inflammatory axes occur and such disruptions are associated with CVD, metabolic syndrome, pulmonary disease, connective tissue disease, mood disorders, frailty, and dementia.[90-100] In a Hormone Restoration Model of Care, women are assessed individually to determine sex steroid hormone levels, and compounded transdermal bioidentical hormone therapy (BHRT) is administered to restore levels to the luteal premenopausal range in efforts to modulate hormone mediated inflammatory and immune signaling pathways.

The Writing Group for the WHI concluded that *transdermal estradiol and progesterone, which more closely mimic endogenous hormones when used in replacement therapy, may have more favorable outcomes as compared to conjugated equine estrogens and medroxyprogesterone acetate.*[3] The Framingham Writing Group has further stated in regard to gender disparity and CVD risk that *future stroke studies with measures of endogenous hormones are needed to inform the underlying mechanisms so that novel prevention strategies for midlife women can be considered.*[9]

From these conclusions, it is plausible that maintaining the premenopausal hormonal milieu at physiologic luteal phase references ranges may potentially offer a benefit and possibly be an effective preventive strategy for age-related decline and CVD risk in peri/postmenopausal women. Published studies on thrombosis and topical estradiol (E2) have demonstrated decreased thrombotic risk as compared to oral conjugated equine estrogens (ESTER trials).[101-102] Our previous work has demonstrated no prothrombotic or proinflammatory effects of monotherapy with transdermal progesterone in a prospective, randomized, placebo-controlled, short-term, cross-over study of postmenopausal women.[103] Because postmenopausal women require endometrial protection, it is essential to investigate the thrombotic and inflammatory effects of progesterone in concert with estrogen. Further-

more, in an individualized model of care, some aging women may require androgen support; therefore, it is essential to investigate the effects of combined therapies on thrombotic, inflammatory, and immune pathways in peri/postmenopausal women.

Progesterone Endogenous Effects and Therapeutic Application

Progesterone is produced by the ovary with surge in the luteal phase with small amounts produced by the adrenal gland, Schwann cells, and glial cells. Cardiovascular effects of progesterone include reduction of cholesterol ester accumulation, inhibition of induced coronary vasospasm, modulation of thromboxane A2 receptors, protection against coronary hyperreactivity, and inhibition of vascular smooth muscle proliferation. In immune pathways, progesterone delays neutrophil apoptosis, and enhances reactive oxygen intermediates. In the uterus, progesterone induces secretory endometrial changes. Metabolic effects of progesterone include promotion of glucose utilization and improved insulin resistance. Progesterone has antagonistic effects to estrogen in breast and endometrial tissue and neurotransmitter pathways. It has been shown to have neuroprotective properties in response to nerve and brain injury. The SWAN study has revealed that progesterone decline heralds the menopausal transition in midlife women, and studies have shown that low progesterone-to-estrogen ratios are associated with mood disorders, breast cancer, migraine, seizure disorders, and premenstrual dysphoric disorder. Progesterone therapy in women has shown beneficial effects on: perinatal outcomes, mood, seizure activity, migraine events, urogenital atrophy, coronary reactivity, and quality-of-life measures. Progesterone is *distinctly different* from synthetic progestins including medroxyprogesterone acetate in its pharmacology, and pharmacotherapeutic effects in all tissues and pathways excluding the endometrium.[104-172]

DHEA Endogenous Effects and Therapeutic Application

DHEA is a steroid hormone produced by the adrenal glands and ovarian stroma. DHEA is chemically similar to—and can be converted into—testosterone and estrogen. The DHEA production level is highest in early adulthood and then decreases with age. Many age-related disorders correlate with low levels of DHEA production. Research has demonstrated that a decline in the DHEA secretory pattern is clearly age related and is an additive factor for both dementia and major depression. The incidence and prevalence of AD are higher in postmenopausal women than age-matched men, and some research suggests that changes in neurohormonal milieu characterized by lowered estrogen and DHEA and elevated IL-6 may predispose to an increased risk of neurodegeneration. Research reveals a correlation of increased Cortisol/DHEA-S Ratios with normal aging, and elevated ratios >10 have been associated with depression, CVD, and dementia. DHEA therapy in women has been shown to have beneficial

effects on insulin regulation, neurocognitive measures, and immune function.[92,104,173-185]

Testosterone Endogenous Effects and Therapeutic Application

Testosterone is secreted by the adrenal glands and ovaries and may be converted to estrone (E1) via aromatase. In the central nervous system (CNS), testosterone is involved in amine synthesis including norepinephrine, epinephrine, dopamine, and acetylcholine. Research reveals that testosterone affects cognition and is associated with spatial-temporal reasoning, assertiveness, and language fluency in females. Testosterone has a role in sexual health and is essential for libido, arousal, and orgasm. It is an anabolic steroid and as such is bone trophic and inhibits fat accumulation. Testosterone levels may increase or decrease during the menopausal transition. Increased androgenicity of the hormonal milieu is associated with increasing waist to hip ratios, depression, and metabolic syndrome, whereas decreased levels are associated with urogenital atrophy and decreased libido. Low-dose transdermal therapy in deficient postmenopausal women has been shown to have favorable effects on cardiovascular, immune, and adrenergic regulation.[104,186-215]

Estrogen Endogenous Effects and Therapeutic Application

E2 is the most potent and active estrogen, and its clinical therapeutic effects are well known and documented. Estriol (E3) is produced endogenously by the hydration of E1 and may not be converted to E2 or E1 via enzyme activity. It is available as a plant-derived compound therapy. It is characterized by decreased binding time to estrogen receptors as compared to E2 with dissociation occurring at 6 hours 24 hours. It exhibits CNS effects with aiding in amine synthesis: norepinephrine, epinephrine, dopamine, and acetylcholine. It aids in glucose transport across the blood-brain barrier and sensitizes neurons to nerve growth factor. In the autonomic nervous system, it decreases sympathetic tone and has favorable effect on estrogen endothelial receptors. Therapeutic E3 effects are weaker than E2 in the liver and endometrium; however, it exhibits full estrogenic responses in vaginal and urethral tissue. Furthermore, research reveals that it is associated with decreased breast cancer risk as E3 does not increase breast density, and, in animal models, E3 prevents breast cancer-promoting effects of E2; E3 protects breast cells against damage by radiation and carcinogens. Epidemiological studies reveal that higher E3 levels are associated with decreased breast cancer risk, and E3 as an estrogenic monotherapy reduces the relative breast cancer risk. E3 therapy has shown benefit for the relief of menopausal symptoms.[104,216-240]

Study Aim

In light of the unfavorable effects observed with conventional HRT, the aim of this study was to prospectively assess women individually during the menopausal transition and postmeno-

pausal period in a hormone restoration model of care, and to restore physiological levels of sex steroids via compounded transdermal BHRT with determination of the effects on hemostatic, inflammatory, and immune signaling factors; quality-of-life measures; and health outcomes in a cohort of women.

METHODS

Study Population

After obtaining permission from the Internal Review Board at our institution, postmenopausal female subjects were recruited from the community. The inclusion criteria stated that the subjects must be 30 to 70 years of age and (1) have stopped menstruating for at least 12 months due to natural menopause, surgical removal of both ovaries with or without a hysterectomy, or (2) have cessation of ovarian function by chemotherapy or radiation, or (3) meet SWAN study criteria for perimenopause. Exclusion criteria included any moderate or severe chronic illness, any acute illness, recent history of alcohol or drug abuse in the last 2 years, cancer diagnosis and treatment within the last 5 years, any treatment with investigational drugs within 12 weeks prior to study entry, or any current therapy of non-permitted medications including: hormonal or steroid preparations; anti-coagulants; chronic antibiotics; lipid lowering drugs; Cox-2 inhibitors; or excessive use of herbal or dietary supplements. Race/ethnicity and educational level of completion were derived from the screening interview. Age, smoking status, physical activity, alcohol use, menopause status, medication use, health conditions, marital status, and number of children in household was derived from questionnaires and interviews that used standard instruments administered during visits. If the patient had an acute illness, phlebotomy for serum markers was postponed until recovery.

Subjects ranged in age from 35 to 70 years, with a combined median age of 52.3 (± 9.6) years. Body mass index (BMI) as calculated for all subjects ranged from 21 to 38 with a mean value of 27.9 (± 5.8). Race/ethnicity characteristics included 78.6% non-Hispanic Caucasian; 14.3% native American; 4.3% Hispanic; and 2.9% African American. Educational levels revealed 25.7% high school/GED diploma; 15.7% with some college; 15.7% with vocational training; and 42.9% with college degrees. Marital status characteristics included: 82.9% currently married; 14.3% divorced; and 2.9% widowed. Alcohol consumption at a frequency of twelve or fewer alcoholic beverages per month was reported by 60% with 40% reporting no alcohol consumption. Tobacco use was reported by 11.4% of the group, previous tobacco use by 18.6%, and no previous tobacco use by 70%. Exercise frequency mean was 3.5 hours per week with a standard deviation of 3.9. Number of children in household mean calculation was 0.8 (± 1.2). Menopause status classification was 38% surgical menopause, 35% natural menopause, and 27% perimenopause (Figure 1). Dietary assessment was performed by Brown University Rate Your Plate evaluation, and mean value was 53.8 (± 7.9) at baseline.

FIGURE 1. Menopause Status.

- Perimenopause 17%
- Surgical Menopause 35%
- Natural Menopause 38%
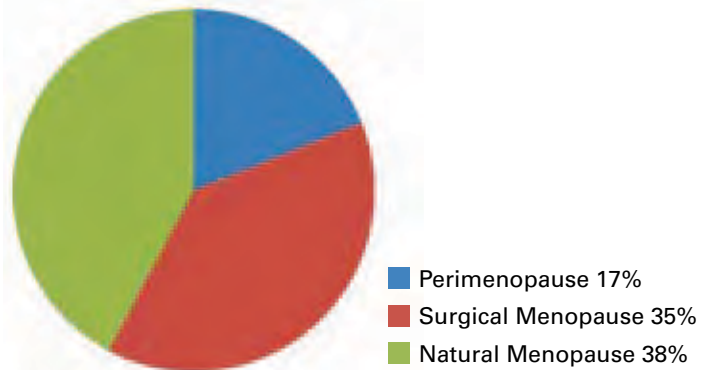
Study Design

Informed consent for study participation was signed by 300 women from the academic medical center and community at large. Seventy-five subjects met the study criteria and were enrolled. Each subject underwent a comprehensive history and physical exam, laboratory assessment, and questionnaire completion at initial visit. Based on hormone levels, subjects were prescribed human identical hormone therapy to be applied transdermally in a total volume of 1 mL daily. Syringes of study drug were code labeled and subjects did not know the specific hormone content or dosages. Daily dosage ranges utilized were: Bi-Est (80% Estriol/20% Estradiol) 0.25 mg to 0.5 mg; progesterone 20 mg to 60 mg; DHEA 1 mg to 2 mg; testosterone 0.2 mg to 0.5 mg. Hormone drug was compounded by two trained compounding pharmacists according to *United States Pharmacopeia* guidelines for compounded drugs and using U.S. Food and Drug Administration (FDA)-approved materials and packaging. The packaging, dose, consistency, and odor of the active drug was kept consistent throughout the study by the compounding pharmacists using HRT base as manufactured by Professional Compounding Centers of America (Houston, Texas). In each case, subjects were instructed to apply 1 mL of transdermal drug daily to a thin-skinned area, and to rotate the site of application so as to apply the transdermal preparation to a different site each day. Subjects were randomly selected throughout the 3-year study to return the syringes containing the transdermal compound to the study coordinator for samples to be sent to an independent testing laboratory to evaluate the compounds for purity, potency, and contaminants.

All subjects underwent an initial medical history and physical exam. Baseline values were obtained for:

- Total factor VII
- Factor VIII
- Fibrinogen
- Antithrombin III

- Plasminogen activator inhibitor-1 (PAI-1)
- CRP
- Tumor necrosis factor-α (TNF-α)
- Matrix Metalloproteinase 9 (MMP-9), and interleukin-6 (IL-6)
- Fasting glucose
- Fasting triglycerides
- Homeostasis Metabolic Assessment of Insulin Resistance (HOMA-IR)
- Insulin-Like Growth Factor-1 (IGF-1)
- BMI
- Blood pressure
- Visual Analog Pain Scale
- Number of prescribed medications
- Number of co-morbidities
- Greene Climacteric Scale
- Hamilton Anxiety Scale (HAM-A) and Hamilton Depression Scale (HDS)
- Holmes Rahe Stress Scale
- NHANES Work and Home Strain
- Sex steroid hormones including

  -E2
  -Progesterone
  -Testosterone
  -DHEAS

Phlebotomy was performed in the morning after an overnight fast for glucose, triglycerides, and all hemostatic, inflammatory, and immune factors. Blood pressure was determined using an automated blood pressure unit to determine resting blood pressure using standard protocols. A measure of weight was taken in the morning in stocking feet on a calibrated digital readout scale situated on a tile floor. Height was measured with a stadiometer and measurements were converted to BMI. Follow-up values were obtained at 2 months, and annually for 36 months. All subjects underwent an end-of-study physical exam. Quarterly telephone interviews were conducted to inquire about any changes in health status, new medication use, and compliance with application of the transdermal drug. There were 17 subjects who dropped out of the study. Of the subjects, 5 were dismissed from the study during year 1 due to ingestion of non-permitted medications or relocation. Of the subjects, 8 discontinued the study in year 2 due to relocation and inability to attend study visits, 1 subject was dismissed due to non-compliance with study protocol, and 3 subjects discontinued the study in year 3 due to inability to attend study visits. No participants discontinued the study due to adverse events or side effects of the study drug.

Statistical Analyses

Means for metabolic factors and health outcomes for group by time analysis were estimated using the least squares approach and were adjusted for covariates. Standard errors are in parenthesis. Spearman's non-parametric correlation was used to examine the relationship between measures of menopausal symptoms and hemostatic, inflammatory, and immune signaling factors. Comparisons with respect to hemostatic factors, inflammatory factors, immune signaling factors, metabolic, and quality-of-life measures were performed using the Student's t-test. All results are reported as mean +/- standard deviations and $P < 0.05$ was considered significant. Statistics were performed using Slide Write Plus.

Measurement of Hemostatic Factors

PAI-1 levels were measured using the Chromolize PAI-1 assay kit (DiaPharma Group, Inc., West Chester, Ohio), which measures active PAI-1. Assays were done according to the manufacturer's directions, and the standard line was linear out to 50 IU/mL with a minimal detectable concentration of roughly 2 IU/mL.

Factors VII:C, VIII, antithrombin III, and fibrinogen were all measured by modified clotting assays that are described separately below. The factor-deficient plasmas used in these assays were obtained as congenitally deficient plasmas from George King Biomedical, Inc. (Overland Park, Kansas). The clotting time for each assay was measured at 37°C in duplicate using a Coag-A-Mate XM (BioMérieux, Durham, North Carolina), and each sample was assayed 3 times on separate days. Clotting times were converted into concentrations by comparison to standard curves performed daily for each assay with pooled normal human plasma using at least 4 different plasma dilutions. All samples were diluted appropriately to obtain clotting times within the standard curve for each respective assay. The concentration obtained was then corrected for the dilution to obtain the actual level present in the sample. Typical dilutions required ranged from 1:10 to 1:75 (indicated). All dilutions were in Tris-buffered saline containing 0.1% bovine serum albumin used as a carrier.

Descriptions of Modified Clotting Assays
Factor VII:C

Diluted sample (typically 1:25) was added to an equal volume of factor VII-deficient plasma and allowed to warm for 1 minute. A bolus of 2 volumes of thromboplastin reagent (Pacific Hemostasis Thromboplastin-D; Fisher Diagnostics, Middletown, Virginia) was then added to initiate clotting. When plotted on a log-log scale, the standard curve was linear between 2 and 100 ng/mL factor VII (using a normal plasma concentration of 500 ng/mL).

Fibrinogen

Diluted sample (typically 1:10) was pre-warmed for 1 minute before the addition of 0.33 volumes of 50 U/mL topical bovine thrombin (Thrombin-JMI; Jones Pharma Inc., St. Louis, Missouri) to initiate clotting. When plotted on a log-log scale, the standard line was linear between 0.08 and 0.6 mg/mL fibrinogen (using a normal plasma concentration of 3 mg/mL).

Antithrombin III

Diluted sample (typically 1:75) was prewarmed for 1 minute before the addition of 1 U/mL thrombin (topical bovine thrombin). For this assay, the sample dilution buffer additionally contained 0.6% PEG 8000 and 1.5 U/mL heparin (Grade IA from porcine intestine; Sigma). After a 30-second incubation, 0.33 volumes of 8 mg/mL purified fibrinogen (from bovine plasma; Sigma) were added to initiate clotting. This assay measures functional antithrombin III and is based on the ability of the heparin-antithrombin III complex to proportionally inhibit thrombin that has been added in excess. When plotted on a log-log scale, the standard curve obtained was valid between 50 and 125 nM antithrombin III (using a normal plasma concentration of 5 microM).

Measurement of Inflammatory and Immune Signaling Factors

CRP levels were measured using an ELISA assay kit (Life Diagnostics, Inc., West Chester, Pennsylvania) according to the manufacturer's instructions. The minimum detectable concentration of CRP in this assay is 0.1 mg/L. Plasma concentrations of human TNF± and IL-6 were measured using ELISA assays (Cyto-Sets, Biosource, Camarillo, California) specific for each cytokine according to manufacturer's recommendations. The sensitivity of both assays is approximately 15 pg/mL. Plasma concentrations of MMP-9 were measured by ELISA assay. The specific methodology was that recommended by the manufacturer (Calbiochem, San Diego, California).

Measurement of Sex Steroids

The measurement of progesterone, DHEAS, E2, and testosterone involved the collection of 3 to 5 milliliters of saliva from the subjects at the designated intervals. The subjects were instructed to abstain from eating, drinking, brushing their teeth, and using facial and lip creams for at least 2 hours prior to the sample collection. After collection at each interval, the samples were shipped at room temperature to ZRT Labs in Beaverton, Oregon, for analysis. Tube contents were transferred to a 96-well grid plate according to predetermined locations specified by the computer-generated grid and the corresponding tube barcode. The tube contents underwent an extraction process via column chromatography to remove any mucins or impurities in order to minimize interference with the binding of the reagents. The quantifying chromogen/substrate was added and incubated for a specified time, followed by the arrestment of the reaction by adding the stop solution. Within 1 hour, the well contents were read using luminescence technology at 450 nm. Quality-control measures were as follows. Each assay grid (96 wells) contained one set of calibrators (two wells), two sets of controls (four wells), and one standard curve generator (eight wells). The resulting coefficient of variation was consistently less than 10% (intra-assay variability). The process was semi-automated, with robots controlling the pipetting of well contents; thus, the inter-assay variability was also consistently less than 10%.

Measurement of the Green Climacteric Scale

Permission was obtained for use of this standardized questionnaire which has been shown to have internal and external validity in the quantitative assessment of menopausal symptoms. The scale is a standardized 4-point ordinal scale of 21 questions based on factor analysis studies of perimenopausal and menopausal symptoms affecting quality of life. These factors included vasomotor, somatic, and psychological factors divided into moods of anxiety or depression. The Greene Climacteric Scale was developed to provide a standard measure of common climacteric symptoms experienced by the majority of menopausal women.[241] This scale is psychometrically sound and has high content validity and test-retest reliability.

Hamilton Anxiety Scale and Hamilton Depression Scale

The HAM-A and HDS scales are widely recommended for clinical use and are included as a routine part of a mood assessment for patients with dysphoria.[242-244] These instruments include questions related to somatic concerns, affect, outward expression of emotions, cognitive changes, reduced motivation, lack of future orientation, severity of mood changes, and reduced self-esteem. A HAM-A score 0 to 17 is considered to be mild, 18 to 25 mild-moderate, and 26 to 30 moderate to severe. According to FDA guidelines for anxiolytic therapy classification, a drug must show reduction of HAM-A scores to seven or less in clinical studies. For HDS scores, 0-7 is considered to be normal; 8-19 is mild depression, 20 or higher indicate moderately severe depression.

Holmes Rahe Stress Scale

The Holmes-Rahe Scale was used as measures of the research participants' endured experiences and events of long-term stress—experiences and events that had potentially contributed to the participants' current and imminent level of health.[245] The Holmes-Rahe Scale, which was developed by Thomas Holmes and Richard Rahe, is a social readjustment rating scale that assesses the level of stressful experiences and 43 events in one's life over the previous 12 months. By assigning a weighted value to each event (e.g., the death of a spouse is a greater stress-producing event than a household move, or change in sleeping habits), the Holmes-Rahe Scale produces an overall stress-experienced score. The higher the score, the greater the level of stress and the more likelihood that the individual will experience an adverse health change. A score greater than 300 predicts a high probability of adverse health change, and a score of 150-299 predicts a moderate risk of adverse health status (30% reduction from 300 plus group). A score less than 150 correlates with a slight risk of illness.

Work and Home Strain

Levels of strain at work and at home were measured using the University of North Carolina Alumni Heart Study (UNCAHS) Strain Scale, which assesses the amount of demands, control, and

decision latitude experienced in each environment This scale was developed for use in the UNCAHS.[246]

RESULTS

No adverse events or effects occurred during the 3-year study. Sex steroid hormone levels were monitored during treatment,

TABLE 1. Sex Steroid Levels.

SEX STEROID	BASELINE	36 MONTHS	P VALUE	GOAL REFERENCE RANGE
Progesterone	27.53(38)	1035(1240)	<0.005	200-3000
Estradiol pg/ml	1.9(1.5)	3.15(3.1)	<0.007	2-5
Pg/E ratio	21(20	400(498)	<0.005	200-1000
Testosterone pg/ml	26.14(14.6)	30.76(15.3)	0.1	20-40
DHEAS ng/ml	5.46(4.9)	4.57(2.79)	0.14	2-10

FIGURE 2. Hormone Regimen.

- Pg/BiEst
- Pg/BiEst/T
- Pg/BiEst/T/DHEA
- Pg/DHEA
- Pg/BiEst/DHEA
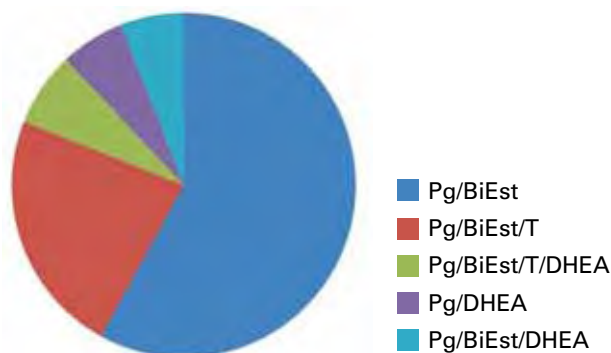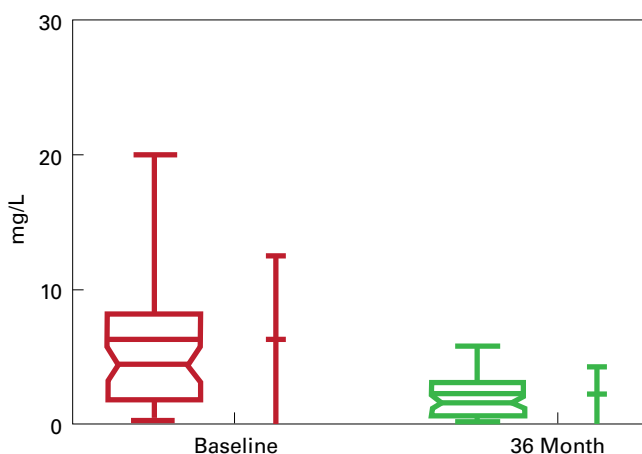
and results demonstrated subject compliance with transdermal application of the study drug (Table 1). Drug dosages were titrated to maintain physiological reference ranges and luteal phase estrogen/progesterone ratios. All study subjects required progesterone therapy, and over half received Progesterone/Bi-Est with less than 20% requiring additional androgen support (Figure 2).

Inflammatory and Immune Signaling Factors

There were statistically significant measurable changes in inflammatory and immune signaling factors with the application of transdermal hormone therapy. Baseline levels of CRP were 6.21 ($\pm$ 6.2) mg/L. Follow-up CRP level ranges were 2.12 ($\pm$ 2.2) mg/L for placebo and P value <0.005 (Figure 3). The large standard deviations in these group range numbers is a result of the wide variation between individuals and is not indicative of poor assay sensitivity.

Baseline levels of TNF-$\pm$ and IL-6 were 47.75 ($\pm$ 78.3) pg/mL and 1.07 ($\pm$ 3.7) pg/mL, respectively. Post-treatment levels of TNF-$\pm$ was 25.25 ($\pm$ 55.4) pg/mL with a P value of 0.08. For IL-6, the values was 0.19 ($\pm$ 0.38) pg/mL P value 0.06 (Figure 4).

MMP-9 baseline level was 81.5 ($\pm$ 48.8) pg/mL and follow-up level was 36.3 pg/mL ($\pm$ 2.9) P value <0.005 (Figure 5). IGF-1 baseline mean value was 170.96 ng/mL ($\pm$ 57.7) and follow-up level was 151.1 ng/mL ($\pm$ 40) P value 0.01 (Figure 6).

Hemostatic Factors

Factor VIII levels at baseline were 1.4 ($\pm$ 0.86) U/mL and 1.1 ($\pm$ 0.37) at follow up, P <0.024 (Figure 7).

Total factor VII:C levels at baseline were 1096 ($\pm$ 370.61) ng/mL and 659 ($\pm$ 158.17) ng/mL at follow up, P <0.001 (Figure 8).

Fibrinogen baseline level was 4.5 ($\pm$ 1.34) mg/mL, follow-up values were 4.2 ($\pm$ 1.08), P <0.026, showing a significant decrease (Figure 9).

Antithrombin III level at baseline was 340 ($\pm$ 40.36) ×/mL (compared to a mean normal value of 500 × M in normal plasma). Fol-

FIGURE 3. C-Reactive Protein p<0.005.
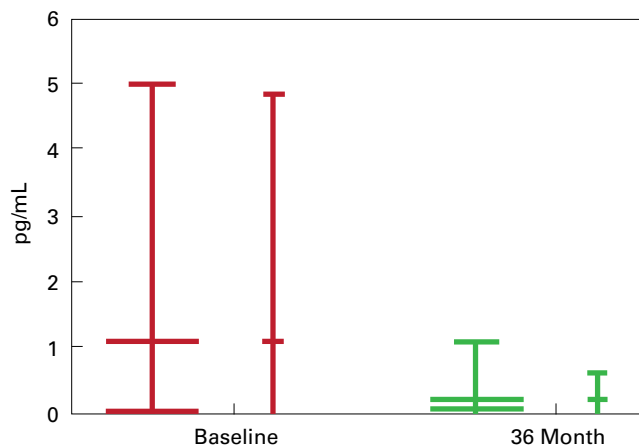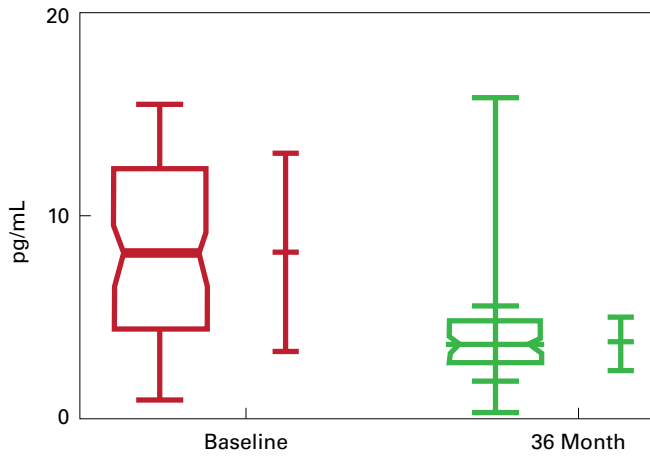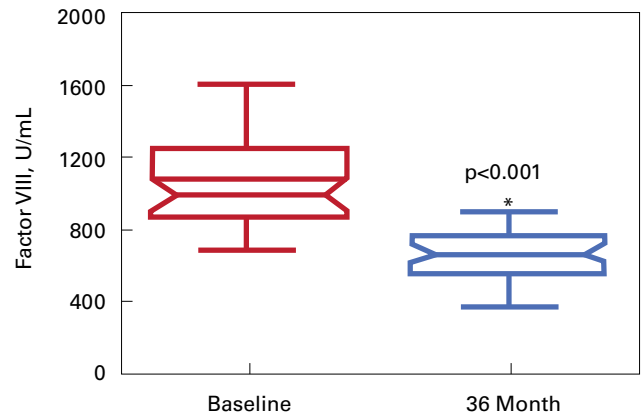

FIGURE 4. Interleukin-6 p=0.06.

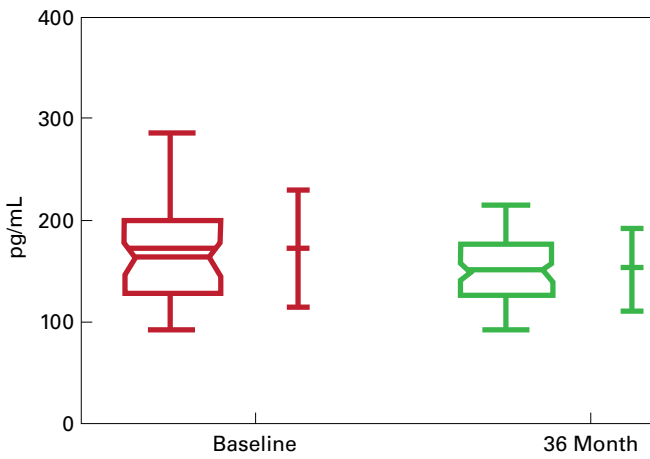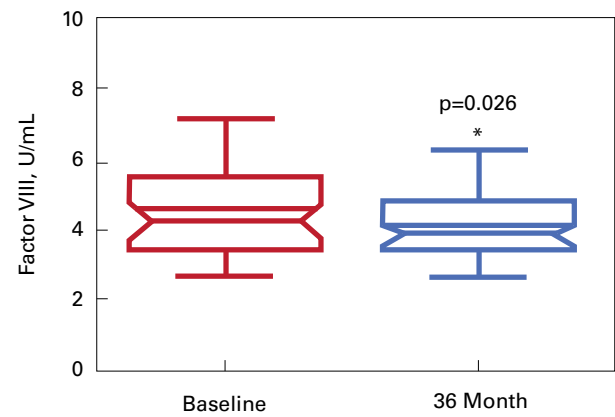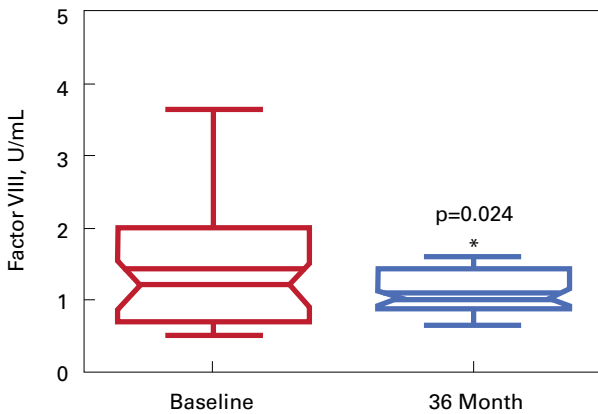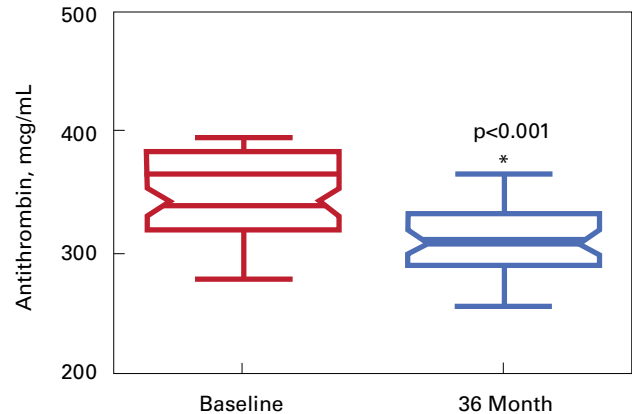**FIGURE 5. MMP9 p<0.005.**

FIGURE 6. IGF-1 p=0.01.

FIGURE 7.

FIGURE 8.

FIGURE 9.

FIGURE 10.

low-up levels of antithrombin were 310 (± 33.22) ×/mL, *P* <0.001 (Figure 10).

PAI-1 level at baseline was 9.6 (± 14.55) U/mL, and follow-up level was 9.2 (± 12.72) U/mL, which was not a significant decrease (Figure 11).

Quality-of-Life Measures

Statistically significant decreases in Greene Climacteric Scale scores were found. Baseline value was 17.45 (± 9.2) and follow-up values were 12.9 (± 6.71) with *P* <0.005 (Figure 12). HAM-A scores were reduced significantly with mean baseline score of 9.29 (± 5.1) consistent with mild anxiety, and a reduction to 6.31 (± 4.15) at follow up with *P* value <0.001. Significant decreases were observed in HDS scores with a baseline mean of 6.25 (± 4.9) and follow-up mean of 4.01 (± 3.29) with *P* <0.05. Work Strain scores were elevated at baseline and follow-up with means of 11.04 (± 6.63)

FIGURE 11.
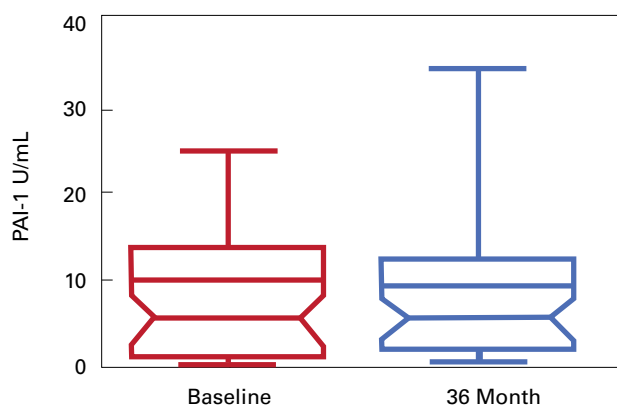
FIGURE 12. Green Climateric Scale *P* <0.005.
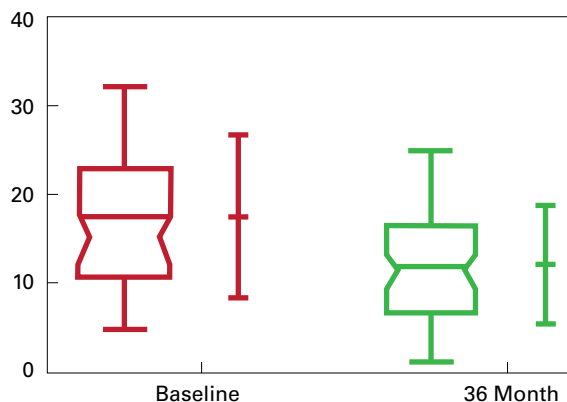
and 11.88 (± 5.3), respectively, *P* value=0.48. Home Strain scores remained elevated at follow up with a mean value of 11.23 (± 4.2) at baseline, and a follow-up mean of 10.8 (± 4.5), *P* value=0.68. Visual analog bodily pain scores were significantly decreased at follow up with a baseline mean of 1.41 (± 0.9) and a follow-up mean of 0.86 (± 0.7), *P* <0.001. Holmes-Rahe Stress Scale score mean at baseline was 472 (± 385), which correlated with a high risk of adverse health status over the following 12 to 24 months.

Relationship Between Menopausal Symptoms and Hemostatic and Inflammatory Factors

Spearman's non-parametric correlation was used to examine the relationship between Greene Climacteric Scale Scores and biomarkers of fibrinogen, TNF alpha, CRP, IL-6, and MMP-9 as recent studies have explored potential contributions of these factors to the etiology of vasomotor symptoms via effects on thermoregulatory mechanisms and endothelial communication. There were no significant correlations with these biomarkers at baseline and follow up.

Cardiometabolic Measures

Fasting Glucose decreased significantly with a baseline mean value of 107.2 (± 24.8) mg/mL and a follow-up mean value of 89.96 (± 16.8) *P* <0.001. Fasting Triglycerides also revealed a significant decrease with a baseline mean value of 163.88 (± 102.7) mg/dL and a follow-up value of 131.43 (± 105.1) *P* <0.03. No significant changes were noted for Systolic Blood Pressure (SBP) or Diastolic Blood Pressure (DBP) with baseline mean values of 129.5 (± 19.74) and 79.56 (± 9.8), respectively. Follow-up mean levels for SBP was 126.47 (± 14.75) and DBP was 76.92 (± 9.39) with *P* values of 0.38 and 0.16, respectively. HOMA IR decreased but was not significant from a baseline mean of 1.97 (± 2.45) to 1.43 (± 1.18) with a *P* value of 0.16. BMI did not change significantly with a baseline of 26.66 (± 4.1) and follow up of 26.49 (± 4.65) *P*=0.84. Dietary assessment was unchanged with a mean of 53.8 (± 7.9) at baseline and follow up of 55.2 (± 6.5) *P*=0.32.

Health Outcomes

Number of prescribed medications was 1.7 (± 0.1) at baseline and unchanged at 1.7 (± 0.2) at 36 months. Number of co-morbidities increased from 2.9 (± 0.2) at baseline to 3.2 (± 0.3) at follow up, but this was not statistically significant. For cardiometabolic and health-outcome measures, each study subject was age and ethnicity matched to a control subject receiving the usual model of care in women's health, including conventional HRT and statin therapy. Statistical analysis of group comparison of the hormone restoration model of care versus usual care showed statistically significant benefits in fasting blood glucose, quality-of-life measures, number of prescribed medications, and number of co-morbidities over three years for the hormone restoration model of care group. The control group had five adverse cardiovascular events

compared to no adverse events in the compounded bioidential transdermal therapy treatment group. Transvaginal ultrasound did not reveal any significant endometrial thickness in postmenopausal women with intact uteri undergoing transdermal hormone therapy with progesterone at a dose of 20 mg in concert with estrogen in continuous therapy at 36 months. Mean endometrial thickness was 3.3 mm (± 1.5). No episodes of postmenopausal vaginal bleeding or spotting occurred in study subjects.

Quality of Compounded Bioidentical Hormone Therapy

Throughout the 3-year study, random samples of compounded transdermal BHRT were obtained from study subjects after dispensing by the compounding pharmacist. Samples were analyzed by an independent testing laboratory (Eagle Analytical Laboratory, Texas) to determine quality, potency, and presence of contaminants. All samples met or exceeded *USP* quality standards as recognized by the FDA policies for compounded medications. There were no errors in compounded dosages, types of hormone therapy, nor any contaminants throughout the 3-year study.

CONCLUSION

Compounded transdermal BHRT may be considered as an important therapeutic option in peri/postmenopausal women due to its net beneficial effect on hemostatic and inflammatory factors, cardiovascular biomarkers, quality-of-life measures, and health outcomes. In contrast to medroxyprogesterone acetate and conjugated equine estrogens, this therapy and model of care is unlikely to increase the risk of thrombotic events or adverse events. These findings are consistent with animal models of menopause which demonstrate a protective effect of exogenous BHRT in pro-inflammatory states, immune dyregulation, and ischemic induced injury. Cytokines are increasingly recognized as significant contributors to immune system functions as well as some endocrine functions, and an imbalance of proinflammatory and anti-inflammatory cytokines has been associated with CVD and cancer risks. This study reveals beneficial effects of transdermal BHRT on these factors within the hormone restoration model of care. As supported by the SWAN study, decreases in estrogen sensitivity of the HPA axis and decreases in absolute progesterone levels occur as women age though the menopausal transition. Previous investigations have called for novel approaches to hormonal changes in the menopausal transition and menopause, as hormonal changes have been identified in both CVD and neuropsychiatric disease risks, both of which exhibit gender disparity in incidence and morbidity. The action of compounded transdermal BHRT on significantly improving quality of life measures, depression and anxiety in subjects supports the neuroactive role of sex steroid hormones. It is plausible that restoration of progesterone/estrogen luteal phase ratios in the brain and peripheral nervous system may induce a stress buffering effect despite high life stress events and high perceived stress as our

cohort exhibited significant elevations in home and work strain as well as signature of life stress compared to national norms.

This study was limited in that it was a small population based study, and the cohort may not be representative of a larger population of midlife women. Although potential confounders were assessed, genetic factors were unmeasured and residual confounding may exist. The study had several strengths in that it is the first study to examine the longitudinal association between BHRT combination therapy in a hormone restoration model of care and numerous inflammatory/hemostatic markers and immune signaling factors, in addition to health outcomes over time, including endometrial thickness. It allowed investigation of associations between BHRT and inflammatory/hemostatic markers within women transitioning thorough menopause rather than solely conduction of *between-woman* comparisons which are more vulnerable to confounding. The study also assessed a wide range of inflammatory/hemostatic markers across several time points, allowing the investigation of the consistency of association over time and across markers.

Finding therapies to alleviate menopausal symptoms without inducing prothrombotic and inflammatory potential and immune dysregulation is of utmost importance in addressing the needs of an estimated 1.2 billion menopausal women in the next two decades. Our study supports the efficacy of compounded transdermal BHRT for both symptom reduction and benefits on inflammatory, immune and cardiometabolic pathways without a net increase in thrombotic risks. Although it has been asserted that compounded transdermal BHRT should be thought of as inducing the same adverse health risks as medroxyprogesterone acetate and conjugated equine estrogens, our data does not support this with respect to the hemostatic, inflammatory, immune, and cardiovascular factors examined (Table 2). Compounded transdermal BHRT with targeted physiological reference ranges for peri/postmenopausal women warrants consideration as a safe and effective therapy for relief of menopausal symptoms, and it offers potentially protective effects on age- and stress-related dysregulation of hemostatic, inflammatory, and immune pathways.

Author Contributions

Dr. Stephenson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Dr. Stephenson contributed the concept and design of the study, obtained funding, and supervised the study. Each author contributed to the acquisition of the study data, the analysis and interpretation of the data, the drafting of the manuscript, the critical revision of the manuscript for important intellectual content, and for the administrative, technical, or material support.

Roles of the Sponsors

No sponsor had any role in the design and conduct of the study; in the collection, management, analysis, and interpretation of

TABLE 2. Effects of HRT and BHRT on Cardiovascular Biomarkers.

BIOMARKER	HRT	COMPOUNDED BIOIDENTICAL TRANSDERMAL HORMONE THERAPY
C-Reactive Protein	Unfavorable	Favorable
MMP-9	Unfavorable	Favorable
IL-6	Unfavorable	Favorable
Triglycerides	Unfavorable	Favorable
Fasting Glucose	Favorable	Favorable
Fibrinogen	Favorable	Favorable
PAI-1	Favorable	Favorable
Anti-thrombin III	Unfavorable	Unfavorable
Factor VIII	Unfavorable	Favorable
Factor VII	Unfavorable	Favorable
TNF alpha	Unfavorable	Favorable
IGF-1	Unfavorable	Favorable

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At the time this research was conducted, Dr. Stephenson was the medical director of the Women's Wellness Center and Associate Professor of Family Medicine at the University of Texas Health Center at Tyler, Tyler, Texas. Currently, she serves as Medical Director at Tejas Missions and Team-5 Foundations, and is a locums physician in New Zealand.

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data; or in the preparation, review, or approval of the manuscript. The authors assume full responsibility for the experimental design and the collection, analysis, and interpretation of the data.