Postmenopausal Hormone Therapy Increases Retinal Blood Flow and Protects the Retinal Nerve Fiber Layer

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Purpose. To investigate whether postmenopausal hormone therapy (HT) increases retinal and ONH blood flow (BF) and protects ONH topography and the function of retinal ganglion cells in postmenopausal women (PMW). The effect of estradiol (E_2) treatment on retinal tissue perfusion was also investigated in ovariectomized rats, an animal model for menopause.

Methods. Sixty-four healthy PMW were recruited, 29 of whom never used HT (∅HT) and 35 of whom had used HT (+HT) continuously since the onset of menopause. Blood flow of the inferotemporal retinal artery (ITRA), peripapillary retina, and ONH rim were measured in one eye. The ONH stereometric parameters and the pattern electroretinogram (PERG) were also measured. In ovariectomized rats, the retinal tissue perfusion was assessed using the BF tracer *N*-isopropyl-p-[^{14}C]-iodoamphetamine ([^{14}C]-IMP) in rats treated with either E_2 ($n = 7$) or placebo ($n = 5$).

Results. Compared with the ∅HT group, the +HT group presented significantly greater BF of the ITRA ($P = 0.006$), greater rim volume for the entire ONH region ($P = 0.032$), and greater rim volume ($P = 0.042$), height variation contour ($P = 0.011$), mean thickness ($P = 0.033$), and cross-sectional area ($P = 0.020$) of the retinal nerve fiber layer for the inferotemporal region of the ONH when adjusted for age, ocular perfusion pressure, and age at menarche. In ovariectomized rats, E_2 treatment significantly increased retinal perfusion in a range of 22% to 45%.

Conclusions. These findings indicate that estrogens and HT increase retinal blood flow and protect the retinal nerve fiber layer. (*Invest Ophthalmol Vis Sci.* 2010;51:2587–2600) DOI: 10.1167/iovs.09-3710

Menopause is the permanent cessation of menstruation resulting from the loss of ovarian follicular activity[1] that leads to a decline in endogenous estrogens and progesterone (P) production in aging women. Postmenopausal hormone therapy (HT), consisting of estrogens alone or combined with progestogens, is frequently prescribed to women to alleviate postmenopausal symptoms.

Estrogens have the ability to influence the vascular tone and blood flow in organs and tissues[2] and to have neuroprotective effects via nonvascular mechanisms.[3] In postmenopausal women, the use of HT has been shown to increase blood flow in the femoral artery[4] and brain parenchyma,[5,6] and to improve the pulsatility and/or resistivity indexes in the common carotid,[7] internal carotid,[8–10] and middle cerebral arteries.[9] The use of HT has also been shown to have a protective effect in some regions of the brain in postmenopausal women by preventing cerebral tissue atrophy.[11,12]

There is increasing evidence that impaired ocular blood flow is a contributing factor in the etiology and progression of glaucoma and age-related macular degeneration (ARMD).[13,14] Recently, there has been an increasing interest in investigating possible associations between endogenous estrogens and the use of HT with glaucoma and ARMD. Epidemiologic population–based studies have indicated that increased duration of exposure to endogenous estrogens (early menarche, late menopause onset, and increasing reproductive years) significantly decreases the odds ratio of developing primary open-angle glaucoma[15,16] and ARMD.[17] With regard to the use of HT, it has been shown to decrease the odds ratio of open-angle glaucoma by half, but the decrease did not reach statistical significance,[15] and no associations were found with intraocular pressure (IOP), cup/disc ratio, or the prevalence of increased IOP and glaucoma.[18] In ARMD, HT has been reported to significantly decrease the odds ratio of advanced[19] and neovascular ARMD,[20] whereas no associations were found between the use of HT or its duration and ARMD risk.[21] These somewhat divergent findings regarding the possible protective effect of HT against glaucoma and ARMD have also been reported in cardiovascular diseases.[22,23] The divergent findings in cardiovascular disease have been explained by the variable time of HT initiation after menopause. To be effective against cardiovascular disease, HT must be prescribed to menopausal women during the "*window of opportunity*" (i.e., in the first five to six

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years of menopause).[22,23] Nevertheless, a possible protective role of endogenous estrogens and HT against glaucoma and ARMD remains to be investigated in depth and may be the result of increased blood flow and a neuroprotective effect in the retina and optic nerve head (ONH).

Estrogen receptors (ERs) are present in the human retina,[24–26] as are progesterone receptors (PRs),[25] and postmenopausal women taking HT have been shown to present improved pulsatility and/or resistivity indexes in the retrobulbar vessels.[10,27–30] However, whether estrogens have a vascular effect on the retinal and ONH blood flow in postmenopausal women is unknown. In addition, as HT has a protective effect in some brain regions and ERs are present in the ganglion cell layer and axons in human retinas,[24,26] it is of interest to know whether estrogens have a protective effect on ONH topography and retinal ganglion cell (RGC) function.

In an attempt to answer these two questions, we designed an observational study investigating retinal and ONH blood flow, ONH topography, and the function of RGCs in healthy postmenopausal women who had been HT users (+HT) since menopause onset. Results were compared with those obtained from healthy postmenopausal women of similar age and clinical, gynecologic, and ophthalmic characteristics who had never used HT (∅HT) since menopause onset. The advantage of this approach over a prospective drug trial is that we could ascertain a potential effect of several years of estrogen therapy. It also would have been impossible to recruit patients for a prospective estrogen trial in the wake of the 2002 report of the Women's Health Initiative Randomized Controlled Trial,[31] when our study was in the planning stages. In a second study, we used ovariectomized (OVx) rats, a model for human menopause, to determine the pure contribution of estrogens to blood flow changes by exposing the rats to 17β-E_2 treatment and measuring the retinal tissue perfusion using the diffusible tracer *N*-isopropyl-p-[^{14}C]-iodoamphetamine ([^{14}C]-IMP).[32,33] Rats were chosen, as they are widely used in ovarian aging research with well-documented pathobiologic, cardiologic, and reproductive endpoints, and because ERs are also present in their retina.[25,34]

Methods

Observational Study

Subjects. This study was approved by the local Institutional Review Board (IRB) of Maisonneuve-Rosemont Hospital and adhered to the tenets of the Declaration of Helsinki. A signed informed consent was obtained from all subjects before enrollment in the study.

Eligibility Criteria. Healthy postmenopausal women were recruited from the CHUM-Notre-Dame and Maisonneuve-Rosemont Hospital Menopause Clinics (Montreal, QC, Canada) and by posting advertisements. To be eligible for the study, all prescreened healthy menopausal women had to meet the following inclusion criteria: 45 years of age or older, body mass index ≤30, a non- or former smoker, natural or surgically induced menopause (bilateral ovariectomy), and not HT (∅HT) or HT (+HT) users (as prescribed by a physician) since menopause onset. Natural menopause was determined retrospectively and diagnosed by 12 months of amenorrhea. For women who had menopause induced by bilateral ovariectomy, with or without hysterectomy, the time of the surgery was considered as the time of onset of menopause. Women who had undergone hysterectomy only and were identified as menopausal by documented elevated follicle-stimulating hormone [FSH] levels of >30 IU were also included. Menopausal women had to be free of systemic hypertension and cardiovascular and central nervous system diseases and had to have normal ocular histories and results in eye examinations. Subjects on vasoactive or anti-inflammatory medications and allergic to eye drops were excluded. The ∅HT postmenopausal women served as a control group.

Study Visit. Twelve hours before the study visit, the subjects were asked to avoid caffeine and alcohol so as not to alter the basal hemodynamic level. As well, they were asked to avoid food with high fat content, which alters the results of serum E_2 level assays. Also, so as to confirm the use of HT and rule out the effects of vasoactive drugs, the subjects were asked to bring all their medications, including any herbal medicine with them. The type of HT regimen (estrogens alone or combined with progestogens) and active ingredients were documented for HT users.

Medical, Gynecologic, and Ocular Histories and Eye Examination. At the time of the study visit, demographic, medical, gynecologic, and ocular histories were obtained from each subject by standardized questionnaire. Weight (in kilograms), height (in meters), and blood pressure (BP; mm Hg) at rest for 30 minutes were measured and used for calculating the body mass index (BMI = weight/height^2) and mean BP [($BP_{diastolic}$) + ⅓($BP_{systolic}$ − $BP_{diastolic}$)]. Gynecologic history included the following variables: age at menarche, age at last menses (natural or induced by ovariectomy), and duration of reproductive years (RY; age at last menses minus age at menarche). Menopause duration (age at study visit minus age at last menses) and HT duration (age at study visit minus age at initiation of HT) were calculated. For menopausal women who had hysterectomy only, the age at last menses, reproductive year duration, and menopause duration could not be determined and were consequently tabulated as missing data.

Ocular histories and eye examinations were obtained that included best corrected Snellen visual acuity expressed as its numerator divided by its denominator, refraction (spherical equivalent), and biometry (OcuScan ver. 3.02; Alcon Surgical, Inc., Ft. Worth, TX). Slit-lamp examination, evaluation of peripheral anterior chamber depth, Goldmann tonometry (IOP mm Hg), and direct ophthalmoscopy were performed. Ocular perfusion pressure (OPP mm Hg) was calculated with the following formula: OPP = ⅔(BP_{mean} − IOP).[35]

Serum E_2 and P Assays. A blood sample was obtained from all subjects and analyzed for documenting total serum E_2 (the most biologically active endogenous estrogen[1]) and P concentrations by chemiluminescent immunoassay technique (ADVIA Centaur; Siemens Health-Care Diagnostic, Tarrytown, NY). Some active ingredients present in the HT regimens and used by postmenopausal women were not 17β-E_2- and/or P-based. Therefore, measures of the serum E_2 and P concentrations not detected by the assay technique were tabulated as 0. As well, in some postmenopausal women who were not HT users, the endogenous serum 17β-E_2 and P concentrations were below the level of detection by the assay technique. Consequently, their serum concentrations were also tabulated as 0.

Selection of the Investigated Eye. All retinal and ONH blood flow (BF) measurements, ONH topography, and PERG measurements were conducted in one eye (i.e., the eye with the clearest media). If poor fixation or lid ptosis was present, the fellow eye with good media clarity was chosen. When both eyes were eligible for the study, one eye was randomly chosen. For the BF and ONH topography measurements, the eye was dilated with tropicamide (mydriacyl 1%; Alcon Canada, Inc., Mississauga, ON, Canada).

Evaluation of the Inferotemporal Retinal Artery BF. BF of the inferotemporal retinal artery (ITRA) was measured with a laser blood flowmeter system[36,37] (CLBF; model 100, software ver. 2.1.23; Canon, Tokyo, Japan) that measures simultaneously the blood column velocity (vel_{mean}) by bidirectional laser Doppler velocimetry[38,39] and the blood column diameter (D),[40] thus allowing an automated calculation of flow according to Poiseuille's law. The D measurement is corrected for the axial length of the eye (operator input) and refractive error of the eye, which is measured by the CLBF itself. There are two motives for choosing the ITRA. First, it is a region that is technically accessible for obtaining CLBF measurements, and second, the inferotemporal region is the most common site for early onset of focal atrophy of the neural rim in early glaucoma damage.[41] The location of the measurement on the ITRA was performed on a straight section of

the vessel before the first bifurcation between 0.5 and 2.0 disc diameters from the edge of the optic disc. Ten consecutive flow measurements were obtained. After excluding incomplete flow measures and discarding the highest and lowest flow values, we used the three closest flow values for averaging D, $vel_{mean,}$ and flow. This recording protocol gave us an intrasession coefficient of variation of 3.2% ± 1.9% for D, 6.7% ± 3.2% for vel_{mean}, and 7.1% ± 4.4% for flow obtained from the ITRA in 19 normal women (Deschênes MC, et al., unpublished data, 2003).

Evaluation of the ONH and Peripapillary Retinal BF. The capillary BF of the ONH rim and both nasal and temporal peripapillary retinal areas were measured by scanning laser Doppler flowmetry (SLDF; Heidelberg Retinal Flowmeter [HRF] v1.02; Heidelberg Engineering, Heidelberg, Germany). Briefly, the HRF combines a confocal laser scanning technique and laser Doppler flowmetry[42] and generates a perfusion image (10° wide × 2.5° height) in which flow (distance traveled by all moving red blood cells per unit of time), volume (number of moving blood cells), and velocity (mean of blood cell speed) is computed for each pixel by the automatic full-field perfusion image analysis software (AFFPIA, ver. 3.3)[43] and expressed in arbitrary units (AU).

Our HRF images were centered on the ONH, optimizing the photodetector sensitivity to obtain a strong signal from both nasal and temporal peripapillary retinas, while avoiding overexposure. Ten SLDF images were obtained from each subject, and the five best images in terms of focusing, ONH centration, optimum exposure, brightness, and lack of major eye saccades were kept for AFFPIA. The obtained flow, velocity, and volume from these five images for the nasal, temporal, and rim areas were then averaged. This SLDF measurement protocol, developed in our laboratory, yields intrasession coefficients of reliability ranging from 0.93 to 0.95, and coefficients of variation ranging from 11.4% to 16.4% for the flow among the three regions of interest.[44]

ONH Topography. The ONH topography was evaluated by measuring the 12 standard stereometric parameters (i.e., disc area, cup area, cup-to-disc [c/d] ratio, rim area, cup volume, rim volume, mean cup depth, maximum cup depth, cup shape measure, height variation contour, mean retinal nerve fiber layer [RNFL] thickness and the RNFL cross-sectional area) by confocal laser scanning ophthalmoscopy (Heidelberg Retina Tomograph I, ver. 2.01; [HRT I], Heidelberg Engineering). Between 5 and 10 ONH images were acquired, and the average of the three closest images (chosen in an unmasked fashion based on standard protocols[45]) in terms of alignment, brightness and clarity was obtained. The stereometric parameters were obtained for the entire ONH (0°–360°) and for the inferotemporal region (270°–360°), which is the region most commonly affected by early glaucomatous damage.

Functionality of the RGC. The pattern electroretinogram (PERG) represents an objective and direct measure of retinal ganglion cell function.[46] The PERG was recorded (VERIS Multifocal System, ver. 5.0; Electro-Diagnostic Imaging, Inc., Redwood, CA) according to the guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV).[46] Transient (4.7 reversal/s [rps]) PERGs (bandwidth, 1–100 Hz) were evoked by a black-and-white reversing checkerboard stimuli of 50° × 38° with a check size of 0.8° generated by a 9-in. monochrome monitor (75-Hz frame rate). The luminance of the white squares was set at 353 cd/m^2 and that of the black squares at 30 cd/m^2 (of 192 cd/m^2 average luminance, 84% contrast). A DTL electrode (DTL Plus; Diagnosys LLC, Littleton, MA) placed in the lower conjunctival sac served as the active electrode in reference to a gold cup electrode (Grass Technologies, West Warwick, RI) placed at the ipsilateral outer canthus. A separate gold cup electrode was placed on the subject's forehead and grounded. Electrode impedances were checked for 5-kΩ or less. Two replicable PERG waveforms were obtained. The N35, P50, and N90 waveform components were identified, and latencies (N35 lat, P50 lat, and N95 lat) and amplitudes (P50 Amp, N95 Amp) were measured.

OVx Rat Study

Animals. All experimental methods and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Montreal Animal Care Committee in accordance with the Canadian Council on Animal Care. Twelve middle-aged (11-month-old), retired, female breeder Brown Norway (*Rattus norvegicus*, 225.5 ± 7.9 g) rats (Harlan Sprague–Dawley, Horst, The Netherlands) were used in the study. This age in rats corresponds approximately to the time when 65% of female rats present irregular estrous cycles, comparable to the perimenopausal period in women. Rats were housed individually and placed in a room at 23°C with a 12-hour light/dark adapted photoperiod, with food and water provided ad libitum.

Ovariectomy and E_2 Treatment. Bilateral ovariectomies (OVx) were performed in rats under isoflurane (3.0%), to normalize serum estrogen concentrations to the lowest level. Silastic capsules (ID 0.058, OD 0.077; 0.5 cm in length, 508-006; Dow Corning, Midland, MI) filled with a crystalline preparation of either 25% E_2 (Sigma-Aldrich, St. Louis, MO) plus 75% cholesterol (OVx + E_2, $n = 7$, ~2 mg E_2 per capsule) or 100% cholesterol (placebo; Sigma-Aldrich, St. Louis, MO) (OVx, $n = 5$) were implanted in the nape of the neck. These capsules have been validated as a means of delivering steady state amount of E_2 in mature OVx rats.[47] After the ovariectomy, 5 mL of Ringer's lactate was injected SC for rehydration, and repeated SC doses of 0.1 mL of buprenorphine hydrochloride (0.1 mg/mL; Temgesic; Reckitt Benckiser Health Science, Ltd., Slough, UK) were injected every 12 hours over a 2-day period for postoperative pain management. The rats were exposed to E_2 or placebo treatment for a period of 6 weeks, which was chosen to establish the groundwork in a future study.

Autoradiographic Tissue Perfusion Investigation. The retinal blood perfusion was assessed by using the quantitative autoradiography after the injection of [^{14}C]-IMP, a diffusible BF tracer. [^{14}C]-IMP binds to the amine sites[48] within the tissue, which avoids further postmortem diffusion.[32,49] Six weeks after OVx surgery and E_2 or placebo treatment, polyurethane catheters (ID 0.6 mm, OD 0.9 mm; Harvard Apparatus, Holliston, MA) were inserted into the femoral vein and artery under 1.5% isoflurane (induction of anesthesia with 3% isoflurane for 5 minutes). At the end of this procedure, 2 mL of Ringer's lactate were given SC for rehydration. Rats were then installed in a hammock and left under minimal restraint over a 2-hour period to recover from anesthesia. Body temperature was maintained at 37°C with a heat lamp, and both blood pressure and heart rate were monitored from the tail with a noninvasive blood pressure cuff system (BP1000; Kent Scientific Corp., Torrington, CT), until the initiation of measurement experiment for retinal perfusion. As well, blood pH and gases were measured with a veterinarian clinical blood gases and electrolytes analyzer (i-STAT; Heska, Fort Collins, CO), from arterial blood samples collected via the arterial catheter, to confirm the recovery to normal physiologic level before the initiation of the experiment measuring retinal perfusion.

The [^{14}C]-IMP (100 μCi/kg; ARC, St. Louis, MO) was dissolved in 600 μL of saline (injectable 0.9% NaCl solution) and infused in fully conscious rats over a 30-second period at a constant rate of 1.2 mL/min using an infusion pump (PHD 2000; Harvard Apparatus) through the femoral vein. At the end of the infusion, the rats were immediately killed by decapitation, and a blood sample was collected for serum E_2 assays. Both eyes were immediately harvested and immersed in a solution of 4% paraformaldehyde. Only the left eye was used for retinal dissection. Two hours later, the anterior segment of the left eye was excised to ease the penetration of the paraformaldehyde toward the posterior segment. On the following day, the retina was removed and wholemounted on a glass slide with the ganglion cell layer facing up. The wholemount retina was then exposed to x-ray film (Biomax; Kodak-Eastman Inc., Rochester, NY) for 10 days with a set of [^{14}C] standards (GE Healthcare, Ltd., Little Chalfont, UK). The autoradiograms were analyzed by the computerized image analysis (MCID Basic Software, ver. 7.0; Interfocus Imaging, Linton, UK).

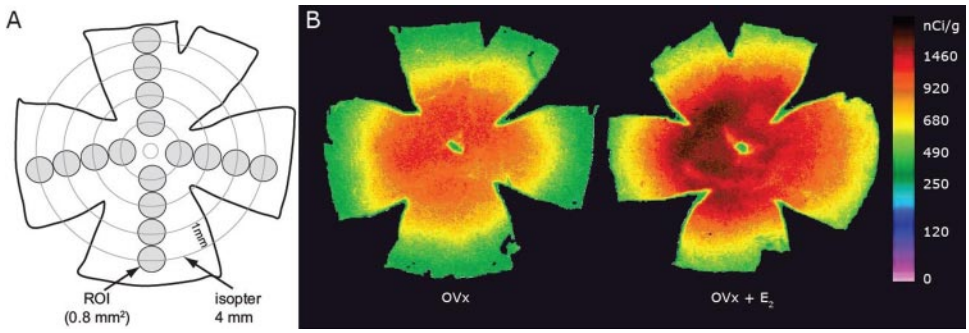

FIGURE 1. (**A**) A wholemount retina, showing the regions of interest (ROI) measured. The retina was divided into four quadrants (the notch indicates the superior quadrant). A circular ROI (*shaded circles*, area 0.8 mm^2) was measured in each quadrant at each isopter (*light concentric lines* at 1, 2, 3, and 4 mm away from the center of ONH). The four values of $C_{IMP}(T)$ in each quadrant were averaged for each isopter. Representative wholemount retina autoradiograms (**B**) displayed in pseudocolor from an OVx rat treated with placebo silastic capsules and an OVx+E_2 rat treated with E_2 silastic capsules. The tissue concentration of [^{14}C]-IMP was higher in the OVx+E_2 rat, indicating that retinal blood perfusion was greater in E_2-treated animals than in placebo-treated ones. *Right*: correspondence between the pseudocolor scale and $C_{IMP}(T)$ values.

Calculation of Retinal Tissue Perfusion. Retinal tissue perfusion was evaluated by using the principle of indicator-fractionation technique.[33,49] The retinal uptake index (RUI) was calculated with the following equation[32]:

$$\text{RUI} = C_{IMP}(T)/A \times \text{BW}$$

where A is the injected dose (nCi), BW is the body weight (g) and $C_{IMP}(T)$ is the radioactivity measured on the retinal autoradiogram at the time (T) of death (nCi/g). $C_{IMP}(T)$ was read from circular regions of interest of 0.8 mm^2 (1-mm diameter) distributed at the 1-, 2-, 3-, and 4-mm isopters away from the center of the ONH in all retinal quadrants (Fig. 1A). Although the uptake index of the [^{14}C]-IMP was measured in wholemount retina free of the choroid, it cannot be excluded that some [^{14}C]-IMP may also have partly diffused from the choroid before retinal dissection. This possibility remains to be investigated.

Rat Serum Assays. The total serum E_2 levels were measured with a third-generation E_2 RIA kit (Diagnostic Systems Laboratories, Webster, TX).

Statistical Analysis

Unpaired Student's two-tailed t-tests were performed for clinical, gynecologic, and ophthalmic variables between ∅HT and +HT postmenopausal women. Linear regression analysis was performed for the outcome variables for the BF, ONH stereometric parameters and PERG between ∅HT and +HT postmenopausal women using unadjusted and adjusted models. The adjusted model was set to control for potential confounding or predicting age and OPP effects on the outcome variables, as well as to control for age at menarche, which was found to be associated with the risk of OAG.[16] Age at last menses, reproduction year, and menopause durations were also found to be associated with the risk of OAG and ARMD, but since they were unknown in postmenopausal women who had undergone hysterectomy only, they were not included in this adjusted model. Consequently, a secondary analysis was performed in postmenopausal women with known menopause duration and the results were added to the adjusted model. Menopause duration was chosen to be included in this adjusted model instead of the age at last menses and duration of reproductive year, as menopause duration is closely linked to the HT duration and its effectiveness when initiated in the "*window of opportunity*".[22,23] As postmenopausal women on HT were either on estrogens alone or combined with progestogens, subsequent linear regression analysis was performed to compare the effect of the type of HT treatment (estrogen therapy versus ∅HT, estrogen+progestogen therapy versus ∅HT, and estrogen versus estrogen+progestogen therapies) to isolate the effects of estrogen therapy only. In OVx rats, the RUIs measured in each quadrant were averaged for each isopter. Nonparametric Mann–Whitney U tests were performed between the OVx group and OVx+E_2 group for the physiological parameters, the E_2 plasma concentrations, and the RUI of each isopter (STATA/IC software; StataCorp LP, ver. 10.0; College Station, TX). A significance level of $\alpha = 0.05$ was chosen.

RESULTS

Observational Study

Study Population Characteristics. A total of 64 subjects (mean age, 56.7 ± 5.2 years; age range, 46.3–70.6) met the study criteria, among whom 29 (44.6%) had never used HT since menopause onset and 35 (55.4%) were using HT currently and continuously since menopause onset. Among ∅HT and +HT women, one and two of them, respectively, had undergone OVx combined with hysterectomy, whereas 1 and 10 of them, respectively, had undergone hysterectomy only.

Clinical, gynecologic, and ophthalmic characteristics for the whole study population are given in Table 1. The duration of HT was 8.3 ± 6.1 years for the +HT group of postmenopausal women. As expected, the only significant overall differences between the ∅HT and the +HT subjects were the serum E_2 concentration (62.2 ± 60.9 pM vs. 260.5 ± 161.0 pM, $P < 0.001$) and serum P concentration (1.9 ± 0.9 nM vs. 12.1 ± 20.3 nM, $P = 0.010$). E_2 and P assays may have measured some E_2 and P synthesized from nonovarian and nonplacental sites, including the brain,[50,51] which may have spilled out into the blood stream. The large SDs for both E_2 and P concentration are most likely due to a variable endogenous source of these hormones that differs between women and with menopause duration. Moreover, since active ingredients present in the HT regimens used by some postmenopausal women were not 17β-E_2- and/or P-based, this could also have contributed to the large SD.

Regarding their clinical, ophthalmic, and other gynecologic characteristics, the two groups of women were not significantly different, in their age, BMI, BP, and menopause duration. For postmenopausal women who were naturally or surgically menopaused, the sample size used for calculating the average age at last menses, year of natural menopause, and reproductive year durations are indicated between parentheses below each value (Table 1).

In the same table, the whole study population is presented in subsets of subpopulations A, B, C, and D, which were investigated for the CLBF, SLDF, retinal tomography (Heidelberg Retinal Tomograph; Heidelberg Engineering), and PERG testing, respectively. Data from some of these tests were either not obtained or were discarded for the following reasons: some subjects presented fixation problems, inability to open the eye wide, and unreliable data measurements for some of the tests conducted. For CLBF testing, data from 14 (21.9%) subjects were discarded because the subjects presented either more than one main inferotemporal retinal artery supplying the in-

Table 1. Clinical, Gynecologic, and Ophthalmologic Characteristics for the Whole Population Study of Women Who Never Used Postmenopausal Hormone Therapy (∅HT) and Who Were HT Users (+HT) and for the Subpopulation Study for CLBF (A), SLDF (B), HRT (C), and PERG (D) Assessments

	Whole Population		Subpopulation							
			A		B		C		D	
Characteristics	∅HT (n = 29)	+HT (n = 35)	∅HT (n = 18)	+HT (n = 21)	∅HT (n = 16)	+HT (n = 27)	∅HT (n = 27)	+HT (n = 32)	∅HT (n = 21)	+HT (n = 30)
Clinical										
Age, y	56.7 ± 5.8	56.6 ± 4.8	57.2 ± 5.9	56.4 ± 4.3	56.6 ± 4.7	56.5 ± 4.9	56.6 ± 5.7	56.6 ± 4.9	56.2 ± 5.4	56.0 ± 4.3
BMI	26.5 ± 3.5	25.4 ± 3.4	27.0 ± 3.7	25.9 ± 3.6	27.5 ± 3.8	25.6 ± 3.4	26.8 ± 3.5	25.4 ± 3.4	26.7 ± 3.9	25.9 ± 3.2
Blood pressure										
Systolic, mm Hg	124.9 ± 12.4	119.9 ± 15.5	123.8 ± 13.1	120.0 ± 15.7	127.1 ± 11.9	119.7 ± 16.4	124.6 ± 13.8	119.1 ± 15.6	125.2 ± 12.5	119.0 ± 15.1
Diastolic, mm Hg	76.7 ± 8.9	73.1 ± 8.1	76.4 ± 10.1	73.6 ± 9.3	79.6 ± 9.0	73.0 ± 8.8*	77.3 ± 8.2	73.3 ± 8.4	76.9 ± 8.0	73.2 ± 8.1
Mean, mm Hg	92.6 ± 9.1	88.6 ± 9.4	92.1 ± 10.3	88.9 ± 10.5	95.3 ± 8.9	88.4 ± 10.1*	92.7 ± 9.7	88.4 ± 9.6	92.8 ± 8.4	88.3 ± 9.4
Pulse amplitude, mm Hg	48.2 ± 9.5	46.7 ± 12.4	47.3 ± 9.1	46.4 ± 11.5	47.5 ± 10.1	46.7 ± 13.4	47.6 ± 10.0	45.8 ± 12.6	48.4 ± 10.4	45.8 ± 11.9
Gynecologic										
Age at menarche, y	12.3 ± 1.3	12.6 ± 1.6	12.3 ± 1.5	12.3 ± 1.7	12.2 ± 1.5	12.8 ± 1.7	12.2 ± 1.4	12.7 ± 1.6	12.3 ± 1.4	12.6 ± 1.6
Age at last menses, y (n)	49.3 ± 4.0 (28)	47.8 ± 5.9 (25)	49.4 ± 4.7 (18)	46.9 ± 6.8 (14)	49.9 ± 4.5 (16)	47.7 ± 5.7 (18)	49.7 ± 3.7 (26)	48.0 ± 5.8 (23)	49.1 ± 3.7 (20)	47.1 ± 6.1 (21)
Duration of RY, y (n)	37.0 ± 4.3 (28)	34.9 ± 6.4 (25)	37.1 ± 4.8 (18)	34.2 ± 7.7 (14)	37.7 ± 4.8 (16)	34.5 ± 6.6 (18)	37.5 ± 3.9 (26)	35.0 ± 6.3 (23)	36.8 ± 3.9 (20)	34.2 ± 6.8 (21)
Duration of menopause, y (n)	7.6 ± 6.4 (28)	8.7 ± 6.3 (25)	7.8 ± 7.1 (18)	9.5 ± 7.4 (14)	6.8 ± 5.1 (16)	8.8 ± 7.2 (18)	7.0 ± 5.6 (26)	8.4 ± 6.5 (23)	7.3 ± 5.4 (20)	8.5 ± 6.6 (21)
HT duration, y	0.0 ± 0.0	8.3 ± 6.1†	0.0 ± 0.0	8.5 ± 6.7†	0.0 ± 0.0	7.9 ± 6.7†	0.0 ± 0.0	8.3 ± 6.2†	0.0 ± 0.0	8.1 ± 6.0†
Serum E_2, pmol/L	62.2 ± 60.9	260.5 ± 161.0†	70.1 ± 63.6	252.1 ± 173.8†	61.8 ± 65.9	253.4 ± 154.1†	62.7 ± 64.2	244.9 ± 162.0†	57.4 ± 51.5	264.6 ± 162.7†
Serum P, nmol/L	1.9 ± 0.9	12.1 ± 20.3†	1.8 ± 0.7	11.7 ± 18.1*	1.6 ± 0.8	11.4 ± 19.1*	1.9 ± 1.0	14.2 ± 22.4†	1.9 ± 1.0	10.5 ± 17.1*
Ophthalmic										
VA (ratio)	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.2
Spherical equivalent	0.0 ± 2.9	−0.9 ± 2.7	0.3 ± 3.4	−0.9 ± 2.5	−0.3 ± 2.9	−1.0 ± 2.6	−0.2 ± 2.8	−0.8 ± 2.8	−0.1 ± 2.8	−0.8 ± 2.7
IOP, mm Hg	14.5 ± 1.7	14.1 ± 2.2	14.1 ± 1.6	13.8 ± 2.0	14.6 ± 1.2	14.6 ± 2.2	14.6 ± 1.6	14.3 ± 2.3	14.6 ± 1.4	14.1 ± 2.1
OPP, mm Hg	47.3 ± 6.3	44.9 ± 6.7	47.3 ± 7.0	45.5 ± 7.1	49.0 ± 5.9	44.4 ± 6.9*	47.2 ± 6.3	44.7 ± 7.0	47.3 ± 5.9	44.8 ± 6.7

Values are the mean ± SD. Number of subjects is in parentheses. HT, hormone therapy; BMI, body mass index; RY, reproductive year; E_2, estradiol; P, progesterone; VA, visual acuity; IOP, intraocular pressure; OPP, Ocular prefusion pressure.

* $P \leq 0.05$.

† $P \leq 0.01$, significance level determined by unpaired Student t-test between the +HT and ∅HT for the whole and the subpopulations.

Table 2. Frequency Distribution of +HT (n = 35) Categorized by Their Active Ingredients

Therapy Type	Active Ingredients	n (%)
Monotherapy	17β-Estradiol	10 (28.6)
	17β-Estradiol micronized	1 (2.9)
	Conjugated estrogens	1 (2.9)
Combined therapy	17β-Estradiol+progesterone micronized	1 (2.9)
	17β-Estradiol+levonorgestrel	2 (5.7)
	17β-Estradiol+norethindrone acetate	2 (5.7)
	17β-Estradiol micronized+progesterone micronized	1 (2.9)
	17β-Estradiol micronized+medroxyprogesterone	1 (2.9)
	17β-Estradiol micronized+norethindrone acetate	1 (2.9)
	17β-Estradiol hemihydrate+progesterone micronized	5 (14.3)
	17β-Estradiol hemihydrate+medroxyprogesterone	1 (2.9)
	Estropipate+progesterone micronized	2 (5.7)
	Ethinyl estradiol+norethindrone acetate	3 (8.6)
	Conjugated estrogens+progesterone micronized	1 (2.9)
	Conjugated estrogens +medroxyprogesterone	3 (8.6)
	Total	35 (100.0)

ferotemporal retinal quadrant or the main inferotemporal retinal artery divided itself into second-order arterioles near the outer edge of the ONH before the site of measurement. Regarding the ∅HT and +HT groups for all subpopulations, they were also similar in all their characteristics except for serum E_2 and P levels. In subpopulation B, the diastolic BP (79.6 ± 9.0 mm Hg vs. 73.0 ± 8.8 mm Hg; P = 0.03), the mean BP (95.3 ± 8.9 mm Hg vs. 88.4 ± 10.1 mm Hg; P = 0.03), and the OPP (49.0 ± 5.9 mm Hg vs. 44.4 ± 6.9 mm Hg; P = 0.03) were significantly higher in the ∅HT group.

In the postmenopausal population, the HT regimens were heterogeneous in their active ingredients, routes of administration, and dosage. Table 2 indicates the frequency distribution of the active estrogen and progestogen ingredients present in the various HT regimens prescribed and used by postmenopausal women. In our whole study population of 64 subjects, 35 (54.7%) of them were +HT users, including 12 (18.8%) who were on monotherapy (estrogens alone) and 23 (35.9%) who were on combined therapy (estrogens+progestogens). Of the +HT users 25 (39.1%) were taking the active ingredient 17β-E_2, which is one of the human bioidentical ingredients.

Flow of Inferotemporal Retinal Artery. The diameter, vel_{mean}, and flow of the ITRA were obtained from 18 ∅HT and 21 +HT postmenopausal women (Table 1, subpopulation A). Table 3 shows diameter, vel_{mean}, and flow data. The flow was significantly greater in the +HT group (P = 0.005), and this difference was primarily due to a significantly greater diameter (P = 0.006) of the blood column when adjusted for age, perfusion pressure, and age at menarche. Age at menarche was found to be a significant predictor of diameter and flow (P = 0.045 and P = 0.044, respectively). Similar findings were observed in a subset group of women excluding the ones who had a hysterectomy only. Diameter (P = 0.015) and flow (P = 0.029) were also significantly greater in 14 +HT postmenopausal women than in 18 ∅HT, when adjusted for age, perfusion pressure, age at menarche, and menopause duration. Age at menarche was a significant predictor of BF (P = 0.046).

ONH and Peripapillary Retinal BF. Table 4 represents the capillary blood volume, velocity, and flow measurements for the rim of the ONH and both temporal and nasal peripapillary retinas obtained from 16 ∅HT and 27 +HT postmenopausal women (Table 1, subpopulation B). The volume and flow were significantly greater (P = 0.029 and 0.042, respectively) in the temporal retina in the +HT group in the unadjusted model, but these differences were no longer significant in the linear regression model when age, ocular perfusion pressure, and age at menarche were accounted for. Ocular perfusion pressure was found to be a significant predictor (P = 0.049) of temporal volume, and both age and ocular perfusion pressure were significant predictors (P = 0.014 and P = 0.022, respectively) of temporal flow. Similar findings were observed in a subset group of women, excluding the ones who had hysterectomy only. Volume (P = 0.019), velocity (P = 0.038), and flow (P = 0.016) were also significantly greater in 18 +HT postmenopausal women than in 16 ∅HT in the unadjusted model. Again, these differences were no longer significant in the linear regression model when age, ocular perfusion pressure, age at menarche, and menopause duration were accounted for, in which only ocular perfusion pressure was a significant predictor of temporal volume (P = 0.041) and flow (P = 0.040).

ONH Topography. Significant findings of the stereometric parameters of ONH topography obtained for both the entire (0°–360°) and the inferotemporal (270°–360°) regions from 27 ∅HT and 32 +HT subjects (Table 1, subpopulation C) are

Table 3. Diameter, Vel_{mean}, and Flow of the Inferotemporal Retinal Artery Measured in ∅HT and +HT Postmenopausal Women

	Mean		Linear Regression Model			
			Unadjusted*		Adjusted†‡	
	∅HT	+HT	D (SE)	P	D (SE)	P
39 Subjects, n	18	21				
Diameter, μm	120.7	133.2	12.5 (4.1)	0.005*	12.3 (4.1)	0.005†
Vel_{mean}, mm/s	36.4	37.0	0.6 (2.2)	0.803	0.9 (2.2)	0.687
Flow, μL/min	12.5	15.4	2.9 (1.0)	0.008*	3.0 (1.0)	0.006†
32 Subjects, n	18	14				
Diameter, μm	120.7	129.9	9.2 (4.1)	0.033*	11.0 (4.1)	0.015‡
Vel_{mean}, mm/s	36.4	36.7	0.3 (2.5)	0.908	0.2 (2.5)	0.936
Flow, μL/min	12.5	14.5	2.0 (0.9)	0.045*	2.2 (1.0)	0.029‡

D, difference between the means.

* Significance level determined by unadjusted linear regression model.

† Significance level determined by adjusted linear regression for age, ocular perfusion pressure, and age at menarche.

‡ Significance level determined by adjusted linear regression for age, ocular perfusion pressure, age at menarche, and menopause duration.

TABLE 4. Volume, Velocity and Flow of the Temporal Peripapillary Retinal, Neuroretinal Rim and Nasal Peripapillary Retinal Areas of the Optic Nerve Head Measured in ∅HT and +HT Postmenopausal Women

	Mean		Linear Regression Model			
			Unadjusted*		Adjusted†‡	
	∅HT	+HT	D (SE)	*P*	D (SE)	*P*
43 Subjects, *n*	16	27				
Volume, AU						
Temporal	19.6	22.3	2.7 (1.2)	0.029*	1.9 (1.2)	0.120
Rim	17.8	18.7	0.9 (1.1)	0.413	0.8 (1.2)	0.509
Nasal	19.1	19.2	0.1 (1.0)	0.947	0.2 (1.1)	0.817
Velocity, AU						
Temporal	0.98	1.11	0.13 (0.08)	0.093	0.08 (0.08)	0.333
Rim	0.99	1.06	0.07 (0.07)	0.324	0.06 (0.07)	0.391
Nasal	1.01	1.01	−0.001 (0.06)	0.982	0.02 (0.07)	0.780
Flow, AU						
Temporal	266.0	312.0	46.0 (21.9)	0.042*	29.4 (21.6)	0.181
Rim	251.4	256.8	5.4 (20.4)	0.794	2.5 (22.7)	0.912
Nasal	274.3	274.7	0.4 (17.6)	0.984	7.6 (19.3)	0.697
34 Subjects, *n*	16	18				
Volume, AU						
Temporal	19.6	22.8	3.2 (1.3)	0.019*	1.5 (1.5)	0.316
Rim	17.8	18.2	0.4 (1.2)	0.767	−1.2 (1.4)	0.384
Nasal	19.1	18.5	−0.6 (1.0)	0.567	−1.5 (1.3)	0.253
Velocity, AU						
Temporal	0.98	1.16	0.18 (0.08)	0.038*	0.08 (0.10)	0.422
Rim	0.99	1.03	0.04 (0.07)	0.581	0.04 (0.09)	0.671
Nasal	1.01	0.97	−0.05 (0.06)	0.477	−0.07 (0.08)	0.376
Flow, AU						
Temporal	266.0	327.0	60.9 (24.0)	0.016*	30.0 (26.4)	0.266
Rim	251.4	248.9	−2.4 (22.8)	0.916	−39.7 (26.9)	0.150
Nasal	274.3	263.4	−10.9 (18.3)	0.554	−21.1 (23.5)	0.375

D, difference between the means.

* Significance level determined by unadjusted linear regression model.

† Significance level determined by adjusted linear regression for age, ocular perfusion pressure, and age at menarche.

‡ Significance level determined by adjusted linear regression for age, ocular perfusion pressure, age at menarche, and menopause duration.

presented in Table 5. For the entire ONH region, the rim volume was significantly greater in the +HT group ($P = 0.032$) than in the ∅HT group, when adjusted for age, perfusion pressure, and age at menarche. Age at menarche was a significant predictor of rim volume ($P = 0.014$). In the inferotemporal ONH region, the rim volume ($P = 0.042$), height variation contour ($P = 0.011$), mean RNFL thickness ($P = 0.033$), and cross-sectional area ($P = 0.020$) were significantly greater in the +HT group than in the ∅HT group. Somewhat similar findings were observed in a subset of women excluding the ones who had hysterectomy only. In both the entire ONH and the inferotemporal region, height variation contour ($P = 0.049$, $P = 0.003$), mean RNFL thickness ($P = 0.046$, $P = 0.006$), and RNFL cross-sectional area ($P = 0.045$, $P = 0.011$) were significantly greater in 23 +HT than in 26 ∅HT postmenopausal women when adjusted for age, perfusion pressure, age at menarche, and menopause duration. Ocular perfusion pressure was a significant predictor for height variation contour ($P = 0.046$) and so was age at menarche for the mean RNFL thickness ($P = 0.032$) of the entire ONH, whereas ocular perfusion pressure ($P = 0.049$) was a predictor for mean RNFL thickness of the inferotemporal region. For both the entire ONH and the inferotemporal region, all other parameters did not reach statistical significance (data not presented).

Functionality of the RGC. The PERG findings from 21 ∅HT and 30 +HT (Table 1, subpopulation D) postmenopausal women are presented in Table 6. P50 latency was shorter by 1.1 ms in the +HT group in the unadjusted model ($P = 0.041$), but this difference was no longer significant in the adjusted model. The remaining PERG components were similar between the ∅HT and +HT groups. Similar findings were observed in a subset group of women excluding the ones who had undergone hysterectomy only. P50 latency ($P = 0.042$) was also significantly smaller in 21 +HT postmenopausal women than in 20 ∅HT in the unadjusted model. Again, this difference was no longer significant in the linear regression model when age, ocular perfusion pressure, age at menarche, and menopause duration were accounted for. No significant predictors were found for P50 latency in both adjusted models.

Effects of Estrogen versus Estrogen+Progestogen Therapies. Table 7 displays the results of linear regression analysis comparing the effects of the type of HT treatment (estrogen therapy versus ∅HT, estrogen+progestogen therapy versus ∅HT, and estrogen versus estrogen+progestogen therapies) for the significant outcome variables. For the inferotemporal retinal artery, postmenopausal women taking either estrogen therapy or estrogen+progestogen therapy presented significantly greater diameters ($P = 0.016$ and $P = 0.023$, respectively) and flow ($P = 0.010$ and $P = 0.042$, respectively) than did the control group. The diameter and flow were slightly greater in the estrogen therapy group than in the estrogen+progestogen therapy group, but the differences were not statistically significant.

Women taking either estrogen or estrogen+progestogen therapy had more favorable stereometric parameters than did the ∅HT group, but the differences did not always reach statistical significance in the subgroups.

Table 5. Standard Stereometric Parameters of the Entire and Inferotemporal Regions of the ONH Measured in ∅HT and +HT Postmenopausal Women

	0–360°						270–360°					
	Mean		Unadjusted*		Adjusted†‡		Mean		Unadjusted*		Adjusted	
	∅HT	+HT	D (SE)	*P*	D (SE)	*P*	∅HT	+HT	D (SE)	*P*	D (SE)	*P*
59 Subjects, *n*	27	32					27	32				
Rim volume, mm^3	0.380	0.440	0.059 (0.037)	0.112	0.080 (0.036)	0.032†	0.050	0.066	0.016 (0.009)	0.100	0.020 (0.009)	0.042†
Height variation contour	0.397	0.417	0.019 (0.023)	0.396	0.030 (0.024)	0.210	0.289	0.355	0.066 (0.023)	0.014*	0.072 (0.027)	0.011†
Mean RNFL thickness, mm	0.256	0.275	0.019 (0.017)	0.272	0.028 (0.017)	0.100	0.167	0.194	0.027 (0.015)	0.076	0.033 (0.015)	0.033†
RNFL cross-sectional area, mm^2	1.235	1.343	0.108 (0.079)	0.177	0.151 (0.081)	0.066	0.198	0.239	0.041 (0.019)	0.031*	0.046 (0.019)	0.020†
49 Subjects, *n*	26	23					26	23				
Rim volume, mm^3	0.387	0.409	0.023 (0.036)	0.530	0.049 (0.038)	0.208	0.050	0.056	0.005 (0.008)	0.488	0.009 (0.008)	0.267
Height variation contour	0.399	0.422	0.023 (0.027)	0.401	0.058 (0.029)	0.049‡	0.292	0.365	0.073 (0.030)	0.018*	0.103 (0.032)	0.003‡
Mean RNFL thickness, mm	0.257	0.274	0.017 (0.020)	0.399	0.043 (0.021)	0.046‡	0.164	0.195	0.031 (0.017)	0.067	0.051 (0.018)	0.006‡
RNFL cross-sectional area, mm^2	1.234	1.321	0.078 (0.093)	0.402	0.120 (0.097)	0.045‡	0.200	0.237	0.037 (0.021)	0.087	0.060 (0.023)	0.011‡

D, difference between the means.

* Significance level determined by unadjusted linear regression model.

† Significance level determined by adjusted linear regression for age, ocular perfusion pressure and age at menarche.

‡ Significance level determined by adjusted linear regression for age, ocular perfusion pressure, age at menarche and menopause duration.

TABLE 6. Components of the Pattern Electroretinogram Measured in ∅HT and +HT Postmenopausal Women

	Mean		Linear Regression Model			
			Unadjusted*		Adjusted†‡	
	∅HT	+HT	D (SE)	*P*	D (SE)	*P*
51 Subjects, *n*	21	30				
N35 latency, ms	21.6	21.0	−0.6 (0.5)	0.268	−0.7 (0.5)	0.233
P50 latency, ms	43.3	42.1	−1.1 (0.5)	0.041*	−1.1 (0.6)	0.063
N95 latency, ms	80.2	80.8	0.6 (1.1)	0.598	0.3 (1.1)	0.777
P50 amplitude, μV	7.3	7.0	−0.3 (0.5)	0.438	−0.2 (0.5)	0.741
N95 amplitude, μV	9.4	9.1	−0.3 (0.5)	0.610	−0.1 (0.5)	0.867
41 Subjects, *n*	20	21				
N35 latency, ms	21.8	21.1	−0.7 (0.6)	0.250	−0.6 (0.6)	0.370
P50 latency, ms	43.4	42.1	−1.2 (0.6)	0.042*	−1.2 (0.7)	0.064
N95 latency, ms	80.2	80.6	0.4 (1.2)	0.687	−0.4 (1.2)	0.725
P50 amplitude, μV	7.3	6.9	−0.4 (0.5)	0.459	−0.2 (0.6)	0.778
N95 amplitude, μV	9.4	9.0	−0.4 (0.6)	0.466	−0.3 (0.7)	0.672

D, difference between both means.

* Significance level determined by unadjusted linear regression model.

† Significance level determined by adjusted linear regression for age, ocular perfusion pressure and age at menarche.

‡ Significance level determined by adjusted linear regression for age, ocular perfusion pressure, age at menarche and menopause duration.

Ovariectomized Rat Study

Effect of Ovariectomy and E_2 Treatment on the Physiological Parameters and Serum E_2 Levels. Physiological parameters in the OVx and OVx+E_2 groups are summarized in Table 8. The E_2 treatment did not alter most physiological parameters, except body weight. As expected, OVx rats gained weight ($P = 0.009$), since estrogens play a role in food intake and energy expenditure.[52]

After 6 weeks of treatment, serum E_2 levels were approximately five times higher in OVx rats treated with E_2 than in OVx rats treated with placebo (71.1 ± 7.6 pM vs. 370.4 ± 57.0 pM, $P = 0.005$). The serum E_2 levels in both groups were consistent with values reported in other studies.[47]

Retinal Uptake Index of [^{14}C]-IMP in Wholemount Retina. Pseudocolor autoradiograms of wholemount retinas (Fig. 1B) displayed a decreasing gradient of tracer from the center to the periphery of the retina, with a fairly homogeneous distribution throughout each isopter. Quantified data expressed in RUIs show significantly elevated tissue perfusion values in the OVx+E_2 rat in the three furthermost peripheral isopters compared with control rats (Fig. 2). This increase in the RUI ranges from 32% to 45%, reaching 45% in the third isopter.

DISCUSSION

This observational study in postmenopausal women provides the first clinical evidence that HT (estrogens alone or in com-

TABLE 7. Effect of Estrogens Therapy and Estrogens Combined with Progestogens Therapy on Different Outcome Variables

	Mean			Adjusted *P**					
		+HT		a		b		c	
	∅HT	Estrogens	Estrogens + Progestogens	D (SE)	*P*	D (SE)	*P*	D (SE)	*P*
CLBF, *n*	18	9	12						
Diameter, μm	120.7	134.6	132.1	13.2 (5.2)	0.016*	11.6 (4.9)	0.023*	1.6 (5.8)	0.786
Flow, μL/min	12.5	16.1	14.9	3.6 (1.3)	0.010*	2.6 (1.2)	0.042*	1.0 (1.4)	0.485
Stereometric parameters, *n*	27	11	21						
(0–360°) Rim volume, mm^3	0.380	0.480	0.418	0.109 (0.048)	0.028*	0.062 (0.042)	0.146	0.048 (0.052)	0.369
(270–360°) Rim volume, mm^3	0.050	0.082	0.057	0.033 (0.013)	0.012*	0.012 (0.011)	0.287	0.022 (0.014)	0.127
Height variation contour, mm	0.289	0.321	0.373	0.032 (0.036)	0.377	0.099 (0.031)	0.003*	0.067 (0.039)	0.096
Mean RNFL thickness, mm	0.167	0.184	0.198	0.017 (0.020)	0.391	0.043 (0.017)	0.016*	−0.026 (0.022)	0.242
RNFL cross-sectional area, mm^2	0.198	0.233	0.241	0.036 (0.026)	0.170	0.053 (0.022)	0.022*	−0.017 (0.029)	0.552

D, difference between the means.

a, Estrogen therapy versus ∅HT.

b, Estrogen+progestogen therapy versus ∅HT.

c, Estrogen therapy versus estrogen+progestogen therapy.

* Significance level determined by adjusted linear regression for age, ocular perfusion pressure, and age at menarche.

TABLE 8. Effects of 17β-E_2 Treatment on the Physiological Parameters Monitored in the Conscious OVx Rats after 2 Hours of Recovery from Anaesthesia and before the Injection of [^{14}C]-IMP

	OV_x (n = 5)	OV_x + E_2 (n = 7)	P
Weight, g	247.4 ± 7.5	224.0 ± 13.0	0.009*
Body temperature, °C	37.3 ± 0.3	37.4 ± 0.5	0.52
Blood pressure			
Systolic, mm Hg	121.0 ± 12.6	128.9 ± 8.5	0.19
Diastolic, mm Hg	95.8 ± 6.2	102.1 ± 10.9	0.34
Mean, mm Hg	104.2 ± 8.3	110.8 ± 9.8	0.26
Arterial pH	7.44 ± 0.03	7.44 ± 0.02	0.57
Arterial pO_2, mm Hg	80.4 ± 5.6	80.3 ± 5.6	0.94
O2 saturation, %	96.2 ± 0.8	96.1 ± 0.7	0.87
Arterial pCO_2, mm Hg	37.1 ± 1.8	37.8 ± 1.2	0.42
Arterial HCO_3, mmol/L	25.4 ± 1.1	25.6 ± 1.4	0.87

Data are expressed as the mean ± SD. pO_2, pCO_2, partial gas pressure of oxygen and carbon dioxide respectively; HCO_3, bicarbonate. Data are the mean ± SD.

* Significantly different from OV_x placebo, Mann–Whitney U test.

bination with progestogens) increases BF in a retinal artery and has a protective effect on the ONH and RNFL. We observed a greater BF in the inferotemporal retinal artery, mostly caused by its greater diameter, and a thicker neuroretinal rim and RNFL. BF was somewhat increased in the temporal peripapillary retina in the +HT group, but this difference did not reach statistical significance in the adjusted regression models. In addition, the retinal blood perfusion was increased by E_2 treatment in rats, indicating that parts of the effects observed in the +HT group resulted from the pure contribution of estrogens to the retinal BF.

Effect of HT on Retinal Circulation

The findings of a greater BF in retinal arteries in postmenopausal women treated with HT are in agreement with other clinical studies that used color Doppler imaging and reported improved pulsatility and/or resistivity indexes in retrobulbar vessels[10,27–30] in women taking HT.

We demonstrated that the significantly greater BF measured in the inferotemporal retinal artery in the +HT group was mostly due to a significantly larger diameter of the ITRA. This finding is the opposite of those reported by Leung et al.[53] and Wong et al.,[54] where the use of HT did not affect the retinal vessel diameters or had a constricting effect on them. However, study limitations have been reported by the authors of these two studies such as a confounding-by-condition bias (i.e., a disease condition prompts the use of HT and may be the causal association rather than the HT use itself). In both studies, women with a history of hypertension or diabetes were included. These women may have been more likely to use HT for its cardioprotective role, and the observed narrower retinal vessels in HT users may simply reflect the effects of elevated blood pressure rather than from HT.[53–55] Moreover, women with a history of hypertension and diabetes are likely to have an impaired endothelium and to be on vasodilator treatment or insulin. Therefore, a potentially impaired endothelium in patients with vascular diseases may not be fully responsive to the HT treatment, and the endothelium of patients taking vasoactive medications may not be responsive to additional vasomotor stimulation from HT treatment. As well, the authors[53,54] reported the lack of E_2 assays to confirm the use of HT, interaction of age, and elevated BP that may not have been completely adjusted in the logistic regression analysis; variations in clarity of retinal photographs; different graders; and slight retinal changes in vessel diameters with the cardiac cycle. In the present study, confounding by condition would have been less significant, since women with hypertension or diabetes were excluded, and this may explain why our results differed. Also, the digitized technique used in both the Blue Mountains Eye Study and the Beaver Dam Eye Study populations to document vessel diameter from photographs has not been directly compared with the vessel diameter obtained with the CLBF system, but since the diameter measurement by the CLBF accounts for cardiac pulsation, the CLBF system may have provided a more accurate vessel diameter measurement. We cannot rule out the possibility of a selection bias, as healthy postmenopausal women who use HT to relieve their postmenopausal symptoms may have a different vascular reactivity to HT than those who are not HT users. A significant portion of postmenopausal women who underwent hysterectomy only were HT users. It is conceivable that these women had an altered vascular reactivity to estrogens as the underlying cause of symptoms leading to hysterectomy, and this could have led to a selection bias. However, in the secondary analysis excluding postmenopausal women who underwent hysterectomy only, significant findings reported on the ITRA diameter, flow, and nerve fiber layer were similar to the ones reported in the first adjusted model that did not control for menopause duration.

Systemic action of HT in postmenopausal women cannot be ruled out, in that there are several reports of the vasomotor effect of estrogens on various vascular beds.[4–10] A longitudinal study monitoring the blood pressure changes in HT users and nonusers indicated that systolic blood pressure increased less in HT users than in nonusers over a period of 10 years, while the diastolic blood pressure remained stable.[56] These findings are somewhat in agreement with ours, where the systolic blood pressure was greater in women who were not HT users, but the diastolic blood pressure and mean blood pressure were also greater in these women than in the HT users. These differences reached a significance level only for the subpopulation investigated for ONH topography. The resulting ocular perfusion pressure was also higher in the women who were not HT users compared with those who were, reaching the level of significance in this subpopulation. Higher ocular perfusion pressure has a positive vascular effect by providing better BF. However, although higher ocular perfusion pressure was greater in postmenopausal women not on HT, their infero-

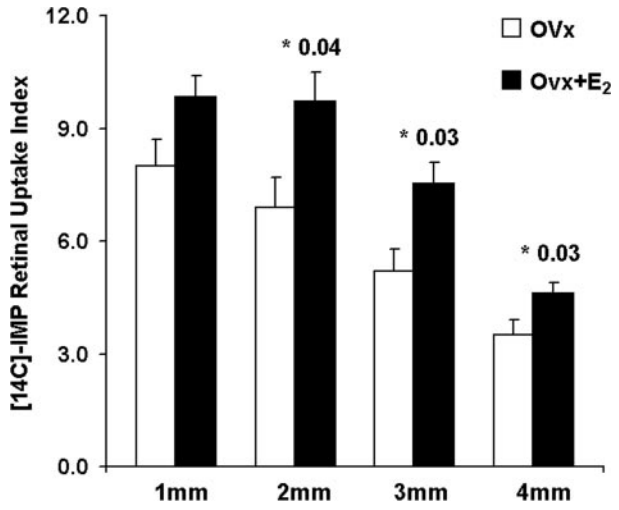

FIGURE 2. Mean retinal uptake index of [^{14}C]-IMP in OVx conscious rats at 1-, 2-, 3-, and 4-mm away from the center of the ONH in wholemount retinas. Mean retinal uptake indexes were obtained from five OVx control rats and seven OVx rats treated with E_2. *Significantly different from OVx placebo (Mann–Whitney U test). Error bars represent standard deviations.

temporal retinal arteries and the temporal peripapillary retinal BF was smaller. Based on these observations, it is most likely that the increased BF we observed in the retina results from a local action of HT on retinal circulation and not from a systemic action.

As well, our findings of greater retinal BF in OVx rats, where SC administration of E_2 increased the tissue perfusion in the retina, support a preponderant action of estrogen in these BF increases and suggest a local action of estrogens in the retina. Indeed, E_2 treatment increased the blood perfusion in all quadrants of rat retinas with higher uptake increases in the peripheral retina. As seen in other species,[57] blood tissue perfusion was higher in the central compared with the peripheral retina, yet E_2 treatment elevated BF in the periphery to a greater degree than in the central retina. This finding may indicate a local effect of estrogens at this level, allowing a better uptake of [^{14}C]-IMP or a concentration of the ERs or downstream target enzymes in this part of the retina. Alternatively, the capillary network in the peripheral retina is denser than in the central retina, as shown in corrosion casts in rats,[58] and could provide better tissue perfusion.

It has to be noted that at the time of death, serum E_2 levels were nearly fivefold higher in the OVx rats receiving the E_2 capsules than in the rats receiving the placebo capsules, which is within the 3.1- to 11.8-fold range of increases in E_2 levels observed in postmenopausal women using typical transdermal E_2 regimens.[8,9] Also the SC release of E_2 from silastic capsules was similar to transdermal application of E_2, as both approaches bypass the hepatic degradation of E_2.

Possible Vasomotor Pathways of Estrogens in Retinal Circulation

The effect of HT on retinal BF is most likely caused by the vasodilating properties of estrogens mediated by the endothelium-derived relaxing factors, primarily the nitric oxide (NO) and prostacyclin (PGI_2) pathways, which have been identified in the retinal vascular bed.[59–62] This effect could be mediated through the ERα or ERβ expressed in the retina.[24–26,34] Qualitatively, the expression of ERα mRNA in retinas from older female donors (74- and 77-year-old) has been reported to be weak or absent compared with retinas from two female donors still having estrus cycles (35 and 49 years old),[24] as documented by Ogueta et al.[24] In contrast, Munaut et al.[26] observed some variation in the expression of ERα mRNA in retinas from five female donors independent of age, whereas the expression level of the EPβ mRNA remained relatively constant. Ogueta et al.[24] indicated that a possible age effect influences the expression of the ERα in the presence of a reduced amount of circulating estrogens after menopause. However, although these observations made in both studies were from few eye donors, with no information on whether they were HT users, it corroborates the observation made by Christian et al.,[63] who observed that the level of expression of ERα but not ERβ declined with age, as documented in intact coronary arteries from women who were HT users, whereas age had no effect on both ERα ERβ expression in non-HT users. The effect of HT on retinal BF that we observed in postmenopausal women who had used HT continually since menopause most likely depends on both ERα and ERβ expression, the level of which remains to be investigated.

A large body of evidence indicates that estrogens upregulate endothelial NO production.[64] With regard to the prostacyclin pathway, studies have shown the stimulating action of estrogens on PGI_2 synthesis,[65,66] and intravitreal injection of PGI_2 has been shown to increase retinal BF.[61] Finally, studies have indicated that estrogens also antagonize the effects of endothelium-derived contracting factors,[2] such as endothelin-1, which has been documented to be a potent vasoconstrictor of the ophthalmic and retinal arteries.[67]

Possible Vasomotor Pathways of Progestogens in Retinal Circulation

Progestogens are prescribed with estrogens as a combined HT in menopausal women with an intact uterus, to regulate the action of estrogens on endometrial cells and to prevent endometrial cancer. In our study population of 64 subjects, 35 (54.7%) were HT users, among which 23 (35.9%) were on estrogen+progestogen therapy. The type of progestogen regimen used was heterogeneous in terms of active ingredients, where 11 (17.2%) subjects were using P, 6 (9.4%) were using norethindrone acetate (NETA), 4 (6.3%) were using medroxyP (MPA), and 2 (3.1%) were using levonorgestrel.

However, divergent vasomotor effects of progestogens have been reported. In animal models, P has been shown to antagonize the estrogen-induced BF in a combined therapy,[68–70] whereas MPA has been shown to antagonize[71,72] or not antagonize[73] the estrogen-induced vasodilating effects in a combined therapy. In clinical trials, P administration has been shown to antagonize[74] or not[75,76] estrogen-induced flow-mediated dilatation (FMD), whereas MPA has been shown to antagonize estrogen-induced FMD.[77] Therefore, possible antagonizing vascular interactions of progestogens on estrogens in our study population cannot be ignored in our findings. In our study population, we observed that when only estrogen therapy was used, the diameter and flow of the inferotemporal retinal artery were slightly greater than in the estrogen+progestogens therapy, but these differences were not statistically significant.

Like estrogens, the vasomotor effects of progestogens can result from an interaction with both the NO[78–80] and PGI_2 pathways[81] that are present in the retinal vascular bed[59–62] and are mediated through the PRs,[25] which have been identified in the retina.

Regional Effect of HT on ONH BF

Using the SLDF technique, we demonstrated greater capillary BF in the +HT group in the temporal peripapillary retina and, to a lesser extent, in the rim area, but these differences did not reach statistical significance. These findings are consistent with our findings on the inferotemporal retinal artery, but a larger sample size would have been necessary to show the statistical significance of greater capillary BF. However, the SLDF technique has been reported to present a 0 offset reading.[82–84] This offset reading may account in a part for our SLDF flow measurements and may have masked potential significant increases in capillary blood velocity and flow in the postmenopausal woman who were HT users. The 0 offset problem also generates some uncertainty as to the accuracy of the SLDF findings as presented, since the numbers generated by the SLDF reflect both BF and imaging noise.

HT Effects on ONH Topography

In the +HT group, we observed that the rim volume, height variation contour, mean thickness of the RNFL, and RNFL cross-sectional area were greater than in the ∅HT group. The rim volume was significant for both the entire and the inferotemporal region of the ONH, whereas the height variation contour, mean RNFL thickness, and cross-sectional area of the RNFL, reached significance in the inferotemporal region. The differences observed in these stereometric parameters indicate a thinner RNFL in postmenopausal women not on HT compared with those on HT. This finding is similar to the sparing of some brain regions observed in postmenopausal women using HT.[11,12] A sparing of the RNFL may result from the vascular

properties of estrogens and progestogens, as described earlier, by providing a better BF and nutrients to the RGC and the NFL. In addition, sparing of the RNFL may result from the protective properties of estrogens on neurons independent of BF.[3] These protective effects have been documented to increase neuron survival in several toxicity models of cultured neurons, such as glutamate, β-amyloid, hydrogen peroxide, and excitatory amino acid toxicity models.[3] Of interest, recent studies have reported a protective effect of estrogens on retinal ganglion cells in glutamate toxicity[85] and axotomized optic nerve rodent models.[86]

Similar to its vasomotor effects, progestogens also present divergent effects in terms of protective effects on neurons. When coadministered with E_2, P has been shown to increase neuron survival beyond that of E_2 alone in a hippocampal glutamate toxicity model.[87] MPA has been shown not to attenuate estrogen-induced neuroprotection in the same hippocampal glutamate toxicity model.[87] Therefore, possible positive or antagonizing protective effects of progestogens in combined therapy cannot be ruled out in our findings. In our study population, we observed that when only estrogen therapy was used, the rim volume, height variation contour, mean thickness, and cross-sectional area of the RNFL measurements were greater than in those in the estrogen+progestogen therapy. These differences reached significance only for the rim volume.

For the height variation contour, mean thickness, and cross-sectional area of the RNFL, the ∅HT group of postmenopausal women presented a decrease of 5.0%, 7.4%, and 8.7%, respectively, compared with the +HT group for the entire optic nerve region, whereas for the inferotemporal quadrant, this decrease was 22.8%, 16.2%, and 20.7%, respectively. Preferential thinning of inferotemporal neural structures is also seen in early glaucoma and several causes have been proposed.[88,89] It is interesting to speculate that the vulnerability of this region may explain some of our findings.

Because our findings on the ONH topography related to RNFL differences between the two groups of postmenopausal women, we could have chosen a test to specifically assess the RNFL, such as optical coherence tomography. However, we were primarily interested in investigating ONH topography, which was obtained with retinal tomography (HRT; Heidelberg Engineering).

The Effect of HT on the Functionality of the RGCs

We observed a borderline significantly shorter P50 latency (1.1 ms) in the +HT group. Although a shorter P50 latency may indicate an improvement in the function of ganglion cells, it is unlikely that such a small improvement would be clinically significant. We also observed a nonsignificant small increase in the N95 amplitude (3%) and a small decrease in P50 amplitude (6%) in the +HT group. Since N95 amplitude correlates with the amount of RNFL, the difference in the RNFL thickness we observed between the two groups of postmenopausal women may not be large enough to induce a significant change in the PERG amplitudes.

Study Limitations

In postmenopausal women, the diameter, vel_{mean}, and flow measurements acquired with the CLBF were obtained from a single retinal arterial vessel: the main inferotemporal retinal artery. Methodologically, it would have been ideal to measure the diameter, vel_{mean}, and flow of all major retinal arterial vessels, to assess the effects of postmenopausal therapy on the entire retinal arterial vasculature. This method would have eliminated "variations among individuals of the number and pattern of branching of the larger arteries of the retina" as reported by Parr and Spears,[90] therefore allowing valid cross-sectional comparisons. To increase measurement accuracy and avoid fatiguing the subject, we opted to measure from a single major vessel, the main inferotemporal retinal artery before it branched off in secondary vessels. Anatomically and as a general rule, the inner retina receives its blood supply from the central retinal artery, which divides into four major vessels, each supplying a quadrant. In the present study, the inferotemporal retinal artery was carefully identified as being the only major vessel supplying the inferotemporal quadrant. We excluded subjects presenting more than one main inferotemporal artery or subjects in whom the main inferotemporal artery divided itself into second order arterioles near the outer edge of the ONH before the site of measurement. Therefore, we believe that the method used in the present study most likely eliminated individual variations in the number and pattern of branching of the inferotemporal retinal artery, still allowing valid cross-sectional comparisons of artery. It is likely that the effects of postmenopausal therapy we observed on diameter, vel_{mean}, and flow of the inferotemporal artery apply to all major retinal arterial vessels and consequently to all retinal quadrants; this assumption remains to be confirmed.

In the heterogeneous group of postmenopausal women whom we investigated, the HT regimens, in terms of their active ingredients, routes of administration, dosage, and duration of administration, were also heterogeneous and may have yielded different degrees of vasomotor effects on the retina and protective effects on the RNFL. In addition, the women were at various postmenopausal times. As well, the outcome variables measured were collected at a single time point, which does not provide any information on the progress of the outcome variables in both groups of postmenopausal women. Finally, postmenopausal women may not all exhibit similar cardiovascular characteristics and risks, which could affect the efficacy of HT. The ideal study design would be a longitudinal randomized clinical trial in which cardiovascular characteristics and risks, BF, structure, and function would be monitored over a 5- to 10-year period.

Conclusion

This is the first observational study in which the long-term effects of a substantial prolonged decrease in estrogen level compared to the use of postmenopausal HT was investigated in the retina and nerve fiber layer in a population of women. The findings of this study indicate that compared to postmenopausal women who had never used HT, postmenopausal women who had used HT since their menopause onset presented (1) an increase in BF through the inferotemporal retinal artery and (2) a greater rim volume, height variation contour, mean RNFL thickness, and cross-sectional area of the RNFL. Greater retinal BF observed in postmenopausal women on HT is in agreement with our findings observed in OVx rats, an animal model of human menopause where SC administration of E_2 increased tissue perfusion in the retina.

These findings very likely reflect the vasomotor and protective properties of estrogen or estrogen and progestogen combined on the retinal circulation and the RNFL in postmenopausal women.

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